

The role of neural specific Src splice variants in neuronal specification and differentiation

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Abstract

C-Src is a ubiquitously expressed non-receptor tyrosine kinase that functions in cell growth, adhesion, movement and differentiation. N1-Src is an alternatively spliced variant of c-Src, evolutionarily conserved in vertebrates, with expression enriched in neural tissues. N1-Src is formed by the insertion of a 15-18 base pair microexon, depending on species, in the SH3 domain of Src. Expression of N-Srcs is correlated with a favourable prognosis in neuroblastoma, a rare childhood cancer, leading to spontaneous differentiation and resolution of the cancer.

Xenopus models, and mammalian cell culture, were used to investigate the role for n1-Src in early vertebrate development. It was found that n1-Src acts between the neural prepattern and proneural genes during the hierarchy of transcriptional activation and repression. The activity of the mitogen activated kinase pathway is regulated by n1-Src, and it is potentially through this modulation that n1-Src directs the transcriptional programme required for neural development. RNA-Seq was utilised to examine the transcriptional programme downstream of n1-Src. Gene Ontology analysis identified that transcripts significantly downregulated following *n1-src* knockdown are required for development of the nervous system and the neural crest. *In situ* hybridisation and RT-PCR analysis confirmed that *n1-src* expression is required for neural crest induction. Transcripts significantly upregulated following *n1-src* knockdown are enriched for Gene Ontology terms spanning RNA processing and splicing. The requirement for n1-Src in regulating splicing was investigated using Illumina short read and Nanopore long read RNA-Seq technology, which revealed that n1-Src regulates the alternative splicing of a variety of mRNA processing and splicing factors.

This study shows that n1-Src is essential for neuronal differentiation and neural crest development, and it potentially drives these processes through splicing machinery regulation. Splicing regulation by n1-Src could be partly responsible for the favourable prognosis in some neuroblastoma patients as aberrant alternative splicing has been associated with cancers.

Table of Contents

Abstract	2
Table of Contents	3
List of figures.....	8
List of tables.....	11
List of accompanying material	12
Acknowledgements.....	14
Declaration	15
1. Introduction	16
1.1. Phosphorylation	17
1.1.1. Receptor tyrosine kinases	18
1.1.2. Non-receptor tyrosine kinases	18
1.2. Src family kinases	19
1.2.1. The SH4 domain and membrane localisation	20
1.2.2. The unique domain	21
1.2.3. The SH3 domain and peptide binding	22
1.2.4. The Src SH2 domain and phosphotyrosine binding	24
1.2.5. SH1/kinase domain	25
1.2.6. C-terminal domain	26
1.2.7. Src regulation	27
1.3. Roles of c-Src	28
1.3.1. Roles of c-Src in the nervous system.....	30
1.3.2. Src family kinases in early development	35
1.4. Identification of neuronal Srcs	36
1.4.1. Splicing of the <i>n1-src</i> microexon	37
1.4.2. Effects of neuronal microexons on the Src SH3 domain	39
1.4.3. Expression of neuronal Srcs in neuroblastomas	40
1.4.4. Expression and function of <i>n1-src</i> in the mature brain.....	42
1.4.5. Expression and function of <i>n1-src</i> in the developing nervous system	42
1.5. This thesis	44
2. Materials and methods	45
2.1. Embryological methods	46
2.1.1. <i>X. tropicalis</i> fertilisation and embryo culture.....	46
2.1.2. <i>X. laevis</i> fertilisation and embryo culture	46

2.1.3. Microinjections	46
2.1.4. Animal cap explants	47
2.1.5. Photography.....	47
2.2. Molecular biology methods.....	48
2.2.1. Gel electrophoresis	48
2.2.2. Total RNA extraction	48
2.2.3. cDNA synthesis for RT-PCR.....	48
2.2.4. Polymerase chain reactions	49
2.2.5. Plasmid ligation.....	49
2.2.6. Plasmid amplification.....	49
2.2.7. Plasmid isolation	50
2.2.8. Linearisation of plasmid DNA.....	50
2.2.9. DNA purification.....	52
2.2.10. Cloning of <i>n1-src</i> into PCS2+	53
2.2.11. Determining nucleic acid concentration	53
2.2.12. Synthetic mRNA synthesis.....	53
2.2.13. Western blot analysis.....	54
2.2.14. Transcription of Digoxigenin labelled antisense probes	55
2.2.15. Whole-mount <i>in situ</i> hybridisations.....	56
2.2.16. Whole-mount immunostaining.....	57
2.3. RNA-Seq analysis	59
2.3.1. RNA-Seq sample preparation.....	59
2.3.2. RNA-Seq library preparation	59
2.3.4. Differential expression analysis of Illumina RNA-Seq data	60
2.3.5. Gene ontology analysis of Illumina RNA-Seq data	61
2.3.6. Gene set enrichment analysis of Illumina RNA-Seq data	61
2.3.7. DEXSeq analysing differential exon usage in the Illumina RNA-Seq data	61
2.3.8. Generation of a novel transcriptome using Nanopore long reads.....	62
2.3.9. Identification of changes in splicing using IsoformSwitchAnalyzeR.....	62
2.3.10. Identification of coordinates for alternative splice sites.....	62
2.3.11. Identification of enriched RNA binding protein motifs	62
2.4. Cell culture methods	63
2.4.1. Culture of cell lines.....	63
2.4.2. Plating out of cells.....	63
2.4.3. Induction of <i>N1-Src-FLAG</i> expression.....	63
2.4.4. N1-Src-FLAG - dpErk time series	64
3. The role of n1-Src in neuronal development in <i>Xenopus</i>	65

3.1. Introduction.....	66
3.1.1. <i>Xenopus</i> neural induction	66
3.1.2. <i>Xenopus</i> neuronal differentiation	68
3.1.3. N1-Src and <i>Xenopus</i> neural development.....	69
3.1.4. Aims	70
3.2. Results	71
3.2.1. Inhibition of bone morphogenic proteins induces <i>n1-src</i> expression in ectodermal tissues.....	71
3.2.2. Knockdown of <i>n1-src</i> causes a malformed tail phenotype and an aberrant touch response.....	72
3.2.3. <i>N1-src</i> knockdown inhibits the differentiation of primary neurons.....	76
3.2.4. <i>N1-src</i> knockdown and regional specificity markers.....	79
3.2.5. N1-Src functions between the neural prepattern and proneural genes.....	81
3.2.6. <i>N1-src</i> overexpression does not affect the expression of neuronal markers	83
3.2.7. <i>N1-src</i> overexpression does not affect the expression of proneural genes.....	85
3.2.8. Overexpression of <i>n1-src</i> leads to posteriorisation of <i>Xenopus</i> embryos.....	87
3.2.9. N1-Src regulates Erk phosphorylation in <i>Xenopus</i>	88
3.2.10. N1-Src can activate the mapk signalling pathway independently of Fgf ligands	91
3.2.11. <i>N1-src</i> expression upregulates Erk phosphorylation in mammalian cells.....	93
3.3. Discussion	94
3.3.1. <i>N1-src</i> knockdown causes morphological defects	94
3.3.2. Knockdown of <i>n1-src</i> causes locomotive defects.....	95
3.3.3. N1-Src is required for differentiation of <i>X. tropicalis</i> primary neurons	95
3.3.4. N1-Src's position in the neurogenic pathway	96
3.3.5. <i>N1-src</i> overexpression causes a loss of anterior structures.....	98
3.3.6. N1-Src and Fgf signalling	98
3.3.7. Conclusion	100
4. Transcriptomic analysis of <i>n1-src</i> knockdown in <i>X. tropicalis</i> development.....	102
4.1. Introduction.....	103
4.1.1. Aims of this chapter	104
4.2. Results	105
4.2.1. Sample generation and read alignment.....	105
4.2.2. Differential expression resulting from <i>n1-src</i> knockdown	110
4.2.3. <i>N1-src</i> knockdown downregulates expression of neuronal differentiation genes	112
4.2.4. Gene ontology analysis of downregulated transcripts.....	112
4.2.5. Gene ontology analysis of upregulated transcripts	124
4.2.6. Identifying enriched gene sets in the RNA-Seq data.....	130

4.3. Discussion	133
4.3.1. <i>N1-src</i> knockdown lacked statistical significance.....	133
4.3.2. N1-Src and nervous system development	134
4.3.3. N1-Src and neural crest development	136
4.3.4. N1-Src and alternative splicing.....	138
4.3.4 Conclusion.....	139
5. N1-Src regulates alternative splicing in <i>X. tropicalis</i>	141
5.1. Introduction.....	142
5.1.1. Alternative splicing.....	142
5.1.2. Alternative splicing in the nervous system	145
5.1.3. Protein phosphorylation and alternative splicing	147
5.1.4. Aims of this chapter	148
5.2. Results	149
5.2.1. DEXSeq analysis of the Illumina RNA-Seq data	149
5.2.2. <i>Hnrnpa1</i> and <i>tra2a</i> intron retention events identified by DEXSeq	154
5.2.3. Nanopore long read RNA-Seq sample generation	160
5.2.4. IsoformSwitchAnalyzeR identifies a genome-wide gain in intron retention.....	163
5.2.5. IsoformSwitchAnalyzeR identified splicing changes in <i>hnrnpa1</i> and <i>tra2a</i>	168
5.2.6. Alternative splicing of <i>hnrnpa1</i> and <i>tra2a</i> in the Nanopore long read RNA-Seq data	171
5.2.7. RT-PCR analysis verifies the <i>hnrnpa1</i> and <i>tra2a</i> intron retention events	173
5.2.8. Overexpression of <i>n1-src</i> mRNA rescues the <i>hnrnpa1</i> splicing changes following <i>n1-src</i> knockdown.....	175
5.2.9. 14 motifs for RNA binding proteins were enriched in the sequences surrounding the alternative splicing events	177
5.2.10. N1-Src regulates <i>n1-src</i> splicing	184
5.3. Discussion	186
5.3.1. N1-Srcs regulation of alternative splicing	186
5.3.2. hnRNPA1 in the nervous system.....	189
5.3.3. Tra2a in the nervous system	191
5.3.4. Conclusions	193
6. The role of n1-Src in neural crest development in <i>X. tropicalis</i>.....	194
6.1. Introduction.....	195
6.1.1. Neural plate border specification	195
6.1.2. Neural crest specification.....	198
6.1.3. Neural crest migration	199
6.1.4. Neural crest and neuroblastoma	201
6.1.5. Aims	201

6.2. Results	202
6.2.1. Knockdown of <i>n1-src</i> downregulates the expression of neural plate border specifiers.....	202
6.2.2. Knockdown of <i>n1-src</i> downregulates the expression of neural crest specifier genes	204
6.2.3. <i>N1-src</i> overexpression results in an upwards trend in <i>phox2a</i> expression.....	207
6.2.4. <i>N1-src</i> mRNA overexpression rescues <i>phox2a</i> expression in <i>n1-src</i> knockdown embryos.....	209
6.2.5. <i>N1-src</i> is expressed in neural crest induced animal caps	211
6.3. Discussion	213
6.3.1. <i>N1-src</i> knockdown downregulates the expression of neural plate border specifier genes and neural crest specifiers	213
6.3.2. <i>N1-src</i> overexpression effects on the expression of neural crest genes.....	215
6.3.3. <i>N1-src</i> expression in the neural crest.....	216
6.3.4. Conclusion	219
7. Discussion	220
7.1. Summary of findings.....	221
7.2. <i>N1-Src</i> in vertebrate development	221
7.2.1. <i>N1-Src</i> is required for neuronal differentiation	222
7.2.2. <i>N1-Src</i> is required for neural crest development.....	223
7.3. Mechanisms by which <i>n1-Src</i> regulates development	225
7.3.1. <i>N1-Src</i> regulates intracellular signalling.....	225
7.3.2. <i>N1-Src</i> regulates alternative splicing.....	229
7.4. <i>N1-Src</i> and neuroblastoma therapies.....	231
7.5. Conclusions.....	233
Appendices	234
List of Abbreviations	255
References	258

List of figures

Chapter 1

Figure 1.1	Src family kinase domain structure.....	20
Figure 1.2	Regulation of Src activity.....	27
Figure 1.3	Src mediates integrin signalling via the phosphorylation of FAK and the activation of downstream signalling pathways.....	29
Figure 1.4	Mechanism for <i>n1-src</i> exon splicing.....	39

Chapter 3

Figure 3.1	Schematic of neural induction in blastula stage <i>Xenopus</i> embryos.....	67
Figure 3.2	Differentiation of primary neurons in <i>Xenopus</i>	68
Figure 3.3	RT-PCR analysis for <i>n1-src</i> following BMP inhibition.....	70
Figure 3.4	<i>X. tropicalis</i> <i>n1-src</i> microexon skipping morpholinos.....	72
Figure 3.5	RT-PCR analysis showing effective knockdown of <i>n1-src</i> by splice blocking morpholinos.....	73
Figure 3.6	Representative phenotype of <i>n1-src</i> knockdown embryos.....	74
Figure 3.7	Knockdown of <i>n1-src</i> leads to aberrant touch responses in <i>X. tropicalis</i>	75
Figure 3.8	<i>N1-src</i> knockdown inhibits <i>tubb2b</i> expression.....	76
Figure 3.9	<i>N1-src</i> knockdown inhibits <i>myt1</i> expression.....	78
Figure 3.10	Effects of <i>n1-src</i> knockdown on expression of regional specificity Markers.....	80
Figure 3.11	Effects of <i>n1-src</i> knockdown on expression of neurogenic pathway genes.....	82
Figure 3.12	Effects of <i>n1-src</i> overexpression on expression of neuron Markers.....	83
Figure 3.13	Effects of <i>n1-src</i> overexpression on expression of neurogenic pathway genes.....	85
Figure 3.14	Effects of <i>n1-src</i> overexpression on <i>X.tropicalis</i> development.....	87
Figure 3.15	Knockdown of <i>n1-src</i> downregulates Erk phosphorylation.....	89
Figure 3.16	Knockdown of <i>n1-src</i> downregulates Erk phosphorylation in <i>X. tropicalis</i> embryos.....	90
Figure 3.17	<i>N1-src</i> overexpression upregulates Erk phosphorylation in <i>X. laevis</i> embryos.....	91
Figure 3.18	<i>N1-src</i> functions downstream of Fgf receptors to regulate Erk phosphorylation.....	92
Figure 3.19	<i>N1-Src</i> upregulates Erk phosphorylation.....	93

Chapter 4

Figure 4.1	RT-PCR analysis for <i>n1-src</i> knockdown.....	105
Figure 4.2	Transcripts per million plots for <i>c-src</i> and <i>n1-src</i> reads.....	109
Figure 4.3	Mapping of the RNA-Seq reads to the <i>n1-src</i> microexon.....	110
Figure 4.4	Transcript volcano plot.....	111
Figure 4.5	Average TPM plots for <i>tubb2b</i> and <i>neurod1</i>	112

Figure 4.6	Top 20 biological processes, based on significance, overrepresented within the significantly downregulated transcripts.....	114
Figure 4.7	Top 20 biological processes, based on fold enrichment, overrepresented within the significantly downregulated transcripts.....	120
Figure 4.8	Top 20 biological processes, based on significance, overrepresented within the significantly upregulated transcripts.....	125
Figure 4.9	Top 20 biological processes, based on fold enrichment, overrepresented within the significantly upregulated transcripts.....	128
Figure 4.10	Gene set enrichment analysis revealed neuronal gene sets enriched in the control samples.....	132

Chapter 5

Figure 5.1	Schematic showing the most common forms of alternative splicing.....	143
Figure 5.2	Schematic showing the two transesterification reactions in pre-mRNA splicing.....	145
Figure 5.3	Schematic depicting the relationship between PTB and nPTB in neuronal development.....	146
Figure 5.4	DEXSeq generates counting bins to examine differential exon usage.....	150
Figure 5.5	Top 20 biological processes, based on fold enrichment, overrepresented within the transcripts identified by DEXSeq having differential exon usage.....	152
Figure 5.6	<i>N1-src</i> knockdown leads to an intron retention event in <i>hnmpa1</i> transcripts.....	156
Figure 5.7	<i>N1-src</i> knockdown leads to two intron retention events in <i>tra2a</i> transcripts.....	158
Figure 5.8	RT-PCR analysis for <i>n1-src</i> expression.....	161
Figure 5.9	Flow chart depicting strategy used to analyse alternative splicing events.....	162
Figure 5.10	Functional consequences of splicing changes identified by IsoformSwitchAnalyzeR.....	167
Figure 5.11	IsoformSwitchAnalyzeR identified significant changes in <i>hnmpa1</i> isoform usage.....	169
Figure 5.12	IsoformSwitchAnalyzeR identified significant changes in <i>tra2a</i> isoform usage.....	170
Figure 5.13	Nanopore long reads confirm the splicing events identified by DEXSeq and IsoformSwitchAnalyzeR.....	172
Figure 5.14	RT-PCR analysis verifies the changes in <i>hnmpa1</i> and <i>tra2a</i> splicing following <i>n1-src</i> knockdown.....	174
Figure 5.15	<i>N1-src</i> mRNA rescues the <i>hnmpa1</i> mis-splicing caused by <i>n1-src</i> knockdown.....	176
Figure 5.16	Analysing the sequences around the alternative splicing events for enriched motifs.....	181
Figure 5.17	<i>N1-Src</i> regulates its own splicing.....	185

Chapter 6

Figure 6.1	Schematic of Bmp induction of the neural plate border in blastula stage <i>Xenopus</i> embryos.....	195
Figure 6.2	Schematic of proposed signals to induce the neural plate border in <i>Xenopus</i> embryos.....	198
Figure 6.3	Schematic showing the complexity of gene expression involved in specification of the neural crest.....	199
Figure 6.4	Schematic showing migration of the neural crest cells in <i>Xenopus</i> ..	200
Figure 6.5	<i>n1-src</i> knockdown downregulates expression of neural plate border specifier genes.....	203
Figure 6.6	<i>n1-src</i> knockdown downregulates expression of neural crest specifier genes.....	205
Figure 6.7	<i>n1-src</i> knockdown inhibits neural crest specifier gene expression...	206
Figure 6.8	Effects of <i>n1-src</i> overexpression on neural crest genes.....	208
Figure 6.9	Rescue of <i>phox2a</i> expression in <i>n1-src</i> knockdown embryos.....	210
Figure 6.10	RT-PCR analysis showing <i>n1-src</i> expression in neural crest induced animal cap explants.....	212
Figure 6.11	Detecting <i>n1-src</i> expression in the neural plate border using previously published RNA-Seq data.....	218

Chapter 7

Figure 7.1	A summary of the proposed role for n1-Src in regulating the expression of genes required for neurogenesis and neural crest development.....	228
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List of tables

Chapter 2

Table 2.1	Antisense morpholino oligo sequences.....	47
Table 2.2	Primers used to amplify <i>X. tropicalis</i> cDNA.....	51
Table 2.3	Primers used to amplify <i>X. laevis</i> cDNA.....	52
Table 2.4	Clone information, restriction enzyme and polymerases used to transcribe in situ probes and synthetic mRNA transcripts.....	52
Table 2.5	Primary and secondary antibody dilutions for protein detection by western blotting.....	55

Chapter 4

Table 4.1	Transcripts per million values for <i>n1-src</i> and <i>tubb2b</i>	106
Table 4.2	Transcripts per million values for <i>src</i> transcripts.....	107
Table 4.3	Transcripts per million values for <i>c-src</i> and <i>n1-src</i> using a de novo transcriptome.....	108
Table 4.4	Significantly downregulated transcripts involved in nervous system development.....	115
Table 4.5	Top 20 biological processes, based on fold enrichment, overrepresented within the significantly downregulated transcripts.....	121
Table 4.6	Significantly downregulated transcripts involved in the top 20 overrepresented biological processes (based on fold enrichment)..	122
Table 4.7	Biological processes related to neural crest development that are overrepresented within the significantly downregulated transcript list.....	123
Table 4.8	Significantly downregulated transcripts involved in the three additional neural crest processes.....	123
Table 4.9	Significantly upregulated transcripts involved in RNA splicing.....	126
Table 4.10	Significantly upregulated transcripts involved in biological processes associated with RNA processing and mRNA splicing.....	129
Table 4.11	Gene sets significantly enriched in the control samples.....	131

Chapter 5

Table 5.1	RNA processing genes identified by DEXSeq to be alternatively spliced in <i>n1-src</i> knockdown embryos.....	153
Table 5.2	Genes identified by IsoformSwitchAnalyzeR to have significant changes in isoform usage following <i>n1-src</i> knockdown.....	164
Table 5.3	The type of alternative splicing event occurring and the genome coordinates of each event as identified by AStalavista.....	178
Table 5.4	The RNA binding proteins, their relevant enriched binding motif and the transcripts in which the motifs are located.....	182

List of accompanying material

Supplementary File 1 – Panther Downregulated

Excel spreadsheet that lists the PANTHER biological processes significantly overrepresented within the significantly downregulated transcripts from the transcriptomics experiment. The transcripts associated with the top 20 significant processes and the top 20 most enriched processes are listed in this file.

Supplementary File 2 – Panther Upregulated Top 20 Significant

Excel spreadsheet that lists the PANTHER biological processes significantly overrepresented within the significantly upregulated transcripts from the transcriptomics experiment. The transcripts associated with the top 20 significant processes are listed in this file.

Supplementary File 3 – Panther Upregulated Top 20 Fold Enrichment

Excel spreadsheet that lists the PANTHER biological processes significantly overrepresented within the significantly upregulated transcripts from the transcriptomics experiment. The transcripts associated with the top 20 most enriched processes are listed in this file.

Supplementary File 4 – DEXSeq Panther Analysis

Excel spreadsheet that lists the biological processes identified by PANTHER to be overrepresented amongst the transcripts that DEXSeq identified as showing differential exon usage. The transcripts associated with the top 20 most enriched processes are listed in this file.

Supplementary File 5 – RNA-Seq Dataset

Excel spreadsheet with the transcripts per million data from the Illumina RNA-Seq experiment.

Supplementary Video 1 – Uninjected Embryo Early Tailbud

Touch response of an uninjected control *X. tropicalis* embryo at the early tailbud stage.

Supplementary Video 2 – Control Morpholino Embryo Early Tailbud

Touch response of a control morpholino *X. tropicalis* embryo at the early tailbud stage.

Supplementary Video 3 – *n1-src* AMOs Embryo Early Tailbud

Touch response of an early tailbud stage *X. tropicalis* embryo injected with the *n1-src* splice blocking morpholinos.

Supplementary Video 4 – Uninjected Embryo Late Tailbud

Touch response of an uninjected control *X. tropicalis* embryo at the late tailbud stage.

Supplementary Video 5 – Control Morpholino Embryo Late Tailbud

Touch response of a control morpholino *X. tropicalis* embryo at the late tailbud stage.

Supplementary Video 6 – *n1-src* AMOs Embryo Late Tailbud

Touch response of an late tailbud stage *X. tropicalis* embryo injected with the *n1-src* splice blocking morpholinos.

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Declaration

I declare that this thesis is a presentation of original work and I am the sole author.

Some data from Chapter 3 of this thesis has been published in the following paper:

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All sources are acknowledged as references.

1. Introduction

1.1. Phosphorylation

Intracellular signalling cascades control a range of cellular processes in response to external stimuli, and nearly all the major signalling cascades require phosphorylation of member proteins for full signal transduction. In fact, nearly all processes required for cell survival, such as metabolism, transcription and cell-cycle progression require protein phosphorylation (Johnson, 2009). Phosphorylation is a post-translational, reversible modification in which a protein kinase catalyses the addition of the γ -phosphate from nucleotide triphosphates to specific target residues (Hunter, 1995; Vulpetti and Bosotti, 2004). Protein kinase function is coordinated with that of protein phosphatases, whose role it is to catalyse the removal of the γ -phosphate from the target protein. This transient, reversible nature of protein phosphorylation - mediated by kinases and phosphatases - is an important aspect of cell signalling pathway control, enabling pathways to be activated and deactivated with relative ease.

The importance of protein phosphorylation in eukaryotic life can be seen by examining the human genome which is thought to encode for over 500 protein kinases, responsible for the phosphorylation of nearly one third of cellular proteins (Sefton, 2001; Roskoski Jr, 2015). Furthermore, protein phosphorylation is seen throughout bacterial and archaeal proteomes and much like in eukaryotes, these phosphorylation events have been found to regulate processes like cell division and gene transcription (Getz *et al.*, 2019).

Phosphorylation events were originally observed solely on serine and threonine residues, and it is these residues where most events do occur, with 86.4% and 11.8% of these residues phosphorylated respectively in humans (Ardito *et al.*, 2017). A third amino acid has also been identified as a target for phosphorylation – tyrosine (Eckhart, Hutchinson and Hunter, 1979). Compared to serine and threonine residues, only 1.8% of tyrosine residues are phosphorylated (Ardito *et al.*, 2017). Tyrosine kinase regulated pathways control processes like cell proliferation and differentiation and thus tyrosine kinases have been strongly implicated in cancers (Paul and Mukhopadhyay, 2004). Tyrosine kinase activity is normally tightly regulated, however, mutations in tyrosine kinases can lead to uncontrolled pathway signalling, unrestricted cellular proliferation and malignancy.

Tyrosine kinases can be broken down into two families, receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs).

1.1.1. Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors, which upon activation, facilitate the cells response to extracellular stimuli (Ullrich and Schlessinger, 1990). 64% of tyrosine kinases identified in the human genome are RTKs, and these are classed into 20 subfamilies (Robinson, Wu and Lin, 2000).

All RTKs, from all 20 subfamilies, have the same general structure, consisting of an extracellular glycosylated ligand-binding domain, a single transmembrane helix, and a cytoplasmic protein tyrosine kinase domain (Schlessinger, 1988). RTKs typically, though not always, exist as monomers that dimerise and activate upon extracellular ligand binding. The cytoplasmic tyrosine kinase domain of each monomer then trans-phosphorylates the cytoplasmic domain of the other. Phosphorylation of RTKs in this manner creates recruitment sites for downstream signalling proteins, with both SH2 domain and Phosphotyrosine Binding (PTB) domain proteins capable of binding (Du and Lovly, 2018). These phosphotyrosine binding proteins function to recruit additional SH2 domain containing proteins, and in turn activate a wide range of intracellular signalling pathways such as the Ras/MAP Kinase or the Phosphoinositide 3-Kinase signalling cascades (Schlessinger, 2000).

1.1.2. Non-receptor tyrosine kinases

The remaining 36% of tyrosine kinases are non-receptor tyrosine kinases (nRTKs), most of which can be divided into 9 subfamilies: Abl, Fes, Syk, Jak, Tec, Ack, Fak, Csk and Src (Neet and Hunter, 1996). nRTKs lack the external and transmembrane domains found in RTKs, and instead of spanning cellular membranes the majority are cytoplasmic and work in the transduction of signals from extracellular receptors through the cell (Siveen *et al.*, 2018; Solouki, August and Huang, 2019). In doing so, nRTKs regulate several cellular functions, being important for cell survival, growth and differentiation.

1.2. Src family kinases

One of the most important nRTK families is the Src family of protein tyrosine kinases (SFKs). This family consists of nine proteins; c-Src, Yes, Fyn, Fgr, Lck, Hck, Blk, Lyn and Yrk. These proteins can be grouped into 2 classes, those ubiquitously expressed in mammals (c-Src, Yes and Fyn), and those with more tissue-restricted expression patterns (Thomas and Brugge, 1997).

The Src family of kinases is named after its first discovered member, c-Src. The Rous Sarcoma Virus (RSV) is a retrovirus, which infects chicken cells and can transform them into cancerous sarcoma cells. The 3' end of the viral RNA was identified as the region containing the gene coding for the viruses transforming protein (Wang *et al.*, 1976). This gene was called viral src (*v-src*). Further analysis identified DNA related to *v-src* in the genome of uninfected chickens, and indeed in a whole host of other vertebrates including humans and mice (Stehelin *et al.*, 1976; Spector, Varmus and Bishop, 1978). This DNA encodes for the proto-oncogene, *src*, which is believed was incorporated into the genome of Rous sarcoma virus, where it eventually became oncogenic (Stehelin *et al.*, 1976). Later in 1979, using antisera directed against the v-Src protein, Opperman *et al.* were able to isolate the c-Src protein from uninfected chicken, rat and human cells.

Since then c-Src has become the focus of much research into protein kinases and their function. In fact, in 1979, the first tyrosine phosphorylation event observed by Eckhart *et al.* was the result of c-Src activity. Alongside this, c-Src has been implicated in a variety of human cancers, where its elevated expression and activity levels drive the growth and progression of tumours (Irby and Yeatman, 2000).

C-Src is grouped with its other family members based on their conserved structure, which comprises a succession of Src Homology (SH) domains (Figure 1.1).

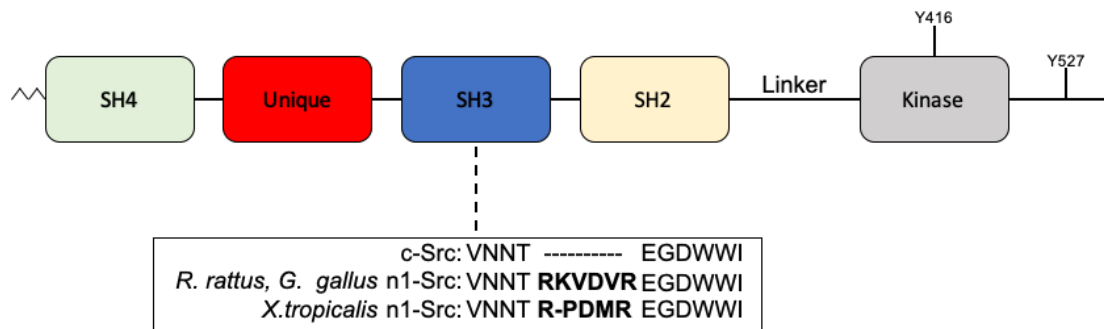


Figure 1.1. Src family kinase domain structure. Src family kinase members share a common domain structure. At the N-terminus is the myristoylated membrane targeting SH4 domain. The unique domain is a variable domain that contains an intrinsically disordered region and can be post-translationally modified. The SH3 domain binds proline-rich substrates, whilst the SH2 domain is a phosphotyrosine-binding domain. At the C-terminus is the kinase domain and two regulatory tyrosine residues. Phosphorylation of Y416 is an activating modification, whilst phosphorylation of Y527 in the C-terminus tail is an inactivating modification. Amino acid positions refer to the chicken Src protein. The SH3 domain is the site in Src where the neural microexon is included. In *X. tropicalis* this is a 15bp microexon that translates to a 5 amino acid insert, whilst mammals have a 6 amino acid insert. Amino acid positions refer to the chicken Src protein.

1.2.1. The SH4 domain and membrane localisation

Src family kinases may localise to the inside of the plasma membrane through their N-terminal SH4 domain (Courtneidge, Levinson and Bishop, 1980; Kaplan, Varmus and Bishop, 1990). The N-terminus of c-Src, and its viral counterpart, is the site of myristoylation (Buss, Kamps and Sefton, 1984). This non-reversible modification, involving the attachment of a myristoyl group to the target protein, is important in its membrane localisation. Analysis following fractionation of infected cells showed that 82% of wild-type v-Src was bound to membranes (Buss *et al.*, 1986). In contrast, less than 12% of myristic acid lacking v-Src mutants were found bound to membranes. Although appearing important for membrane attachment, myristoylation itself is insufficient since cell fractionation found soluble myristoylated v-Src alongside 15 other myristoylated proteins (Buss, Kamps and Sefton, 1984).

Mutational analysis initially identified amino acids 1-14 as the region within the N-terminal half of src that is required for myristoylation (Cross *et al.*, 1984). Of these 14 amino acids, glycine-2 and lysine-7 have been identified as critical determinants of SFK myristoylation. Myristoylation of cellular proteins generally occurs at the N-terminal glycine residue (Udenwobe *et al.*, 2017). In the case of Src this is via an amide linkage to glycine-2 (Buss and Sefton, 1985; Schultz *et al.*, 1985). Whilst not the site of myristoylation, lysine-

7 is an important factor in myristoylation of glycine-2, with v-Src mutants showing near complete abolition (Kaplan *et al.*, 1988).

Further post-translational modifications of the SH4 domain contributes to the membrane localisation of most SFKs. Multiple SFK members are palmitoylated on cysteine-3 in their N-terminal SH4 domain (Koegl *et al.*, 1994; Shenoy-Scaria *et al.*, 1994). Palmitoylation is a reversible modification important for enhancing the trafficking and membrane association of proteins, including SFK members. (Sato *et al.*, 2009; Guan and Fierke, 2011). Interestingly, whilst most SFK members are palmitoylated, Src itself is not (Shenoy-Scaria *et al.*, 1994). A cluster of basic residues - three alternating lysine residues at positions 5, 7 and 9 and three arginines at positions 14, 15 and 16 - in Src act alongside the myristoyl motif to target the protein to the plasma membrane, via electrostatic interactions with acidic phospholipids of membranes. (Kim *et al.*, 1991; Silverman, Sudol and Resh, 1993; Resh, 1994).

The SH4 domain mediated interaction of SFKs with the internal surface of the plasma membrane is an important factor in their correct function. V-Src mutants which have had their glycine-2 substituted show decreased membrane association, and these non-membrane bound mutants lack the transforming ability of their wild-type relatives (Kamps, Buss and Sefton, 1986).

1.2.2. The unique domain

The unique domain of SFKs is a 50-90 base pair intrinsically disordered region, named due to the lack of sequence similarity between the different SFK members (Amata, Maffei and Pons, 2014). The SFKs however do show conservation of their unique domains between different species.

Despite being an intrinsically disordered region, the unique domain of c-Src has a partially structured region called the unique lipid binding region (ULBR). The ULBR can bind acidic lipids within the plasma membrane, aiding the SH4 domain in anchoring c-src to the membrane (Pérez *et al.*, 2009). The unique domain is also post translationally modified, with Ser-37 and Thr-75 both being phosphorylated by p34cdc2 (Shenoy *et al.*, 1992; Pérez *et al.*, 2013). These phosphorylation events disrupt the affinity of the ULBR with the plasma membrane, and coincide with a decrease in the phosphorylation of Src Tyr-527, an inhibitory phosphorylation event, and an increase in Src activity. (Shenoy *et al.*, 1992; Pérez *et al.*, 2013). This repulsion and activation of Src could lead to Src

phosphorylation of cytosolic targets required for cell cycle progression and cell proliferation. Phosphorylation of the unique domain could therefore be a method of regulating Src activity.

NMR analysis identified that the ULBR can also form an intramolecular interaction with the Src SH3 domain (Pérez *et al.*, 2013). The exact function of this interaction is unknown; however, it was postulated that this could be a mechanism to help regulate the proteins activity.

The Src unique domain can therefore bind both acidic lipids and the Src SH3 domain, as well as being a target of post translational phosphorylation, and these appear to be methods to help regulate Src kinase activity.

1.2.3. The SH3 domain and peptide binding

The SH3 domain of SFKs plays a role in substrate binding and in regulating SFK kinase activity. This domain is approximately 60 amino acids and has been found widely throughout nature, from amoeba to humans (Mayer and Eck, 1995). The SH3 domain of Src consists of two three-stranded antiparallel β -sheets connected by the distal, RT and n-Src loops. The β -sheets are packed at right angles to one another and the interface between them forms a hydrophobic core that acts as the domain's binding site (Yu *et al.*, 1992). In the core are two distinct xP dipeptide-binding pockets (Mayer and Eck, 1995). Adjacent to the core binding domain is a negatively charged third pocket - the "specificity pocket" - formed by the RT and n-Src loops (Feng *et al.*, 1995)

The SH3 domain recognises and binds proline rich motifs with a conserved PxxP sequence (Ren *et al.*, 1993). These proline rich ligands adopt a polyproline-2 helix, which is an extended left-handed helical structure (Mayer, 2001). There are two classes of ligands identified containing this SH3 binding motif, class 1 with the consensus sequences K/RxxPxxP and class 2 with the consensus sequence PxxPxK/R (Zarrinpar, Bhattacharyya and Lim, 2003). These sequences bind the SH3 domain in opposite orientations but in the same manner, with each xP dipeptide recognising a dipeptide-binding pocket in the hydrophobic core (Kurochkina and Guha, 2013). The adjacent "specificity pocket" interacts with the positively charged lysine or arginine residue near the PxxP motif (Saksela and Permi, 2012). Whilst much of the early work investigating SH3 substrate binding focused around substrates containing a characteristic PxxP motif, more recent work has indicated that this is not always the case and that the SH3 domain binds a variety of non-canonical

targets (Teyra *et al.*, 2017). Teyra *et al.* used a high-throughput peptide-phage display and deep sequencing to identify that nearly half of 115 human SH3 domains are capable of binding peptides in a non-canonical manner. These non-canonical ligands typically make more extensive contacts with the SH3 domain than canonical ligands, and in doing so can bind with a much greater affinity (Saksela and Permi, 2012).

When not interacting with a substrate, the SH3 domain interacts intramolecularly with other regions of the kinase, in particular the linker between the SH2 domain and the kinase domain (Sicheri, Moarefi and Kuriyan, 1997; Williams *et al.*, 1997; Xu, Harrison and Eck, 1997). This interaction assists the stabilisation of SFKs in their inactive closed states (see 1.2.7 for more information).

Examination of the crystal structures of SFKs revealed that the SH2-kinase linker adopts a polyproline-2 helical conformation, like the SH3 domain substrates (Alvarado *et al.*, 2010). Though the linker adopts a conformation resembling SH3 substrates, the binding to the SH3 domain is by no means as strong, with suboptimal residues located in key positions (Moroco *et al.*, 2014). This weaker interaction allows for the displacement of the linker from the SH3 domain by SFK substrates.

The regulatory interaction between SH3 domains and the SH2-kinase linker is seen in other kinase families. C-Abl is a member of the Abl family of tyrosine kinases, whose structure is similar to that of SFKs, with an SH3 domain, an SH2 domain and then a short linker connecting the catalytic domain (Greuber *et al.*, 2013). Similar to SFKs, the linker forms a polyproline helix, thus enabling interaction with the SH3 domain (Nagar *et al.*, 2006).

In addition to interacting with proline-rich ligands, *in vitro* NMR work identified that the SH3 domain of c-Src can interact with acidic lipids (Pérez *et al.*, 2013). This interaction occurs with residues located in the conserved RT and n-Src loops, on the opposite side of the domain to the PxxP interaction. Allosteric interference caused by the binding of proline-rich ligands to the opposite side of the domain abolishes these interactions (Maffei *et al.*, 2015).

1.2.4. The Src SH2 domain and phosphotyrosine binding

The SH2 domain is a phosphotyrosine-binding domain, approximately 100 amino acids in size, that mediates cellular localisation, substrate recruitment and the regulation of src kinase activity (Filippakopoulos, Müller and Knapp, 2009).

X-Ray crystallography of the v-Src SH2 domain revealed the domain consists of an antiparallel beta sheet - which can be subdivided into two connected beta sheets - flanked by two alpha helices (Waksman *et al.*, 1992). The domain contains two binding clefts; a phosphotyrosine binding pocket and a hydrophobic binding pocket which helps confer specificity for SH2 binding peptides (Pawson and Gish, 1992; Eck, Shoelson and Harrison, 1993).

Synthetic phosphopeptide libraries were screened to determine the optimal sequence around the phosphotyrosine for Src SH2 domain binding and identified preferential selection for peptides with pTyr-Glu-Glu-Ile motifs (Songyang *et al.*, 1993). Peptides that bind to the SH2 domain with high affinity interact with both binding pockets, the phosphotyrosine interacts with its pocket and the isoleucine at position +3 binds to the hydrophobic pocket. High affinity peptides anchor in both pockets and when doing so interactions also occur between the surface of the SH2 domain and the residues at positions +1 and +2. In contrast, low affinity peptides only interact the phosphotyrosine pocket and in these cases the peptide main chain fails to bind to the domain surface (Eck, Shoelson and Harrison, 1993).

Within the phosphotyrosine binding pocket are three positively charged residues - R155, R175 and K203 - that are important for binding of the phosphorylated peptide (Waksman *et al.*, 1992). R155 and K203 both form single hydrogen bonds with the phosphotyrosine ring whilst R175 forms two bonds with the phosphate group. This dual interaction between R175 and the phosphate group is key for binding and its mutation results in a near complete loss of SH2 binding (Bibbins, Boeuf and Varmus, 1993). The corresponding residue in the SH2 domain of c-Abl is R171 and mutation of this abolished phosphotyrosine binding (Mayer *et al.*, 1992).

Like the SH3 domain, the SH2 domain of SFKs can interact intramolecularly with other regions of the kinase. Within the C-terminus is a tyrosine residue (Tyr-527 in chicken c-Src), that when phosphorylated, binds to the SH2 domain (Sicheri, Moarefi and Kuriyan, 1997). The phosphorylation of Tyr527 in Src, or the equivalent tyrosine residue in other SFKs, is a key factor in the inhibition of kinase activity. The interaction between pTyr-527

and the SH2 domain, along with the SH3-kinase linker interaction, holds the kinase in a closed and inactive conformation (Liu *et al.*, 1993).

1.2.5. SH1/kinase domain

The kinase domain is the catalytic domain of the Src protein, responsible for protein phosphorylation. This domain is made up of two lobes; a small N-terminal consisting of five β -strands and a single α -helix, and a larger C-terminal lobe that is predominantly α -helical, with a single β -sheet (Boggon and Eck, 2004). The two lobes are separated by an ATP and substrate binding cleft (Ozkirimli and Post, 2006). The N-terminal lobe is predominantly involved in the anchoring of ATP, whilst the C-terminal lobe is primarily responsible for binding the substrate to be phosphorylated (Roskoski Jr, 2004).

Within the N-lobe are a couple of conserved motifs important for Src kinase activity through their interactions with ATP. The first is the conserved Lys-295 which binds to ATP, and the other is the Glycine-rich loop that functions to position the gamma phosphate of ATP for catalysis (Taylor and Kornev, 2011).

The C-terminal lobe contains the activation, or A loop, that is critical in regulating the activity of Src (Boggon and Eck, 2004). The phospho-status of one residue, Tyr-416, within the A loop has been identified to be a key factor in kinase activity. Phosphorylation of this tyrosine was first observed by Smart *et al.* in 1981 in Srcs viral counterpart. Mutational analysis identified that pTyr-416 is, unlike pTyr-527, an activating post translational modification (Kmiecik and Shalloway, 1987). Mutation of this residue to phenylalanine eliminates the activity of c-Src and suppresses the increased activity seen when Tyr-527 is mutated.

The increase in catalytic activity seen by Tyr-416 phosphorylation is nearly four-fold and its phosphorylation stabilises the kinase domain in its catalytically competent form (Meng and Roux, 2014; Boczek *et al.*, 2019).

The kinase activity of c-Src is tightly regulated and most the time Src is inactive and Tyr-416 remains unphosphorylated. In this state, the activation loop forms two short α -helices positioned between the two lobes (Xu *et al.*, 1999). This conformation buries Tyr-416 to prevent its phosphorylation and works to block substrates from entering the binding cleft. When Src is in an active conformation the A-loop forms a more open and extended structure, freeing up the cleft to permit substrate binding (Ozkirimli and Post, 2006).

Once activated, Src can cycle between an open and closed conformation to catalyse the phosphorylation of the target substrate. Its open active form allows the binding of Mg^{2+} -ATP and the target substrates, and once bound, the domain closure causes the catalytic transference of the gamma phosphate from ATP to the tyrosine target on the substrate (Roskoski Jr, 2015). Once transference is complete the domain reopens, releasing ADP and the phosphorylated substrate from the binding cleft.

Interestingly, analysis of SH2 domain containing protein tyrosine kinases revealed a preference for substrates with either isoleucine or valine at the -1 position, and a glutamic acid or asparagine at the +1 position, in relation to the tyrosine phosphorylation target (Songyang and Cantley, 1995). In addition to these residues, tyrosine kinases prefer peptide substrates with a large hydrophobic amino acid at the +3 position. Therefore, one of the potential motifs for SFK tyrosine phosphorylation is Ile/Val-Tyr-Glu-Glu-Ile, which once the tyrosine is phosphorylated is the target motif for the SH2 domain discussed earlier. Indeed, SFKs and other cytosolic tyrosine kinases do selectively phosphorylate peptides recognised by their own SH2 domains (Songyang *et al.*, 1995). The phosphorylation of a substrate's optimal tyrosine and subsequent binding by the SH2 domain could potentially be enhancing subsequent phosphorylation on multiple non-optimal sites.

1.2.6. C-terminal domain

The carboxy-terminus of c-Src contains Tyr-527. This region and this amino acid in particular are important for regulating src activity. Replacement of the c-Src carboxy-terminus sequence with the sequence from v-Src, which lacks this residue, is sufficient for c-Src to acquire the same transforming ability seen in its viral counterpart (Cooper *et al.*, 1986). Furthermore, when comparing levels of terminal tail phosphorylation in an activated population c-Src, with the levels in an inactive population, the phosphate levels on Tyr-527 in the activated group is less than 1/20th the level in the inactive population. The tail of c-Src therefore plays an important role in suppressing the proteins transforming and protein kinase ability. This is supported by work carried out by Kmiecik and Shalloway (1986) who generated a Tyr-Phe 527 mutant c-Src which had strongly activated transforming and kinase activities compared to wild-type.

With such an important role in controlling SFKs activity, this region is highly regulated. This most prominently occurs through phosphorylation by c-Src kinase (CSK), a protein-

tyrosine kinase that specifically phosphorylates negative regulatory sites on c-Src and other SFKS (Nada *et al.*, 1991; Bergman *et al.*, 1992).

1.2.7. Src regulation

Following identification of the viral Src counterpart as the transforming agent in the avian Rous Sarcoma Virus that is responsible for the development of sarcoma tumours, much work has been carried out to identify potential roles for c-Src in human cancer development. Indeed, a variety of cancers show overexpression or aberrant activity of c-Src, which helps drive the proliferation and metastasis of the tumours (Summy and Gallick, 2003). For this reason, Src activity is tightly regulated, and is predominantly found in its inactive form. This inactive conformation involves multiple intramolecular interactions that locks the kinase domain in an inactive conformation, the first being the interaction between the phosphorylated Tyr-527 and the SH2 domain, and the second between the SH3 domain and the SH2-kinase linker (Figure 1.2).

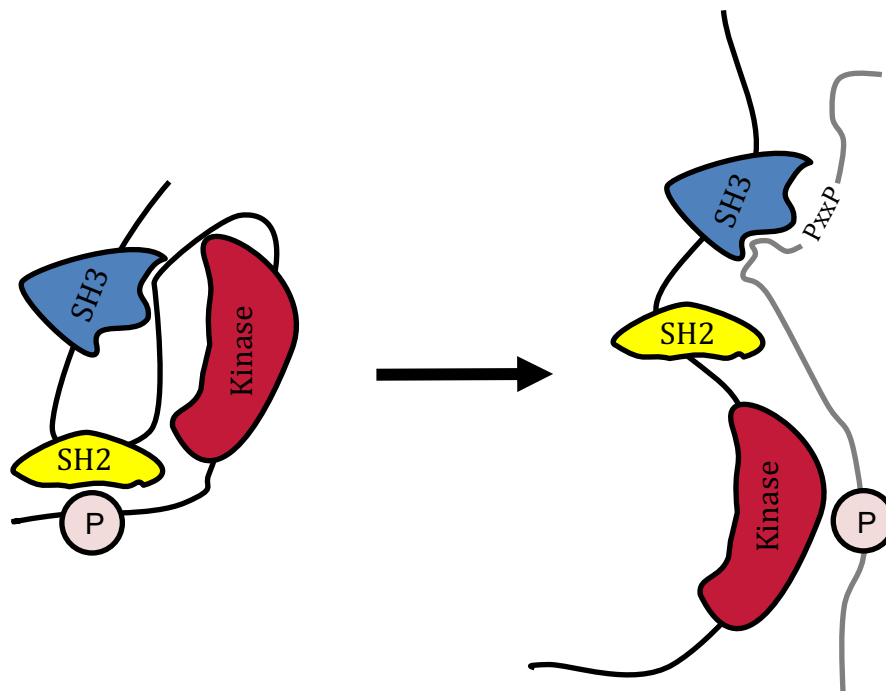


Figure 1.2. Regulation of Src activity. C-Src activity is tightly regulated and as such the majority of the time it is in closed and inactivated conformation. Y527 in the C-terminal tail is phosphorylated, created a phosphotyrosine binding site for the SH2 domain. Whilst this SH2 domain is bound to the phosphorylated tyrosine, the SH3 domain binds to the SH2:kinase linker which resembles a proline-rich ligand. This keeps the kinase domain in an inactivated conformation. However, these intramolecular are suboptimal and they can be displaced by a Src ligand. These ligands can either bind to the SH3 or SH2 domains and partially activate Src. Complete activation occurs when Y527 is dephosphorylated and Y416 within the kinase domain is phosphorylated. Once activated the kinase domain phosphorylates target proteins on specific tyrosine residues.

When active Src is required, these intramolecular interactions will be broken as the protein adopts an open and active conformation. It is important then that the intramolecular interactions occurring here are suboptimal, for example, the SH2-kinase linker lacks the optimal PxxP motif found in SH3 binding substrates (Boggon and Eck, 2004). This suboptimal binding means that when a substrate with a superior SH3 or SH2 binding sequence is in close proximity to Src, the closed conformation is easily undone.

The opening up and activation of Src is a multistep process that can occur in two main ways. The first method begins with the displacement of the intramolecular interactions by substrate proteins, followed by conformational changes in the protein and the opening of the kinase domain (Xu *et al.*, 1999). Displacement of the SH3 or SH2 domains leads to autophosphorylation of Tyr-416 in the kinase domain and full activity is achieved through the dephosphorylation of Tyr-527 in the C-terminal region (Lerner and Smithgall, 2002; Moroco *et al.*, 2014). The other method begins with the dephosphorylation of Tyr-527 by tyrosine phosphatases, and leads to the opening up of the kinase to form a dock for substrates to bind.

1.3. Roles of c-Src

SFKs are involved in the regulation of four main cellular functions: proliferation, adhesion, differentiation, and movement, through the interaction with numerous intracellular signalling pathways (Parsons and Parsons, 2004). They do this through the interaction with cellular receptors and connecting them to the signalling machinery of the cytoplasm.

An example of this is in the migration of cells, which requires complex signalling interactions between the cell and the extracellular matrix (ECM). Integrins are cell surface receptors that recognise extracellular matrix molecules and their activation leads to the phosphorylation of focal adhesion kinase (FAK), an intracellular non-receptor tyrosine kinase (Guan, 1997). FAK autophosphorylates Tyr-397 in response to the integrin-ECM interaction and this generates a high affinity binding site for the SH2 domain of Src, leading to the disruption of the SH2-mediated Src inhibition and the formation of a stable complex between FAK and Src (Schaller *et al.*, 1994) (Figure 1.3). The activated Src is then able to further phosphorylate FAK on various tyrosine residues (Calalb, Polte and Hanks, 1995). This activated FAK/Src complex then activates a host of downstream signalling pathways such the mitogen-activated protein kinase (MAPK)/Erk pathway (Dwyer and Gelman, 2014). Inhibition of *erk* expression or activity leads to the loss of cell

migration, whilst normally Erk can phosphorylate myosin light chain kinase, which subsequently phosphorylates myosin light chain (Klemke *et al.*, 1997). This phosphorylated myosin light chain then regulates actin-myosin motor function and in turn, cell movement.

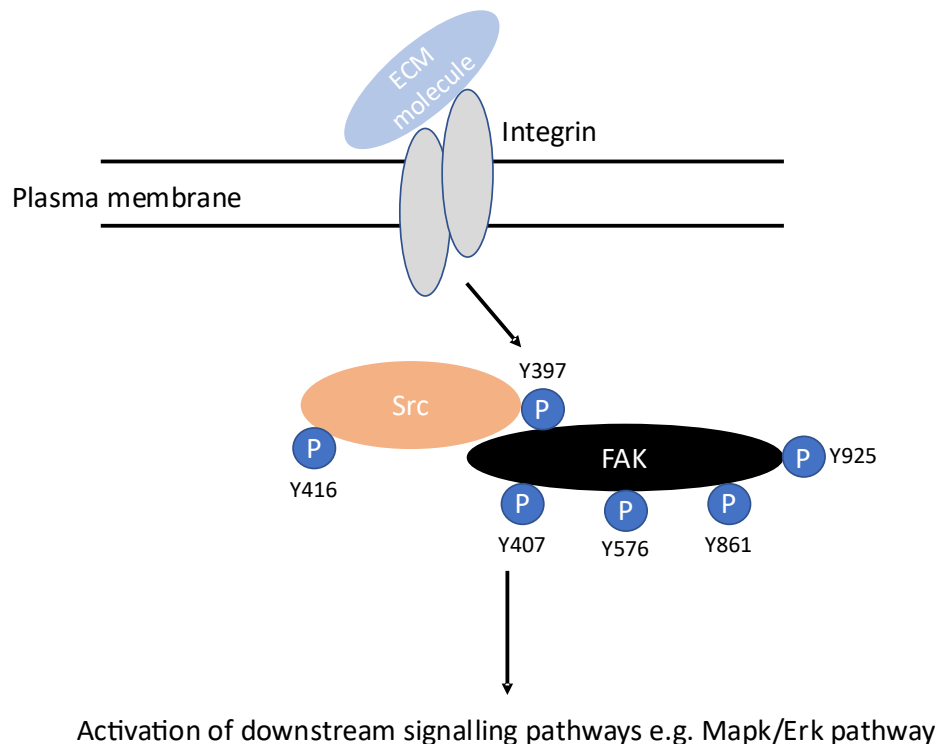


Figure 1.3. Src mediates integrin signalling via the phosphorylation of FAK and the activation of downstream signalling pathways. Extracellular matrix (ECM) molecules bind to and activate integrin receptors on the plasma membrane. Activation of integrin receptors leads to the autophosphorylation of focal adhesion kinase (FAK) on Y397. This acts as a dock for the phosphotyrosine binding SH2 domain of Src. Activated Src is phosphorylated on Y416 and then proceeds to further phosphorylate other FAK tyrosine residues. The activated FAK/Src complex then activates downstream signalling pathways such as the Mapk/Erk pathway.

In addition to transducing signals from integrins, Src also connects activated growth factor receptors, such as the epidermal or fibroblast growth factor receptors, to the MAPK, Phosphatidylinositol 3-kinase/Protein kinase B (PI3K/Akt), or Signal transducer and activator of transcription 3 (STAT3) signalling pathways (Frazer, Irvine and McMullin, 2007). Src is able to positively or negatively regulate signalling pathway activity, for example it is involved in the phosphorylation of the MAPK pathway member, RAF proto-oncogene serine/threonine-protein kinase (Raf1) which upregulates Erk activity, but also interacts with fibroblast growth factor receptor substrate 2 (FRS2) in order to

phosphorylate and activate Sprouty, which attenuates signalling by the Erk pathway (Li *et al.*, 2004; Sandilands *et al.*, 2007).

1.3.1. Roles of c-Src in the nervous system

Src expression is enriched in platelets, osteoclasts and neurons (Brown and Cooper, 1996). Interestingly, despite having enriched neuronal expression, the only noticeable phenotype in *src* knock out mice is osteopetrosis (Soriano *et al.*, 1991). The absence of any neuronal phenotype could be due to the redundant nature of c-Src and Fyn, as *fyn* knockout mice are viable, but the *Src/Fyn* double mutants are lethal (Stein *et al.*, 1994; Morse *et al.*, 1998). Examination of the double mutants revealed that they had neuronal defects.

Within the nervous system, c-Src has been shown to be involved in axon extension and dendrite morphogenesis. Axon extension is directed by the growth cone at the tip of the axon, and the environmental cues that the growth cone receives (Dent, Gupton and Gertler, 2011). The growth cone is a highly dynamic structure whose shape is moderated by the interactions between microtubule bundles and actin filaments, leading to the generation of filopodial and lamellipodial extensions (Geraldo and Gordon-Weeks, 2009).

Src expression has been detected in the axonal growth cones, and its activity is required for filopodial initiation and extension (Maness and Matten, 1990; Robles, Woo and Gomez, 2005).

One major substrate for c-Src within growth cone membranes is tubulin, with the phosphorylation of both alpha- and beta-tubulin detected in rat cortical neurons (Matten *et al.*, 1990). This phosphorylation could be inhibited by the use of antibodies directed against c-Src. The reorganisation of microtubules is an essential part of neurite outgrowth (Williamson *et al.*, 1996) and this suggests the phosphorylation of tubulin by c-Src is likewise essential.

There are multiple further targets of Src phosphorylation in axonal growth cones, whose phosphorylation is required for lamellipodia and filopodia dynamics. Examples of Src targets include cortactin and N-Wasp (Park, Sutsugu and Takenawa, 2005; Tehrani *et al.*, 2007; Ren *et al.*, 2019). Cortactin promotes polymerisation and rearrangement of the cortical actin cytoskeleton whilst N-Wasp induces actin filament nucleation through activation of the Arp2/3 complex (Yamaguchi *et al.*, 2005; Ammer and Weed, 2008). N-

Wasp and cortactin are both essential for axon outgrowth. Along with a key role in regulating axon growth, c-Src has also been connected the regulation of dendrite morphology (Skupien *et al.*, 2014). Within dendrites, CD44 activity is required for Src and FAK activation, and knockdown of CD44 results in structural alterations to the Golgi, which is essential for mediating dendritic growth. Constitutively active c-Src is capable of blocking the Golgi alterations caused by CD44 knockdown.

Further evidence linking Src to neurite outgrowth can be seen when examining the differentiation of PC12 cells. Calcium induced neurite outgrowth in PC12 cells requires Src activity, and dominant-negative forms of Src inhibit neurite outgrowth (Rusanescu *et al.*, 1995). Likewise, neurite induction in PC12 cells by nerve growth factor or fibroblast growth factor was inhibited by antibodies directed against Src (Kremer *et al.*, 1991).

Axon extension is directed by the attraction and repulsion of the growth cone to various extracellular factors. One such factor is brain derived neurotrophic factor (BDNF), a chemoattractant that attracts axonal growth cones through the activation of focal adhesion kinase (FAK) and the downstream protein Cdc42, a Rho GTPase (Yuan *et al.*, 2003; Myers and Gomez, 2011). The stimulation of growth cones with BDNF leads to significant increase in the levels of active c-Src within the growth cones, and an increase in tyrosine phosphorylation levels in response to c-Src activation (Robles, Woo and Gomez, 2005; Myers and Gomez, 2011). The increase in tyrosine phosphorylation in response to BDNF treatment requires the activity of both FAK and Cdc42 as inhibition of either results in a significant reduction in the levels of tyrosine phosphorylation. Interestingly, the tyrosine phosphorylation events are localised to growing filopodia tips, however FAK is never detected at the growing tips (Myers and Gomez, 2011). This suggests that Src activation by FAK, induced by BDNF, occurs within the body of the growth cone, and the activated Src then translocates to the growing filopodia tips to phosphorylate its target proteins.

Whilst BDNF interacts with the receptor tyrosine kinase, Tropomyosin receptor kinase B (Soppet *et al.*, 1991), other axon guidance molecules interact with different receptors such as ephrin receptors, and the deleted in colorectal cancer (DCC) receptor. Activation of ephrin receptors leads to the phosphorylation of intracellular tyrosine residues, that serve as binding sites for the SH2 domain of c-Src (Zisch *et al.*, 1998). Src binds to the activated ephrin receptor and phosphorylates downstream target proteins. In retinal growth cones, ephrin mediated repulsion was inhibited following the inhibition of SFK activity, and this inhibition prevented the phosphorylation of the downstream target,

cortactin (Knöll and Dreshcher, 2004). Meanwhile DCC receptors bind the axon guidance molecule netrin, and netrin binding activates both Src and FAK, which phosphorylate the receptor (Li *et al.*, 2004). Not only does netrin stimulation activate Src, it also activates other SFKs such as Fyn. Examination of the effects of netrin stimulation on Src/Fyn/Yes triple-knockout mouse embryonic fibroblasts found that the phosphorylation of DCC receptors is inhibited without SFK activity. The inhibition of SFKs or FAK in cortical explants inhibited the attractive response of growth cones to netrin (Liu *et al.*, 2004).

Another protein involved in neurite migration is L1 cell adhesion molecule (L1-CAM) (Patzke *et al.*, 2016). Cerebellar granule neurons from *src* knockout mice display impaired neurite outgrowth when plated on surfaces coated with the cell adhesion molecule L1-CAM (Ignelzi *et al.*, 1994). The levels of L1-CAM on the cell surface is dynamic and fluctuates, and this is an important mechanism in regulating growth cone guidance. L1-CAM undergoes endocytosis from the cell surface, however, this process is inhibited if Y1176 of L1-CAM is phosphorylated (Schaefer *et al.*, 2002). *In vitro* work identified Y1176 as a target for phosphorylation by c-Src. This suggests that c-Src activity is required in the regulation of L1-CAM trafficking in neuronal growth cones, and this would lead to the impaired growth seen by Ignelzi *et al.*

There is therefore extensive evidence implicating Src in neurite outgrowth and guidance. In addition to these roles, Src has been shown to function at synapses and regulate neurotransmission. Synapses allow the passing of a signal from one neuron to another, or to a target effector cell. When an action potential travels down the axon of a neuron and reaches the axon terminal, it depolarises the membrane and this leads to the opening of voltage gated calcium channels and the influx of calcium ions into the cell (Catterall, 2011). This triggers the release of neurotransmitters into the synaptic cleft where they then bind to receptors on the postsynaptic surface, leading to the opening of receptor channels and the influx of ions into the cell (Südhof, 2012).

Prior to their release into the synaptic cleft, neurotransmitters are stored in synaptic vesicles, and in a neuroendocrine cell line, Src was found to be associated with these vesicles (Linstedt *et al.*, 1992). Immunofluorescence microscopy found that the distribution of c-Src in the axon terminal matched that of the neurotransmitter containing vesicles, and purification of synaptic vesicles from cells also pulled out c-Src, indicating a physical interaction between the two. Indeed c-Src is specifically enriched in the synaptic vesicles compared to other SFKs and was found to interact with, and phosphorylate synapsins, a family of proteins that are involved in regulating

neurotransmitter release at the synapse (Onofri *et al.*, 2007). Synapsins are involved in regulating neurotransmitter release by reversibly tethering vesicles to the cytoskeleton, thus preventing neurotransmitter release (Cesca *et al.*, 2010). c-Src interacts with synapsin-I through its SH3 and SH2 domains, and Y301 on synapsin-I is a target for phosphorylation by Src, and this phosphorylation event increases synapsin-1 binding to the synaptic vesicle and thus the tethering of the vesicle to the cytoskeleton (Onofri *et al.*, 1997; Messa *et al.*, 2010).

C-Src therefore appears to negatively regulate neurotransmitter release, and the use of PP2, a SFK inhibitor, or siRNAs targeted against *src*, enhanced the levels of neurotransmitter release confirming c-Src plays an inhibitory role in neurotransmitter release (Ohnishi *et al.*, 2001; Zhang *et al.*, 2014). Furthermore, transient overexpression of constitutively active viral Src suppresses neurotransmitter release. The synaptic vesicle protein, synaptophysin, is also a target of tyrosine phosphorylation by Src, within the synaptic cleft (Barnekow, Jahn and Scharlt, 1990). Synaptophysin function is not fully understood but it has been implicated in regulating synaptic vesicle endocytosis (Kwon and Chapman, 2011). C-Src also interacts with dynamin, a GTPase essential for endocytosis, via its SH3 domain (Foster-Barber and Bishop 1998). Src activity therefore appears to regulate the release of neurotransmitter at nerve terminals, both by sequestering synaptic vesicles to the cytoskeleton, and regulating their endocytosis.

On the other side of the synaptic cleft, c-Src interacts with a variety of postsynaptic receptors that mediate synaptic transmission, including N-methyl-D-aspartate (NMDA), Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid (AMPA) and Acetylcholine receptors (Wang and Salter 1994; Hayashi and Huganir, 2004; Wang *et al.*, 2004). NMDA and AMPA receptors are ligand gated ion channels that open upon ligand binding to their extracellular domains. The predominant neurotransmitter in the central nervous system is glutamate, and its binding to NMDA and AMPA channels is followed by an influx of Na⁺, K⁺, and Ca²⁺ into the cell (Kalia, Gingrich and Salter, 2004). NMDA and AMPA ion channels mediate the majority of fast excitatory synaptic transmission. Early studies implicated c-Src in regulating the activity of NMDA receptors and the transmission of synaptic currents. Treatment of neurons with the tyrosine kinase inhibitor, Genistein, reduces the current flow through NMDA receptors, whilst inhibiting tyrosine phosphatase activity, or applying c-Src to neurons, leads to the potentiation of NMDA currents (Wang and Salter, 1994). The c-Src mediated upregulation of NMDA currents is essential for long-term potentiation, which is the lasting enhancement of synaptic transmission required for learning and memory, and the inhibition of c-Src activity in rat hippocampus's

with the inhibitor PP2 prevents memory formation (Lu *et al.*, 1998; Bevilaqua *et al.*, 2003; Kalia, Gingrich and Salter, 2004).

NMDA receptors consist of multiple core NR1 subunits in combination with at least one type of regulatory subunits (Cull-Candy, Brickley and Farrant, 2001). The regulatory subunits include the NR2A and NR2B subunits, which are both phosphorylation targets for SFKs. Three C-terminal tyrosine residues on the NR2A subunit have been identified as phosphorylation targets for Src (Yang and Leonard, 2001). NMDA receptors are allosterically modulated by extracellular ions, including zinc ions, and the phosphorylation of the NR2A subunit by c-Src weakens the inhibitory effects that extracellular zinc has on the receptor (Zheng *et al.*, 1998). The NR2B subunit has five tyrosine phosphorylation sites located on its C-terminal cytoplasmic domain, with Y1472 identified as a key residue for Src phosphorylation (Cheung and Gurd, 2001). Lack of phosphorylation at this site has been linked with depression and cognitive defects, both symptoms experienced by survivors of traumatic brain injuries (Park *et al.*, 2013). Traumatic brain injury leads to a significant decrease in phosphorylation of this tyrosine residue, a significant decrease in the activating Y416 phosphorylation on Src, and a significant increase in the phosphorylation of Src inhibitor Y527. Mutational analysis in mice has also linked phosphorylation of this site with learning, as Y1472F mice displayed impaired learning and synaptic plasticity defects (Nakazawa *et al.*, 2006). Further examination of these mice revealed the improper localisation of the NR2B subunit to the periphery of the synapse, compared to wild-type mice where the subunit is localised to a more central synaptic region.

Src phosphorylation of the NR2A subunit therefore appears to regulate the current flow through the channel, whilst phosphorylation of NR2B is required for correct localisation. This is supported by experiments which revealed that Src could potentiate current flow through recombinant heteromeric NMDA receptors made up off the NR1 and NR2A subunits, but not the NR1 and NR2B subunits (Köhr and Seeburg, 1996). Interestingly, in contrast to NMDA receptors where c-Src promotes current flow, AMPA receptors are Src-phosphorylated on their GluR2 subunit and this phosphorylation promotes receptor endocytosis and thus inhibits the current flow (Hayashi and Huganir, 2004).

The activity of c-Src and glutamate receptor ion channels is important for normal brain functioning, but their activity has also been linked to ischemic strokes, via the over activation of the ion channels by excessive extracellular glutamate (Lewerenz and Maher, 2015). Glutamate induces c-Src activation in neuronal cells and this activation is key in

neurodegeneration (Khanna *et al.*, 2002). The induction of cerebral ischemia in mice leads to tissue necrosis due to inadequate blood supply, but the size of effected region could be reduced by around 50% in *src* knockout mice, indicating that Src contributes to stroke-induced brain damage (Paul *et al.*, 2001). The inhibition of Src activity has also been shown to preserve neurological function in rats following cerebral ischemia (Bai *et al.* 2014). Furthermore, the induction of intracerebral haemorrhages in rats leads to enhanced glucose metabolism, neuronal cell death and behavioural abnormalities (Ardizzone *et al.*, 2007). The treatment of rats with a Src inhibitor prior to haemorrhage induction significantly decreased glucose metabolism and cell death and prevented much of the behaviour abnormalities. As blocking NMDA receptors blocks glucose hypermetabolism (Ardizzone *et al.*, 2004), the authors proposed that, amongst other things, the inhibition of Src activity reduces the activity of NMDA glutamate receptors to preserve neurological function.

Therefore, whilst Src is required for directing neurite outgrowth and in synaptic transmission and normal brain functioning, its activity levels must be tightly regulated to prevent against neurodegeneration.

1.3.2. Src family kinases in early development

Alongside roles in mature animals, SFKs are critical in early developmental steps. One of the earliest stages of vertebrate development sees the generation of the three primary germ layers - the ectoderm, mesoderm and endoderm - that will eventually form all the tissues and organs of the organism and *Xenopus* embryos are often used to study these earliest stages of development. Weinstein *et al.* (1998) identified a *Xenopus* SFK they termed *laloo*, later identified in other species as Hck, that is required for the induction of mesoderm. Mesoderm induction requires the activity of fibroblast growth factor signalling via the MAPK pathway, and *laloo* interacts with this pathway to help mediate induction (Kusakabe *et al.*, 2001). Like *laloo*, other SFK members such as Fyn, have been shown to induce expression of mesodermal markers in pluripotent cell explants (Hama *et al.*, 2002). Other vertebrate models have also illustrated the role of SFKs in germ layer development. Murine embryonic stem (mES) cells are pluripotent, self-renewing cells that can differentiate into the primitive ectoderm, and this transition appears to require c-Src activity (Meyn and Smithgall, 2009). The total inhibition of SFK activity blocks the cell differentiation, however, this inhibition can be circumvented by an inhibitor-resistant c-Src mutant. Other SFK mutants were generated to overcome their inhibition, however these mutants did not drive differentiation of the mES cells into primitive ectoderm. Indeed,

knockdown of Yes activity leads to the differentiation of mES cells (Annerén, Cowan and Melton, 2004). SFK members therefore have a variety of functions during these early developmental stages and the correct balance of their activities is required for successful embryonic development.

In summary, the *src* gene is ubiquitously expressed and plays a key role in a plethora of processes including those in early development and within the mature nervous system. The simple process of phosphorylation by c-Src is able to link extracellular signals to a variety of intracellular signalling pathways to modulate cell behaviour. This simple modification can also have contrasting effects, either promoting or inhibiting processes such as synaptic transmission, depending on which target protein it phosphorylates.

1.4. Identification of neuronal Srcs

As mentioned, c-Src function is incredibly important in the nervous system. Interestingly expression of *src* is considerably enriched in the developing brain and neural tissues (Levy *et al.*, 1984). Expression levels in chicken embryos were detected to be 8- 10-fold higher in neural tissue lysates compared to lysates from other tissues and these levels were seen to fluctuate through the first two weeks of development (Cotton and Brugge, 1983). Furthermore, the expression in rat neurons and astrocytes was 15-20 times higher than in fibroblasts, with the Src kinase activity level specifically elevated more in neurons than astrocytes (Brugge *et al.*, 1985). Interestingly, the Src protein expressed in the rat neurons was seen to migrate slower on SDS-polyacrylamide gels than the protein found in fibroblasts or astrocytes, indicating that it had possibly undergone a structural modification that increases its size. This was the first identification of n1-Src.

Further investigation in mice identified this Src modification as a six-amino acid insert within the N-terminal half of the protein, and this was then mapped onto an 18-nucleotide insertion in the mRNA, around a splice junction in the *src* gene (Martinez *et al.*, 1987). At the same time, work isolating the *src* cDNA clone from a chicken brain cDNA library and comparing it with the genomic *c-src* sequence identified a similar 18 nucleotide insertion (Levy *et al.*, 1987). The 18-nucleotide insert is located between exons three and four of *c-src* and as such leads to alteration in the SH3 domain of the Src protein, with a six residue RKVDVR insert.

N1-Src expression has been detected in a wide range of vertebrates; from mice, humans and fish, all the way to lower vertebrates like the teleost fish *Xiphophorus* (Raulf,

Robertson and Scharf, 1989). Though the teleost amino acid insert sequence is slightly different, RKINCR compared to RKVDVR seen in higher vertebrates, they all have a conserved arginine at each end of the insert. These conserved arginines are present in *X. laevis*, even though in each gene the inserts are only 5 amino acids long, with the sequences RPDIR and RPDMR respectively (Collett and Steele, 1992).

Another Src variant, n2-Src, was identified in 1990 by Pyper and Bolen and unlike n1-Src, it has only been identified in mammals. The *n2-src* microexon is 33 nucleotides long and is mainly used in conjunction with the *n1* microexon to generate a product with a 17-amino acid insert.

1.4.1. Splicing of the *n1-src* microexon

N1-src expression is required at key moments in vertebrate development, with levels peaking during neuronal differentiation and neurogenesis. Inclusion of the microexon must therefore be under strict temporal control. In the early 1990s work began to study the mechanism of inclusion or exclusion of the *n1* microexon in HeLa and LA-N-5 neuroblastoma cell lines (Black, 1991). Exon usage was absent from HeLa cells but was observed in 50% of the mature *src* mRNA in LA-N-5 cells. The *n1* microexon and its flanking introns were then cloned into an expression vector, between two adenovirus exons. When this was expressed, the same ratio of exon usage was observed in both cell lines, indicating that elements that control exon usage are located within the microexon itself, or in the immediate flanking regions. Interestingly, merely increasing the length of the microexon is sufficient for it to insert in HeLa cells. It could therefore be that the exclusion of the microexon in non-neural cells is a passive process with its small size preventing spliceosome assembly, via steric hindrance, whereas the exon inclusion requires the activity of various neural specific binding proteins.

However, it was later found that other mechanisms are involved in the exclusion in non-neural cells (Chan and Black, 1995). Conserved CUCUCU elements were identified in regions flanking the *n1* exon, and introduction in HeLa cells of RNA competitors containing these regions lead to the inclusion of the *n1* exon in mature mRNA transcripts. These CUCUCU elements bind the polypyrimidine tract binding protein (PTB), which functions as a repressor of splicing by binding to elements and preventing the assembly of the spliceosome (Chan and Black, 1997; Saulière *et al.*, 2006). The alleviation of splicing repression in the competitor experiments by Chan and Black (1995) was

therefore due to PTB binding to the RNA competitor elements rather than the elements around the microexon.

Other elements outside the *n1* microexon have been identified to be involved in promoting exon usage. Mutational analysis identified a region 38-142 nucleotides downstream of the *n1-src* exon donor site that is essential for inclusion (Black, 1992). Within this region there are multiple RNA enhancer elements which bind a multiprotein complex comprising at least 6 different polypeptides, including KH-type splicing regulatory protein (KSRP), that promotes exon inclusion (Modafferi and Black, 1997). KSRP appears the most important of the proteins as it induces the assembly of the others into the complex (Min *et al.*, 1997). It is also the only one of the proteins that has noticeable differences in expression between neural and non-neural cells, with levels much higher in the former.

Also expressed in neural tissues is a neural homologue of PTB (nPTB) (Markovtsov *et al.*, 2000). This protein is highly homologous to PTB, however, it is a much weaker repressor of splicing, and in fact it promotes splicing by inducing the binding of KSRP to the downstream enhancer elements.

On top of these flanking sequences, a sequence has also been identified within the 5' half of the *n1* exon that interacts with SF2/ASF and Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) (Rooke *et al.*, 2003). *In vitro* it was observed that SF2/ASF works in conjunction with the proteins bound to the downstream enhancer elements, promoting the microexon inclusion. In contrast, hnRNPA1 was seen to repress the splicing event, and did so in a manner independent of intronic binding.

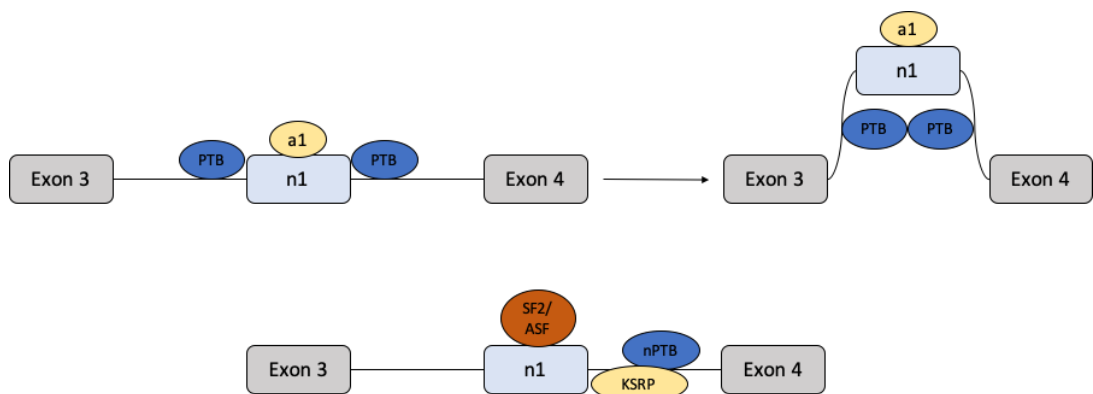


Figure 1.4. Mechanism for *n1-src* exon splicing. In non-neuronal cells, PTB binds to CUCUCU elements flanking the *n1-src* microexon. PTB inhibits exon inclusion, in part by preventing spliceosome formation. Meanwhile, hnRNPA1 binds sequences within the microexon to inhibit exon splicing. In neuronal cells, PTB is replaced by a neural homologue, nPTB, which promotes splicing by inducing KSRP binding to downstream enhancers. KSRP works in conjunction with SF2/ASF, which binds to microexon, to promote microexon inclusion.

Therefore, in non-neural cells PTB binds to repressor elements around the exon and prevents its inclusion, whilst hnRNPA1 binds to the exon itself (Figure 1.4). However, in neural cells, the neural homologue nPTB binds to these elements instead and promotes the binding of KSRP and the multiprotein complex to the enhancers to promote exon usage (Figure 1.4). Meanwhile SF2/ASF binds to the exon and potentially stimulates the binding of components of the spliceosome such as U1snRNP or U2AF.

1.4.2. Effects of neuronal microexons on the Src SH3 domain

SH3 domains function in protein: protein interactions, binding substrates with proline rich motifs. Therefore, the amino acid insertion in the Src SH3 domain could potentially be a mechanism to alter the Src substrate pool in neuronal tissues. SH3 substrates interact with three pockets in the SH3 domain, two of which bind the xP dipeptides. The third pocket is the negatively charged “specificity pocket” - formed by the RT and n-Src loops - that binds a positively charged amino acid adjacent to the proline rich motif.

Insertion of the microexon with the n-Src loop results in a whole host of c-Src substrates not being recognised by n1-Src. For example, dynamin 1 interacts with the SH3 domain of c-Src, but inclusion of the microexon abolishes this interaction (Dergai *et al.*, 2010). The positively charged arginine residues that flank the microexon interfere with the interaction. The interaction could be rescued by the substitution of the arginine at the end

of the insert with a non-polar alanine residue. A further example of this is with the microtubule-associated protein Tau which can bind to the SH3 domain of a variety of SFKs, including Fyn, c-Src, and Lck, however, the neuronal insert prevents the binding of Tau to neuronal Srcs (Reynolds *et al.*, 2008).

In other cases, the insertion of the microexon merely weakens the interaction with a specific substrate rather than ablating it. GST-SH3 binding assays using lysates from immortalised B-cell lines identified Sam68 as a substrate that binds with high affinity to the SH3 domain of c-Src, but with n1-Src this association appeared to be weak (Finan, Hall and Kellie, 1996).

The microexon insertion also affects the regulation of n-Src activity. Phosphorylation of Tyr-416 in Src is a marker of activation and Srcs experience a basal level of autophosphorylation. Enhanced levels of autophosphorylation and hence higher kinase activity levels are seen for both n1- and n2-Src compared to c-Src, with n2-Src being more active than n1 (Brugge *et al.*, 1985; Keenan *et al.*, 2015). Autophosphorylation of Tyr-416 can arise following either displacement of the SH3 domain from the SH2-kinase linker (Lerner and Smithgall, 2002). Research suggests that the elevated autophosphorylation and higher kinase activity of neuronal Srcs is due to the alterations in the n-Src loop, weakening the inhibitory intramolecular SH3:SH2-kinase linker interaction (Keenan *et al.*, 2015).

The substrate binding alterations and increased activity of n1-Src, compared to c-Src, suggest n1-Src may play unique roles in the nervous system.

1.4.3. Expression of neuronal Srcs in neuroblastomas

Since v-Src was discovered as the transforming agent behind chicken sarcomas, much work has been done to determine if its cellular counterpart could potentially be involved in human cancers. C-Src is indeed overexpressed and highly active in a variety of cancers, particularly breast and colon cancers, and is implicated in the growth of the tumour and subsequent metastasis (Irby and Yeatman, 2000). N1-Src activity has also been connected to human cancers, particularly neuroblastomas, however this relationship appears to be the opposite to that seen by c-Src in cancers.

Neuroblastoma is a childhood developmental tumour that arises from precursor cells in the sympathetic nervous system, which is itself derived from the neural crest (Ciccarone

et al., 1989; Brodeur, 2003). Neuroblastoma tumours are characterised by diverse behaviours, with some patients having aggressive, treatment resistant tumours that leave them with very poor survival chances, whilst others experience spontaneous regression of the cancer (Maris, 2010). The clinical outcome of a tumour is largely influenced by the patient age, with those diagnosed within their first 18 months having a better outcome than those diagnosed after (Schleiermacher, Janoueix-Lerosey and Delattre, 2014). Another indicator of neuroblastoma outcome is its MYCN status (Matthay *et al.*, 2016). MYCN is a transcription factor that promotes the proliferation of cells in the nervous system, and its amplification is associated with the most malignant tumours and poor survival chances.

For patients with high risk tumours, one of the main treatments currently is retinoid therapy (Reynolds *et al.*, 2003). Retinoic acid (RA) treatment has long been known to drive the differentiation of many malignant cell types, for example, treatment of the embryonic carcinoma P19 cell line induces differentiation of tumour cells into neurons and glia (Jones-Villeneuve *et al.*, 1982; Lippman, Kessler and Meyskens Jr, 1987). The RA driven differentiation of neuroblastoma cells activates multiple intracellular signalling pathways, including the PI3K/Akt and Erk1/2 pathways, both of which c-Src is known to interact with (Qiao *et al.*, 2012).

Interestingly, the RA induced differentiation of embryonic carcinoma cells is accompanied by an 8-20-fold increase in *n1-src* expression (Lynch, Brugge and Levine, 1986), and has since been associated with neuroblastoma differentiation. Four different cultured neuroblastoma cell lines, at different stages of differentiation, were assayed for the expression and activity of Src (Mellström *et al.*, 1987). The further differentiated the cell line was, and therefore the less malignant, the higher the Src kinase activity, with a 30-fold difference between the least and most differentiated. Furthermore, only the highly-differentiated cell lines expressed *n1-src*. Likewise, a study of eight different neuroblastoma cell lines identified that only those with the ability to differentiate express *n1-src* (Matsunaga *et al.*, 1993). In a similar study, levels of c-Src and n1-Src was examined in neuroblastoma cell lines, and in cases that were estimated to have a good prognosis, n1-Src was identified as the dominant form (Bjelfman *et al.*, 1990). Therefore, n1-Src appears to be an indicator of a favourable prognosis. As mentioned, the patient's age influences the outcome of the tumour, with younger patients having a more positive prognosis. In keeping with this, and a role of n1-Src in neuroblastoma differentiation, tissue samples from patients over the age of one lacked *n1-src* expression (Matsunaga *et al.*, 1991).

1.4.4. Expression and function of *n1-src* in the mature brain

The nervous system can be divided into two main parts, the CNS and the peripheral nervous system (PNS). Immunoblotting for c-Src and n1-Src in tissues of the CNS and PNS detected expression of both Src proteins in the CNS, however, n1-Src appeared absent from the PNS (le Beau, Wiestler and Walter, 1987). The authors proposed that this difference in *n1-src* expression in the CNS and PNS could suggest an important role of n1-Src in neuronal plasticity. Furthermore, they detected within the CNS, regional differences in n1-Src activity, with elevated kinase activity of n1-Src, compared to c-Src, in the forebrain and hippocampus. Kinase activities were similar between the two proteins in the cerebellum and midbrain. These tissue specific differences between c-Src and n1-Src in the CNS were also seen when examining expression levels by *in situ* hybridisation (Ross *et al.*, 1988). *N1-src* expression was elevated in the forebrain compared to *c-src*, but in the hindbrain their levels were similar, reflecting the patterns of activity seen by Le Beau *et al.*, and again the authors proposed that n1-Src could be playing a role in neuronal plasticity.

Taking the work to a cellular and subcellular level, the localisation of n1-Src within neurons of the rat CNS was investigated using an n1-Src specific antibody (Sugrue *et al.*, 1990). The protein was detected in neurons throughout the brain, but interestingly not in all neurons, and those neurons that did express the protein shared no obvious classification. At the subcellular level, immunoreactivity was detected in the cell body and dendritic processes of many n1-Src expressing neurons, along with in the axon and nerve terminals of neurons in some discrete brain regions. The widespread, rather than discrete, localisation of n1-Src in mature neurons suggests it may have multiple different functions depending on where it is situated.

One such function of n1-Src could be neurite extension or guidance. Expression of constitutively active n-Src in mice leads to impaired dendrite morphogenesis, with deformities in microtubules (Kotani *et al.* 2007). Roles for n1-Src in neurite extension are also seen in cultured *Xenopus* cells. In a *Xenopus* epithelial cell line, *n1-src* expression drives the elongation of neurite like processes, whilst knockdown of *n1-src* by shRNAs leads to reduction in neurite elongation in cultured *Xenopus* neurons (Worley, Cornel and Holt, 1997; Keenan *et al.*, 2017).

1.4.5. Expression and function of *n1-src* in the developing nervous system

More work using cultured chick retinal explants identified the presence of n1-Src in mature postmitotic neuronal cells, however it was first detected, using an n1-Src antibody,

at the onset of differentiation of the neurons (Sorge, Levy and Maness, 1984). This link between differentiation of neurons and n1-Src has been seen multiple times in rats and chickens. Immunoprecipitation experiments on neurons from rat embryonic striatal neurons identified c-Src and n1-Src (Cartwright *et al.*, 1987). C-Src was the predominant form in undifferentiated neurons, but upon differentiation of the neurons, n1-Src was detected, alongside a 14-fold increase in kinase activity. Similarly, in developing chicken cerebellums, expression of *n1-src* appeared to coincide with the differentiation of neurons, linking it with a role in differentiation rather than precursor proliferation (Fults *et al.*, 1985).

Further support for a role of Src in differentiation rather than progenitor proliferation can be seen in the work by Scharf and Barnekow (1984). They studied the enzymatic activity of c-Src during embryogenesis of fish, frogs and chickens and observed that kinase activity increased dramatically during organogenesis before decreasing in adult animals. The increase and decrease of activity during and after organogenesis points towards Src functioning more in differentiation than proliferation. Likewise, Levy *et al.* (1984) examined the expression of *src* in chicken embryonic tissues and saw a peak in expression in the trunk of the embryo during the major organogenesis period, and in another study Src activity in the striatum and hippocampus of the developing rat brain was seen to peak during neurogenesis (Cartwright *et al.*, 1988).

In embryonal carcinoma cell lines, this link between neural differentiation and n1-Src is also observed (Lynch, Brugge and Levine, 1986). These cells can be induced to differentiate into neuronlike cells following treatment with retinoic acid, and in treated cells the level of n1-Src protein was seen to increase up to 20-fold.

In summary, in adults, expression and activity of n1-Src is seen throughout the CNS, with particularly high levels relative to c-Src in the forebrain. Furthermore, expression levels of *n1-src* are higher in embryos than in adults and appear to peak around the period of differentiation and organogenesis. These studies however do not elucidate whether n1-Src is expressed and active as a result of neuronal differentiation or it is involved in actually driving differentiation, work in *Xenopus* however has shown that it is in fact the latter. *N1-src* is expressed in the neural plate of *Xenopus* embryos as a rapid response to neural induction (Collett and Steele, 1992). The neural plate is the region of embryo that houses the proliferating neural precursor cells that will differentiate to generate the primary neurons of the *Xenopus* nervous system. Knocking down of *n1-Src* by blocking splicing of the *n1* microexon using antisense morpholino oligos leads to the elimination

of the primary neurons (Lewis *et al.*, 2017). Assaying for differentiated neurons via *in situ* hybridisations for *tubb2b* revealed that the motor, inter and sensory neurons all failed to differentiate following *n1-src* knockdown. Thus n1-Src is a key factor required for the successful differentiation of neurons in this vertebrate developmental model.

1.5. This thesis

The main objective of this project was to investigate the role of the neural specific Src splice variant, n1-Src, in development of the amphibian nervous system. Chapter 3 investigates this, whilst Chapter 4 presents the transcriptional targets of n1-Src. The transcriptomic data revealed roles for n1-Src in the regulation of alternative splicing and development of the neural crest, which are investigated in Chapters 5 and 6 respectively.

The core aims of this thesis are:

- Characterise the role of n1-Src in early amphibian development by examining the effects that blocking *n1-src* splicing has on neuronal development in *X. tropicalis* embryos.
- Investigate the regulation of intracellular signalling cascades by n1-Src.
- Establish the transcripts and their associated biological and developmental processes that are significantly upregulated or downregulated in response to *n1-src* knockdown.
- Determine if there are any changes in alternative splicing during primary neurogenesis as a result of *n1-src* knockdown in *X. tropicalis* embryos and attempt to obtain a mechanistic insight into how n1-Src could be regulating alternative splicing.
- Characterise the role of n1-Src in the induction and development of the amphibian neural crest by examining the effects of *n1-src* knockdown on crest development.

2. Materials and methods

2.1. Embryological methods

All work involving animals was approved by the Biology Ethics Committee, University of York, and performed under the relevant UK Home Office legislation.

2.1.1. *X. tropicalis* fertilisation and embryo culture

Females were primed overnight with a subcutaneous injection of 10 units of Human Chorionic Gonadotropin (HCG; Chorulon). 3-4 hours prior to laying, females were induced with 100 units of HCG. Testes were dissected from males and macerated in 1ml L15+10% fetal calf serum. Eggs were fertilised on L15+10% fetal calf serum covered plates with the sperm suspension. 5 minutes after fertilisation, plates were flooded with 1/9X Modified Ringers solution (MRS/9). After 20 minutes, embryos were de-jellied in cysteine (MRS/9 + 3 % L-cysteine, pH 7.8-7.88) and cultured in MRS/9 on 1.5% agarose coated plates, between 22-25°C. 4-5 hours post fertilisation, embryos were transferred to 1/20X Modified Ringers solution (MRS/20). Embryos were staged according to Nieuwkoop and Faber (1994).

2.1.2. *X. laevis* fertilisation and embryo culture

Females were induced overnight with subcutaneous injection of 450-600 units of HCG. Eggs were fertilised with a suspension of testes macerated in distilled water. 5 minutes after fertilisation, embryos were flooded with 1/3X Normal Amphibian Medium (NAM/3) and prior to first cleavage, were de-jellied in cysteine (10% NAM salts + 2.5% L-cysteine hydrochloride monohydrate, pH7.8-7.88). Embryos were cultured at 14-20°C in NAM/3 and were transferred to 1/10X Normal Amphibian Medium (NAM/10) prior to gastrulation.

2.1.3. Microinjections

X. tropicalis embryos were microinjected bilaterally or unilaterally at the one to two cell stages with the appropriate concentration of control or *n1-src* splice blocking morpholinos, or synthetic *noggin* or *n1-src* mRNA. Sequences for the *n1-src* splice blocking morpholinos are in Table 2.1. Prior to morpholino injection, the morpholinos were heated to 65 °C for 5 minutes. During and after injection, embryos were cultured in MRS/9 + 3% Ficoll. Injected embryos were transferred into MRS/20 prior to gastrulation.

X. laevis embryos were microinjected at the two to four cell stage with the appropriate concentration of synthetic *n1-src*, *fgf4*, *dnfgr1* or *pax3-GR/zic1-GR* mRNA, whilst being cultured in NAM/3 + 5% Ficoll and were transferred to NAM/10 prior to gastrulation.

Injectons for rescue experiments involved the co-injection of the *n1-src* splice blocking morpholinos and synthetic sense *n1-src* mRNA together at the one to two cell stages in *X. tropicalis*.

When carrying out unilateral injections, the orientation of embryos was established based on their pigmentation pattern. Cortical rotation results in reduced pigmentation in the future dorsal side of the embryo. Based on this pigmentation pattern, the left and right sides of the embryo can be determined (Klein, 1987). Two and four cell embryos were all placed in the same orientation and the same hemisphere was then injected in each embryo.

Table 2.1. Antisense morpholino oligo sequences. Antisense morpholinos were designed by Gene Tools LLC (<https://www.gene-tools.com/>) to knockdown *n1-src* expression in *X. tropicalis*. AMO A refers to the splice acceptor targeting AMO and AMO D refers to the splice donor-targeting AMO.

Antisense morpholino oligo	Sequence
AMO A	GTCAGGTCTCCTATGGCACAGCATG
AMO D	GCCGCCGGATGGTCACATACCTCAT

2.1.4. Animal cap explants

X. laevis animal caps were dissected with mounted tungsten needles at late blastula stage (NF Stages 8-9). Caps were dissected and cultured to the desired stage in 1/2X Normal Amphibian Medium (NAM/2).

2.1.5. Photography

Images of embryos were taken using a SPOT 14.2 Colour Mosaic camera and SPOT Advanced software, with a Leica MZ FLIII microscope used for magnification. Images were processed using Photoshop Elements 13 Editor.

2.2. Molecular biology methods

2.2.1. Gel electrophoresis

1-2% Agarose gels stained with Ethidium Bromide were used to run RNA and DNA samples in either 1X Tris-Acetic acid-EDTA (TAE) or 1X Tris-Borate-EDTA (TBE) buffer. Samples were loaded with a 6X gel loading buffer (NEB) and run alongside 2-3 μ l 2-log DNA ladder (NEB).

2.2.2. Total RNA extraction

All centrifugation steps occur at 13000 RPM. Embryos and animal caps were flash frozen on dry ice at desired stages and stored at -80°C. Samples were homogenised on ice in 1ml of Tri-Reagent (Sigma) using a tissue homogeniser in a sterile RNase free tube. Samples were stood at room temperature for five minutes and were then centrifuged for 10 minutes at 5°C. The supernatant was moved to a new tube and left to stand for 5 minutes at room temperature, followed by the addition of 200 μ l of chloroform. Samples were vortexed and stood at room temperature for 5 minutes and were then centrifuged for 15 minutes at 4°C. The aqueous phase was removed to a new tube and an equal volume of chloroform added, the samples were vortexed and centrifuged for 5 minutes at 4°C. Once again aqueous phase was removed, and mixed with 500 μ l of isopropanol. Samples were precipitated for 30 minutes at -20°C, then centrifuged for 15 minutes at 4°C to pellet the RNA. Isopropanol was removed and the pellet washed in 200 μ l 70 % ethanol and centrifuged for 5 minutes at 4°C. Pellets were desiccated before the RNA was resuspended in 100 μ l of nuclease-free water and 120 μ l of 7.5M LiCl/50mM Ethylenediaminetetraacetic acid (EDTA) and stored at -80°C overnight. The following day the samples were centrifuged for 20 minutes at 4°C, washed in 70% ice cold ethanol and desiccated. RNA pellets were resuspended in 20-30 μ l of nuclease-free water and stored at -80°C.

2.2.3. cDNA synthesis for RT-PCR

Total RNA was extracted from embryos as per section 2.2.2. First strand cDNA synthesis was achieved using 0.3-0.5 μ g of RNA and Superscript IV Reverse Transcriptase (Thermo-Fisher). RNA and water were combined with 1 μ l of random hexamer primers and 1 μ l of 10mM dNTPs to a total volume of 13 μ l. Tubes were incubated at 65°C for 5 minutes and then incubated on ice for at least 1 minute. 4 μ l of Superscript IV RT reaction

buffer, 1µl 100mM DTT and 1µl water were added to each tube. 1µl of Superscript IV reverse transcriptase was then added to each tube except for the water control which had 1µl of water. Reactions were then incubated at 23°C for 10 minutes, 55°C for 10 minutes and 80°C for 10 minutes. cDNA was then frozen in aliquots at -80°C or used immediately for PCR amplification.

2.2.4. Polymerase chain reactions

cDNA generated from *X. tropicalis* and *X. laevis* embryos and animal caps was amplified using primers listed in tables 2.2 and 2.3 respectively. Polymerase chain reactions took place in a total volume of 20µl, consisting of 10µl 2X Promega PCR Mastermix, 7µl nuclease free water, 1µl 10µM forward primer, 1µl 10µM reverse primer and 1µl cDNA. Water controls were also set up with water replacing the cDNA to check for contamination. cDNA was amplified using the following cycle: two minutes at 95 °C, then 25-30 cycles of 30 seconds at 95 °C, 30 seconds at the annealing temperature and 45 seconds at 72°C, before a final extension of 15 minutes at 72°C. PCR products were then run on an agarose gel as previously described in section 2.2.1.

2.2.5. Plasmid ligation

Recombinant plasmids were generated using the pGEM-T Easy Vector. Ligation reactions were set up as follows: 5µl 2X Rapid Ligation Buffer, 1µl pGEM-T Easy Vector, 1µl T4 DNA Ligase, 3µl of PCR product. Reactions were incubated overnight at 4°C.

2.2.6. Plasmid amplification

Dam⁻/dcm⁻ competent *E.coli* cells (NEB, #C2925I) were used for transformations. Cells were thawed on ice and 3-4µl of plasmid was mixed with 50µl of competent cells. The mixture was placed on ice for 30 minutes, heat-shocked for 45 seconds at 42°C and then placed back on ice for 2 minutes. 1ml of warm LB media was added to each transformation reaction and reactions were incubated for 1 hour at 37°C whilst shaking. 1/10 and 1/100 dilutions of bacteria were plated out on LB/Agar plates containing 100µg/ml ampicillin and plates were incubated at 37°C overnight.

2.2.7. Plasmid isolation

Single colonies were added to 3ml LB media containing 100µg/ml ampicillin and cultured overnight at 37°C with agitation. Amplified plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen) as per the manufacturer's instructions. DNA was eluted in 50µl of nuclease-free water.

2.2.8. Linearisation of plasmid DNA

Templates for transcription of sense mRNA for injection, and antisense Digoxigenin (DIG)-labelled probes for *in situ* hybridisation, were generated by plasmid linearisation. Reactions were set up as follows and incubated at 37°C for 2 hours: 1-5µg plasmid DNA, 10µl 10X reaction buffer, 3µl restriction enzyme (Table 2.4) and then made to 100µl with nuclease free water.

Table 2.2. Primers used to amplify *X. tropicalis* cDNA.

Primer	Sequence (5'-3')	Annealing temperature (°c)
pan-src forward (philip lewis)	ATCTCGCACCGAGACAGACT	50
c-src specific reverse (isobel bradley)	CAGTCGCCTTCCGTGTTATT	50
n-src forward (philip lewis)	ACTGTGACCTGACGCCTTTT	50
n1-src specific reverse (isobel bradley)	CCTCATGTCAGGTCTCGTGTT	50
rpl8 forward	GGGCTRTCAGCTTYGCTGA	50
rpl8 reverse	ATACGACCACWCCAGCAA	50
sox2 forward	CCCTCAAGAAGGATA	45
sox2 reverse	GAAGAAGAGGTGACTACA	45
sox3 forward	CCAGAGGATAGACACTTAT	45
sox3 reverse	CTACTCTGAAGGGAAGAA	45
irx1 forward	GAGGAAGAGGATGAGAAA	45
irx1 reverse	GAGGAAGAGGATGAGAAA	45
neurod1 forward	CTCTCTCCGAGATTCTAC	50
neurod1 reverse	GGCACTCATTACTCTTC	50
myt1 forward	CCAGAGAAGAGAGACATAA	42
myt1 reverse	CAGCATCATGGGTATAG	42
gsx1 forward	TCCTCAAGACCAATTCC	50
gsx1 reverse	GGTGACACCTCCTTATC	50
msx1 forward	GGGTGAAAGTAGAAGAAAG	45
msx1 reverse	CCAAGAGGAAAGGAGATA	45
nkx6.1 forward	TCTGTACCCTGCTTATAC	45
nkx6.1 reverse	GTGTTTCCTCTTTCCATC	45
snai2 forward	CAGCAAGACCTACTCTAC	45
snai2 reverse	CTACACAACAACCAGATTC	45
twist1 forward	CAGAAAGAAGCGATCCACTAGG	50
twist2 reverse	CTGTAAGACCTGGCAGAGAAG	50
sox9 forward	TGCCAGTCAGAGTTAAT	45
sox9 reverse	AGGTTTCGATGGTAGAG	45
sox10 forward	GTCAATGGTGGCAGCAAATC	50
sox10 reverse	CTGCCTGAAGCTCTGTCTTT	50
pax3 forward	GAGAGAACCCACTACCCAGATA	50
pax3 reverse	GGCCTCCAGTTAAAGGAGATAAG	50
zic1 forward	GATGTGACGGATCGAGAAGTG	50
zic1 reverse	CAGAAGCAGATGTGGTTGGA	50
phox2a forward	CCCTACAAGTTCTTCTCT	42
phox2a reverse	ATTTGCCTTCAGAGTATTG	42
hnrnpa1 forward	GTTATGGTGGAGATGGCTACAA	50
hnrnpa1 reverse	CCACCATAATTGCCACCTTTC	50
tra2a forward	GCGGTGGTGGAGGTAGT	50
tra2a reverse	TATCGGCTGTAATAAGGTGATGG	50
n1-src cloning forward	GAGAAGGCCTACCATGGGTGCCACTAAAAGCAA	55
n1-src cloning reverse	TCTCTCTAGATTACTTGTGTCATCGTCTT	55

Table 2.3. Primers used to amplify *X. laevis* cDNA.

Primer	Sequence (5'-3')	Annealing temperature (°c)
snai2 forward	ACTCACACTGGAGAAAAGCC	45
snai2 reverse	AGCTAGTTTAGCATAGTTTAAGAGT	45
twist1 forward	GTAGACAGTGCGAGTAA	43
twist1 reverse	CCTTCAAGAATGGGAATG	43
odc forward	AAAGCTTGTTCTACGCATAGCAACT	50
odc reverse	AGGGTGGCACCAAATTTTAC	50
n-src forward (Philip Lewis)	ACTGTGACCTGACGCCTTTT	50
n1-src specific reverse (Isobel Bradley)	CCTCATGTCAGGTCTCGTGTT	50

Table 2.4. Clone information, restriction enzyme and polymerases used to transcribe *in situ* probes and synthetic mRNA transcripts

Insert	Plasmid	Enzyme	Polymerase	Source	Use
twist1	Unknown - Supplied linearised		T7	Betsy Pownall	in situ probe
snai2	pSP72	EcoRI	T7	Betsy Pownall	in situ probe
pax3	pGEM T-Easy	SacII	SP6	Own	in situ probe
phox2a	pGEM T-Easy	NcoI	SP6	Isobel Bradley	in situ probe
tubb2b	pGEM-5Zf(-)	Apal	SP6+hydrolysis	Harland Lab	in situ probe
myt1	pGEM T-Easy	NdeI	T7	Own	in situ probe
n1-src	pCS2+	NotI	SP6	Own	mRNA

2.2.9. DNA purification

DNA samples were made up to 400µl with nuclease free water. An equal volume of Phenol-chloroform was added and samples were vortexed for 20 seconds before centrifugation at 13000 RPM for 5 minutes. The aqueous phase was transferred to a new tube and combined with an equal volume of chloroform-isoamyl. Samples were vortexed and centrifuged again. The aqueous phase was once more transferred to a new tube, and 0.1 volumes of 3M sodium acetate and two volumes of 100 % ethanol were added. Samples were vortexed and then incubated overnight at -20°C to precipitate. Samples

were centrifuged for 20 minutes at 4°C, washed in 70% ethanol and desiccated. DNA pellets were resuspended in 25-50µl nuclease-free water and stored at -20°C.

2.2.10. Cloning of *n1-src* into PCS2+

The *X. tropicalis n1-src* gene had been acquired from the IMAGE clone library (IMAGE number: 5572523) by Phillip Lewis, and cloned into an empty pFLAG vector by double digest with *BglII* and *KpnI*. The FLAG-tagged *n1-src* gene was amplified out of the pFLAG by PCR using Phusion DNA Polymerase. The forward primer was designed to have a 5' *StuI* restriction site and a Kozak sequence. The reverse primer was designed to have a *XbaI* site. The reaction set up was as follows: 5µl 5X Phusion GC Buffer, 1µl dNTP mix (10mM each), 2.5µl 10mM forward primer, 2.5µl 10mM reverse primer, 1µg DNA, and nuclease free water to a total volume of 49.5µl. The reaction was heated to 98°C and then 0.5µl of Phusion polymerase was added. The reaction was then 98°C for three minutes, then 35 cycles of 10 seconds at 98°C, 10 seconds at 55°C and one minute at 72°C, followed by 10 minutes at 72°C. The primer sequences are listed in table 2.2.

The PCR product was checked on a 1.5% agarose gel and the product was then cleaned up via the Monarch DNA Gel Extraction Kit, as per the manufacturer's instructions. The clean PCR product and the pCS2+ plasmid were then cut with both *XbaI* and *StuI* restriction enzymes, as per section 2.2.8. Restriction reactions were cleaned up via the Monarch DNA Gel Extraction Kit and the clean products were ligated together as follows using T4 DNA ligase (Promega): 1µl 10x ligation buffer, 1µl T4 DNA ligase, 6µl PCR product, 2µl pCS2+ plasmid, and reactions were left overnight at 4°C. After amplification and isolation, the plasmids were sequenced by GATC Biotech.

2.2.11. Determining nucleic acid concentration

Nuclei acid concentration was determined by loading 1µl samples onto Nanodrop® ND1000 Spectrophotometer.

2.2.12. Synthetic mRNA synthesis

Template DNA was generated through linearisation of full length clones as per section 2.2.8. mRNAs were synthesised from templates using mMessage mMachine SP6 Transcription Kit (Invitrogen). 20µl reactions were set up as follows at room temperature: 10µl 2X NTP/CAP, 2µl enzyme mix, 0.5-1µg linear template DNA, 2µl 10X reaction buffer and nuclease-free water to a total volume of 20µl.

Reactions were incubated at 37°C for 2 hours before being checked by gel electrophoresis. Reactions were treated with 1µl turbo DNase for 15 minutes at 37°C. mRNA was recovered by Phenol:chloroform extraction and isopropanol precipitation. 115µl nuclease-free water and 15µl Ammonium Acetate Stop Solution was added to each reaction. An equal volume of phenol-chloroform was added, reactions were vortexed and centrifuged at 13000 RPM. The aqueous phase was moved to a clean tube and mixed with an equal volume of chloroform before centrifugation. Once again, the aqueous phase was moved to a fresh tube and the mRNA was precipitated through the addition of an equal volume of isopropanol and 1/10 volume 5M ammonium acetate and incubation at -20°C for at least 30 mins, if not overnight. Samples were centrifuged at 4°C for 15 minutes at 13000 RPM and the pellet was then washed in 100µl 70% ethanol, desiccated and resuspended in 20µl of nuclease-free water before storing at -80°C.

2.2.13. Western blot analysis

Samples were collected at the desired stages post microinjection. For whole *Xenopus* embryos, liquid was removed from samples and they were flash frozen on dry ice and stored at -80°C.

For *Xenopus* animal cap explants, liquid was removed and 50µl of 2X Sample Buffer was added per 10 caps. Samples were macerated, boiled at 95-100°C for 5 minutes and then flash frozen on dry ice.

For analysis, *Xenopus* embryos were homogenised in 100µl of Phosphosafe Extraction Reagent (Novagen), and samples were centrifuged for 10 minutes at 4°C. The supernatant was removed and 1X volume 2X Sample Buffer was added. Samples were boiled at 95°C for 5 minutes and samples were loaded onto 10% Acrylamide/SDS-PAGE gels. For animal cap explants already stored in 2X Sample Buffer, samples were boiled at 95°C for 5 minutes before loading onto 10%Acrylamide/SDS-PAGE gels.

Samples were run at 180V for 2 hours alongside 5-10µl of PageRuler Prestained Protein Ladder (ThermoFisher) in Tris-Glycine Running Buffer. After running, gels were soaked for 10-30 minutes in Tris-Glycine Transfer buffer, whilst PVDF membranes (Merck Millipore) were activated in methanol and water. Transfers of protein on PVDF membranes took place at 85V for 2 hours at room temperature, or at 30V overnight at 4°C. Membranes were blocked for 1 hour in 5% Marvel milk powder/1X Phosphate-

Buffered Saline, 0.1% Tween 20 (PBST). Primary antibodies were diluted in 5% Marvel milk powder/1X PBST blocking solution and membranes were incubated at 4°C overnight in the primary antibody solution (Table 2.5). Membranes were washed four times in 1X PBST for 15-30 minutes and then blocked again in 5% Marvel milk powder/1X PBST for 30 minutes at room temperature. Secondary antibody dilutions were made up in blocking solution (Table 2.5) and membranes were washed in antibody solution for 1 hour at room temperature. Membranes were washed four times for 20-40 minutes in 1X PBST, and then developed. Development used either BM Chemiluminescence substrate kit (Roche) or Immobilon Western Chemiluminescent HRP substrate (Merk Millipore) and membranes were exposed to Hyperfilm ECL (Amersham) for visualisation.

Table 2.5. Primary and secondary antibody dilutions for protein detection by western blotting.

Target	Primary antibody dilution	Secondary antibody dilution
Anti-FLAG	1:1000 (cells and <i>X.laevis</i>) 1:500 (<i>X. tropicalis</i>)	1:4000 anti-mouse HRP (62-650, Invitrogen)
dpErk	1:4000	1:4000 anti-mouse HRP (62-650, Invitrogen)
Erk	1:500000	1:2000 anti-rabbit HRP (7074, Cell Signaling Technology)
β-Actin	1:4000	1:4000 anti-mouse HRP (62-650, Invitrogen)
β-Tubulin	1:10000	1:2000 anti-rabbit HRP (7074, Cell Signaling Technology)

2.2.14. Transcription of Digoxigenin labelled antisense probes

Templates were linearised as per section 2.2.8. Transcription reactions were assembled at room temperature as follows: 4µl 5X transcription buffer, 2µl 10X DIG NTP mix, 2µl 100mM DTT, 1µl SP6 or T7 RNA polymerase, 1µg DNA and made up to 20µl with nuclease-free water.

Transcription reactions were incubated at 37 °C overnight. Products were checked on a 1.5% agarose gel before being ethanol precipitated overnight at -20°C by addition of: 50µl of nuclease free water, 50µl of 5M Ammonium acetate, 1µl of Glycoblue Coprecipitant (Invitrogen) and 300µl of 100% ethanol. The next day, samples were centrifuged for 15 minutes at 13000 RPM and 4°C. Pellets were then washed in 70% ethanol and desiccated. Pellets were resuspended in 50µl of nuclease-free water and stored at -80°C.

Due to its long length, the *tubb2b* probe was hydrolysed to enable embryo penetration. 25µl of probe was incubated for 12.5 minutes at 60°C in an equal volume of hydrolysis solution (80mM NaHCO₃ and 120mM Na₂CO₃, pH 10.2) (Silva *et al.*, 2006). Following hydrolysis, 50µl of 5M ammonium acetate and 312.5µl of 100% ethanol was added and the probe was precipitated overnight at -80°C. The incubation time for the hydrolysis reaction was calculated as follows (Willars and Challiss, 2004) :

$$t = \frac{(\text{starting length, kb}) - (\text{desired length, kb})}{0.11(\text{starting length, kb} \times \text{desired length, kb})}$$

The starting length of the probe was 1.6kb and the desired length was 0.5kb.

Following precipitation, the probe was centrifuged at 13000 RPM and 4°C for 30 minutes. The pellet was then washed in 70% ethanol and desiccated. The pellet was resuspended in 50µl of nuclease-free water and stored at -80°C.

2.2.15. Whole-mount *in situ* hybridisations

In situ hybridisations were carried out as per Harland (1991). Embryos were collected at desired stages and vitelline membranes were removed prior to fixing in MEMFA (20ml 10X MEM Salts:1M MOPS, 20mM EGTA, 10mM MgSO₄, 20ml Formaldehyde and 160ml nuclease-free water) for 1 hour. Embryos were then washed in ethanol for 5 minutes and then stored in ethanol at -20°C.

Embryos were returned to room temperature in 100% ethanol and then rehydrated by washing on a roller for 10 minutes in 75% ethanol/PBST, 10 minutes in 50% ethanol/PBST and then 3 x 5 minutes in PBST. Embryos were then treated with 10 µg/ml proteinase K at room temperature with gentle swirling. Stage 14 embryos were treated for 5 minutes, stage 24 embryos for 8.5 minutes and stage 35 embryos for 13 minutes. Embryos were then rinsed for 2 x 5 minutes with 0.1M, pH 7.8 triethanolamine. After the second rinse, 12.5µl acetic anhydride was added to the triethanolamine and vials were swirled for 5 minutes to aid mixing. A further 12.5µl of acetic anhydride was then added and vials were swirled for a further 5 minutes. Embryos were then washed 2 x 5 minutes in PBST whilst rolling and fixed for 20 minutes in 10% formalin/PBST. Following fixation, embryos were washed 5 x 5 minutes in PBST. Embryos were then transferred with 0.5ml of PBST to 1.5ml screw cap Eppendorf tubes containing 0.5ml hybridisation buffer.

Hybridisation buffer consisted of 50% formamide, 5x saline-sodium citrate (SSC) (pH 7) 100µg/ml heparin, 1x Denhart's, 0.1% tween 20, 0.1% CHAPs, 10 mM EDTA. This was warmed to 60°C prior to the addition of 1 mg/ml total yeast RNA. PBST/hybridisation buffer was removed and replaced with 1ml fresh hybridisation buffer and incubated at 60°C for 2 hours while rocking. DIG-labelled probes were heated to 80°C for 2 minutes and then 3µl added to 1ml fresh hybridisation buffer. The hybridisation buffer was then removed from embryos and replaced with probe solution and incubated at 60°C overnight while rocking.

The probe solution was removed the next day and embryos were washed 2 x 10 minutes in 1ml hybridisation buffer at 60°C. Embryos were washed 3 x 20 minutes in 2x SSC + 0.1% tween at 60°C. Embryos were then washed at 60°C for 3 x 30 minutes in 0.2x SSC + 0.1% tween. Embryos were washed 2 x 15 minutes at room temperature in maleic acid buffer (MAB) (100mM maleic acid, 150mM NaCl, pH 7.8, 0.1% tween). Embryos were then pre-incubated with MAB + 2 % BMB + 20% heat treated lamb serum for 2 hours at room temperature. This was then replaced with fresh MAB/BMB/lamb serum + 1/2000 dilution of affinity purified sheep anti-digoxigenin antibody coupled to AP (Roche) and embryos were rocked overnight at 4°C.

Embryos were washed 3 x 5 minutes with MAB and then transferred from 1.5 ml Eppendorf's to scintillation vials and they were then washed 3 x 1 hour with MAB. Embryos were washed for 3 minutes and then 10 minutes in Alkaline Phosphatase (AP) buffer at room temperature (100mM Tris, 50mM MgCl₂, 100mM NaCl, pH 9.5 and 0.1% Tween). AP buffer was then replaced with 1ml BM purple and embryos were left undisturbed until staining developed. Once stained, embryos were washed 2 x 15 minutes in PBST and fixed overnight in 10% Formalin/PBST. Embryos were bleached in 5% H₂O₂/PBST, rolling under bright light for several hours to remove pigmentation. They were then washed 2 x 10 minutes in PBST and refixed in 10 % formalin/PBST.

2.2.16. Whole-mount immunostaining

Embryos were collected at desired stages and fixed in MEMFA (20ml 10X MEM Salts:1M MOPS, 20mM EGTA, 10mM MgSO₄, 20ml Formaldehyde and 160ml nuclease-free water) for 1 hour. Embryos were then washed in methanol for 10 minutes and then stored in methanol at -20°C.

Embryos were returned to room temperature in 100% methanol and then rehydrated by washing on a roller for 10 minutes in 75% methanol/PBS, 10 minutes in 50% methanol/PBS and then 10 minutes in 100% PBS. Embryos were then rocked at room temperature for 40 minutes in Potassium Dichromate solution in 5% acetic acid (7.35g Potassium Chromate, 12.5ml 100% Acetic Acid and 250ml nuclease-free water). Embryos were then washed 3 x 5 minutes in PBS and then 3 x 30 minutes in PBS until the PBS wash is clear. Embryos were rocked for 45 minutes in 5% H₂O₂/PBS. The H₂O₂ was then removed and embryos were washed 3 x 15 minutes in PBS. Embryos were then transferred to 1.5ml screw cap Eppendorf tubes and washed 2 x 1 hour in 1.5ml BBT solution (PBS, 1%BSA, 0.1% Triton X-100; for 200ml use 20ml 10X PBS, 2g BSA, 1ml 20% Triton X-100 and make up to 200ml with nuclease-free water). Embryos were then blocked for 1 hour in BBT solution + 5% horse serum. Embryos were then incubated overnight at 4°C in BBT solution/5% horse serum + 1/10000 dpErk mouse antibody (Sigma).

Embryos were then washed 4 x 1 hour in BBT solution and then washed for 1 hour in BBT/5% horse serum. Embryos were then incubated overnight at 4°C in BBT solution/5% horse serum + 1/1000 anti-mouse igG-AP secondary antibody (Vector Laboratories)

Embryos were washed for 1 hour in BBT solution, then transferred from 1.5 ml Eppendorf's to scintillation vials and washed for 4 hours in PBST. This was then replaced with 1ml BM purple (Roche) and embryos were left undisturbed until staining developed. Once stained, embryos were washed 2 x 15 minutes in PBS and fixed overnight at room temperature in 10% Formalin/PBS.

2.3. RNA-Seq analysis

2.3.1. RNA-Seq sample preparation

For Illumina short read sequencing, sibling *X. tropicalis* embryos were bilaterally injected at the 1-2 cell stage with 20 ng total *n1-src* AMO A+D or with 20 ng control morpholino. Four biological repeats were carried out and embryos flash frozen on dry ice at stage 16. For Nanopore sequencing of full length transcripts, sibling *X. tropicalis* embryos were injected with 20ng total *n1-src* AMO A+D, 20ng control morpholino or 250pg FLAG-tagged *n1-src* mRNA. Four biological repeats were carried out and embryos flash frozen at stage 16. Total RNA was extracted as per section 2.2.2. until the isopropanol precipitation stage. After 30 minutes precipitation at -20°C, RNA was centrifuged for 15 minutes at 13000 RPM and 4°C and pellets were washed in 70% ethanol and desiccated. Pellets were resuspended in 40µl nuclease-free water.

RNA was then DNase treated to remove DNA traces from samples. 5µl DNaseI and 5µl DNA digestion buffer (Zymo) were added to each RNA sample and samples were incubated at 15 minutes at room temperature. RNA was purified using RNA Clean & Concentrator™-25 (Zymo) as per the instructions. 100µl RNA binding buffer was mixed with samples, followed by 150µl 100% ethanol. Samples were run through Zymo-Spin™ IC Columns and flow-through discarded. 400µl Zymo RNA prep buffer was added to columns and flow through discarded after centrifugation. 700µl RNA wash buffer was added and flow through discarded after centrifugation. This last step was then repeated with 400µl of RNA wash buffer. RNA was eluted in 15µl nuclease-free water. RNA concentration was measured using the ND-1000 spectrophotometer and samples were run on Agilent 2100 Bioanalyzer to measure the RNA Integrity.

Samples whose RNA Integrity Number was too low were further cleaned via Lithium chloride precipitation. Samples were precipitated with 2.5M Lithium chloride in a total volume of 50µl at -20°C overnight. Samples were spun down for 15 minutes at 4°C and 13000 RPM. Pellets were washed in 70% ethanol and desiccated. Pellets were resuspended in 15µl nuclease-free water. RNA Integrity was once again checked.

2.3.2. RNA-Seq library preparation

Library preparation was carried out by staff at the Bioscience Technology Facility at the University of York. For the Illumina RNA-Seq library, the NEBNext Poly(A) mRNA Magnetic Isolation Module was used to isolate Poly(A) mRNA from total RNA and the

NEBNext RNA Ultra II Directional RNA Library Prep Kit was used to generate the cDNA library. For the Oxford Nanopore long read sequencing, the Oxford Nanopore PCR-cDNA Sequencing Kit was used to generate the cDNA library.

After cDNA library construction, transcripts were sequenced on the Illumina HiSeq 3000 machine at the University of Leeds or on the Oxford Nanopore Technologies' PromethION at the University of York.

2.3.4. Differential expression analysis of Illumina RNA-Seq data

Initial bioinformatics analysis was carried out by the University of York Bioscience Technology Facility.

Illumina sequencing resulted in ~35-50 million reads per sample. For initial analysis, reads were mapped to the NCBI v9.1 *X. tropicalis* reference transcriptome (<https://www.ncbi.nlm.nih.gov/genome/?term=xenopus%20tropicalis>), with Salmon (Version 0.10.2) (<http://salmon.readthedocs.io>). Differential expression was then analysed using Sleuth (Version 0.30; <http://pachterlab.github.io/sleuth/>). Sleuth fits two separate models; it firstly fits a model including treatment and pair, and then it fits a reduced model including only pair. This should test for any effect of treatment, accounting for variation due to paired samples. The raw reads were normalised as transcript per million values.

As the *X. tropicalis* reference transcriptome contains many transcript models that do not necessarily have any supporting experimental data, a de novo approach was also attempted to construct a transcriptome that reflects the transcripts actually present in *X.tropicalis* embryos at this particular developmental stage. Illumina sequencing reads were aligned to the *X. tropicalis* reference genome by HISAT2 (<http://www.ccb.jhu.edu/software/hisat2>) and StringTie (<https://ccb.jhu.edu/software/stringtie/index.shtml>) was run on these alignments to generate a novel transcriptome. StringTie is a fast and efficient program that assembles RNA-Seq alignments into potential transcripts. These new transcripts were then merged back to the reference annotation with gffcompare in order to link gene names to the new transcripts (<https://github.com/gpertea/gffcompare>). The Illumina reads were then aligned to this novel transcriptome and the Salmon and Sleuth packages were run on them.

2.3.5. Gene ontology analysis of Illumina RNA-Seq data

Gene ontology analysis was carried out on transcripts significantly upregulated or downregulated 1.15-fold. A Q-Value of 0.05 was used to judge transcript significance. Transcript lists were uploaded onto the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (<http://pantherdb.org/>) (Mi *et al.*, 2021). This software identifies statistically over-represented biological processes within the transcript list. Due to better annotation, the mouse gene database was used as the reference database.

2.3.6. Gene set enrichment analysis of Illumina RNA-Seq data

Gene set enrichment analysis (GSEA) was performed using the GSEA 4.0.0 software (Broad Institute). Following the instructions from the Broad Institute website (software.broadinstitute.org/gsea) an expression dataset file (.gct) was created containing the TPM values for each transcript in each sample, and a phenotype labels file (.cls) was created to label the phenotype of each sample. The Illumina_human.chip file was used to translate the transcript names in the expression dataset file to HUGO (Human Genome Organisation) gene symbols. The biological processes gene set file (c5.bp.v6.2) was chosen from the Molecular Signature Database (Broad Institute) for analysis. GSEA was performed to detect gene sets enriched in the control and *n1-src* knockdown samples. The phenotype permutation was originally used to assess the statistical significance of the enrichment scores, however, no gene sets were significantly enriched at a FDR Q-Value<0.25. The gene set permutation was therefore used to assess the statistical significance. As this is a less stringent assessment of significance, a more stringent FDR cut off of 0.05 was used.

2.3.7. DEXSeq analysing differential exon usage in the Illumina RNA-Seq data

To test for differential exon usage between the control and experimental RNA-Seq samples, the Bioconductor package DEXSeq was used by the Bioinformatics team in the University of York Bioscience Technology Facility (Anders, Reyes and Huber, 2012) (<https://bioconductor.org/packages/release/bioc/html/DEXSeq.html>). Transcripts are collapsed into sets of exon units for each gene by the software. These exon units are pieces of exon that reflect the underlying intron-exon boundaries across all the transcripts for the gene. DEXSeq then tests for differential expression across each exon unit.

2.3.8. Generation of a novel transcriptome using Nanopore long reads

Due to a lack of sufficient sequencing depth, differential expression analysis was not carried out on the Oxford Nanopore RNA-Seq reads. The bioinformatics team instead used the full length transcripts to assemble a de novo transcriptome, that would include novel transcripts generated by changes in alternative splicing, which would be absent from the reference transcriptome. Full length transcripts were aligned to the *X. tropicalis* reference genome v9.1 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000004195.3/) and StringTie2 was run on these alignments to assemble a novel transcriptome (Kovaka *et al.*, 2019).

2.3.9. Identification of changes in splicing using IsoformSwitchAnalyzeR

Illumina short reads were then aligned to the novel transcriptome assembled from the Nanopore long reads (2.3.8). IsoformSwitchAnalyzeR was then run on these alignments (<https://www.bioconductor.org/packages/release/bioc/html/IsoformSwitchAnalyzeR.htm>) (Vitting-Seerup and Sandelin, 2019). IsoformSwitchAnalyzeR identifies pairs of isoforms with opposite changes in usage between two experimental conditions, where at least one of the changes is significant. A significance value of $Q < 0.1$ was used.

2.3.10. Identification of coordinates for alternative splice sites

IsoformSwitchAnalyzeR identifies isoforms with significant changes in usage between two experimental conditions. It cannot however identify the underlying splicing event occurring in each isoform change, or the genomic coordinates of events.

Transcripts which were detected by IsoformSwitchAnalyzeR to display significant changes in usage between the two conditions were extracted from the StringTie2 transcriptome GTF file (section 2.3.8). These transcripts were then inputted into the Alternative Splicing transcriptional landscape visualization tool (AStalavista) (<http://astalavista.sammeth.net/>), which characterises the alternative splicing events in a given transcript annotation, identifying the type of splicing event and the genomic coordinates.

2.3.11. Identification of enriched RNA binding protein motifs

Sequences surrounding the alternative splice events identified in section 2.3.10 were analysed for enriched motifs for RNA binding proteins. Regions 50-150 base pairs

upstream and downstream of each splicing event were extracted from the *X. tropicalis* genome v9.1 and uploaded onto the Analysis of Motif Enrichment (AME) tool, which is part of the MEME suite (<https://meme-suite.org/meme/tools/ame>). Random 100 base pair sequences from the *X. tropicalis* genome were uploaded as control sequences. AME searched the experimental and control sequences for RNA binding protein motifs from a motif database available within the MEME suite (Ray *et al.*, 2013).

2.4. Cell culture methods

2.4.1. Culture of cell lines

Stably transfected n1-Src-FLAG HeLa cells (generated by Phil Lewis) were cultured at 37°C and 5% CO₂, in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing glutamate and pyruvate, that was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. 500µg/mL Hygromycin B was added to maintain the selection pressure.

Once reaching ~70% confluence, cells were passaged into fresh flasks. Cell media was removed and cells were washed in 10ml of sterile PBS. Cells were then incubated for 3-5 minutes at 37°C in 1X Trypsin-EDTA. Once cells were dislodged the trypsin was inhibited by addition of fresh cell media. Suspended cells were centrifuged at 2000 RPM for 5 minutes and then the pellet was resuspended in 1ml of fresh media. The appropriate volume of cell suspension was then moved into new 25 or 75cm² flasks containing fresh media and hygromycin.

2.4.2. Plating out of cells

Once resuspended in 1ml of media, cells were quantified using a haemocytometer and plated into 6-well plates containing 2ml of cell media. 70000-75000 cells were plated into each well.

2.4.3. Induction of *N1-Src-FLAG* expression

One day after plating out, stably transfected HeLa cells were treated with 1 µg/ml doxycycline for 48 hours to induce expression of n1-Src-FLAG. Cells were then washed

twice in sterile 1X PBS and then lysed in 100µl 2X Laemmli buffer (Bio-Rad) and stored at -20°C until used in western blots.

2.4.4. N1-Src-FLAG - dpErk time series

N1-Src-FLAG HeLa cells were plated out into 6-well plates. 1µg/ml doxycycline was applied to cells for 48, 24, 12, 6 or 3 hours or cells were untreated. 12 hours before addition of doxycycline the cell media was replaced with “serum free” media supplemented with 0.5% FBS only. When ready for collection cells were washed twice in sterile 1X PBS and then lysed in 100µl 2X Laemmli buffers and stored at -20°C until used in western blots.

3. The role of n1-Src in neuronal development in *Xenopus*

3.1. Introduction

3.1.1. *Xenopus* neural induction

This chapter aims to determine the role of n1-Src in neuronal development, using *Xenopus* as a model. *Xenopus* has been one of the main models used in the study of early vertebrate development, mainly due to them producing a large number of robust embryos that develop rapidly externally (Beck and Slack, 2001).

Xenopus nervous system development begins during the late blastula and gastrula developmental stages. During the blastula stages, the three primary germ layers, the ectoderm, mesoderm and endoderm begin to be established (Figure 3.1). The endoderm arises in the vegetal hemisphere and gives rise to most the internal organs including intestines and lungs (Nowotschin, Hadjantonakis and Campbell, 2019). The animal hemisphere of the *Xenopus* embryo gives rise to the ectoderm that will later differentiate into epithelial and nervous system tissues, and neural crest derived tissues (Yasuo and Lemaire, 2001).

The cells at the interface between the animal and vegetal hemispheres, around the equator of the embryos, give rise to the mesodermal tissues, such as muscle, cartilage, bone, blood and notochord (Kiecker, Bates and Bell, 2016). The model proposed for mesoderm induction is a three signal model, where two signals from the vegetal pole induce mesoderm in the marginal zone, then a third signal in the dorsal marginal zone patterns dorsoventral axis (Slack *et al.*, 1987). The mesoderm inducing factors signalling from the vegetal pole are members of the Tgf- β family, including *Xenopus* Nodal-Related (Xnr) factors and Vg1 (Weeks and Melton, 1987; Agius *et al.*, 2000; Birsoy *et al.*, 2006).

Ectodermal cells in the animal pole are induced towards either the epidermal or neural fates depending on the signals that they receive from cells in the underlying mesoderm and from other ectodermal cells (Hemmati-Brivanlou and Melton, 1997). The “default” state of the ectoderm is to become neural tissue, whilst epidermal tissue requires induction by the extracellular signalling proteins, bone morphogenetic proteins (BMPs), expressed in the ectoderm but mainly released from the underlying ventral mesoderm (Hemmati-Brivanlou and Melton, 1994). In the dorsal ectodermal region, that becomes neuroectoderm, BMP signalling is attenuated by neural inductive signals, like noggin, chordin and follistatin, from the Spemann-Mangold organiser in the dorsal mesoderm (Lamb *et al.*, 1993; Hemmati-Brivanlou, Kelly and Melton, 1994; Sasai *et al.*, 1995; de Robertis, 2006) (Figure 3.1).

BMPs bind to their cell surface receptors and this leads to the intracellular phosphorylation of Smad1, 5 and 8 effector proteins (Wang *et al.*, 2014). These phosphorylated smad proteins associate with co-smad (Smad4), and the complex translocates to the nucleus to regulate gene expression necessary for epidermal differentiation. In the dorsal ectodermal region, antagonists including noggin, chordin and follistatin directly bind to BMPs and prevent them from interacting with their receptors (Ali and Brazil, 2014). Further attenuation of BMP signalling is provided by fibroblast growth factor (Fgf) proteins in the ectoderm (Delaune, Lemaire and Kodjabachian, 2005). Fgfs bind to their receptors, leading to activation of the intracellular mitogen-activated protein kinase (MAPK) signalling pathway and in turn, the phosphorylation of Smad1, which prevents its translocation into the nucleus. Other factors have been found to be involved in neural induction through BMP inhibition, including Wnt proteins and retinoic acid (Patapoutian and Reichardt, 2000; Sheng *et al.*, 2010).

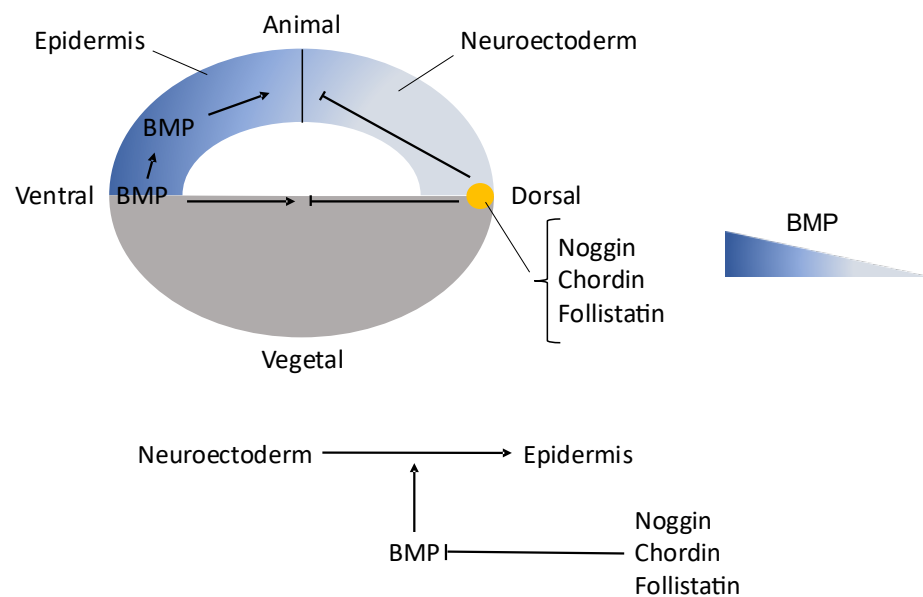


Figure 3.1. Schematic of neural induction in blastula stage *Xenopus* embryos. In the blastula stage *Xenopus* embryo, the endoderm is established in the vegetal hemisphere (grey), the ectoderm in the animal hemisphere (blue) and the mesoderm at the interface of the two. The white space is the fluid filled blastocoel. Cells in the ectoderm can be induced towards an epidermal (dark blue) or neural (light blue) fate depending on the signals that they receive. BMPs expressed from the ventral mesoderm and ventral ectoderm signal to drive the ectodermal tissue from a neural to an epidermal fate. BMP antagonists are released from cells in the Spemann-Mangold organiser (orange) in the dorsal mesoderm. These antagonists, like noggin, chordin and follistatin inhibit BMP activity in the dorsal ectoderm, allowing cells to advance down a neural route.

3.1.2. *Xenopus* neuronal differentiation

The establishment of neurons in *Xenopus* occurs via two waves of neurogenesis. The first wave generates the primary neurons that are required for the embryos early motility. This begins in gastrulation and comes to an end at stage 36/37 (Schlosser, Koyano-Nakagawa and Kintner, 2002). At neurula stage 14, these differentiated primary neurons form as three distinct populations in the neural plate, on either side of the neural midline (Figure 3.2). The three columns are the motor, inter and sensory neurons, that are required for early embryos reflex responses to touch.

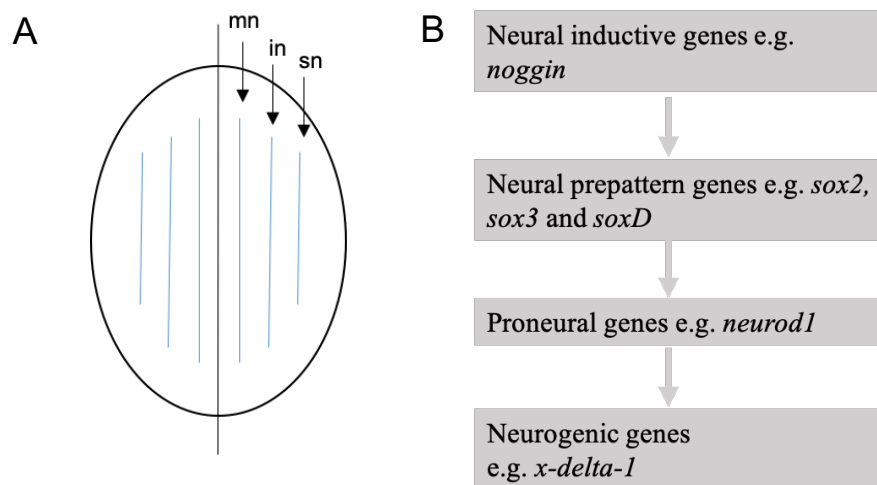


Figure 3.2. Differentiation of primary neurons in *Xenopus*. **A)** Dorsal view, anterior to the top, showing the stereotypical three strips of *Xenopus* primary neurons on either side of the midline. Mn=motor neurons, in=inter neurons, sn=sensory neurons. **B)** The cascade of transcriptional activation and repression within the neural plate that establishes the differentiated neuron populations.

The differentiation of these neuron populations is the result of a cascade of transcriptional activation and repression within the neural plate cells (Figure 3.2). After neural induction and neural plate formation, the plate consists of a population of progenitor cells that express the neural prepattern genes like *sox2* and *sox3*. These genes drive cell proliferation and delay the differentiation of neurons, via the repression of proneural gene expression, ensuring the neural plate reaches sufficient size prior to nervous system development (Bylund *et al.*, 2003). Overexpression of these factors in *Xenopus* embryos leads to an expansion of the neural plate at the expense of epidermis and delays the onset of neurogenesis (Archer, Jin and Casey, 2011).

The inhibition of BMP signalling, and the activity of *sox3*, induces *soxD* expression (Mizuseki *et al.*, 1998; Rogers *et al.*, 2009). Once the neural plate has reached sufficient

size, *soxd* expression activates the proneural gene, *neurogenin* (*xngr1*), which in turn activates expression of the neural differentiation factor, *neurod1*, committing the cells to differentiation into neurons (Mizuseki *et al.*, 1998)

The differentiated primary neuron populations form as three distinct columns on either side of the neural midline. The generation of these distinct domains from a group of developmentally equivalent cells is controlled by the expression of neurogenic genes like *notch* and *delta* via lateral inhibition (Appel, Givan and Eisen, 2001). Those cells that are programmed to develop into neurons express high levels of the membrane bound ligand, delta, which interacts with the cell surface receptor, notch, on adjacent cells (Revinski *et al.*, 2010). Expression of *delta* in those cells that will become neurons is activated, in part, by the activity of the proneural protein, neurogenin (Ma, Kintner and Anderson, 1996). Activation of the notch receptor on adjacent cells by delta, leads to the expression of target genes that suppress neuronal cell fate and the generation of the distinct neuron domains separated by regions of non-differentiated cells.

3.1.3. N1-Src and *Xenopus* neural development

N1-src expression has been detected in *X. laevis* embryos during the neurula stages (Collett and Steele, 1992). RT-PCR analysis first detected *n1-src* expression in stage 15 (neural plate stage) embryos and specifically in dissected neural plate tissue. It was then hypothesised that *n1-src* plays a role in the elaboration of neuron structure (Collett and Steele, 1992). More recently, work in our lab has detected *n1-src* expression, via RT-PCR analysis, in gastrula stage 11 *Xenopus tropicalis* embryos, with expression levels then increasing throughout the early neural developmental stages (Lewis *et al.*, 2017). We have detected expression levels decrease after primary neurogenesis, but increases again during stages when secondary neurogenesis occurs. Spatial expression was also analysed via *in situ* hybridisation analysis, revealing that *n1-src* expression is enriched throughout the neural plate in neurula stage 14 embryos (Lewis *et al.*, 2017). Furthermore, we have shown that the blocking of *n1-src* splicing inhibits the differentiation of primary neurons in *X. tropicalis* embryos, and work focusing on this in this chapter has contributed to Lewis *et al.* (2017) paper. It remains unknown however, how and where *n1-Src* functions to drive neuronal differentiation.

3.1.4. Aims

The aims of this chapter are:

1. Characterise the effects that blocking *n1-src* splicing has on neuronal development in *X. tropicalis* embryos.
2. Identify where in the cascade of transcriptional expression and repression n1-Src functions in the process of neuronal differentiation.
3. Identify intracellular signalling pathways regulated by n1-Src activity.

3.2. Results

3.2.1. Inhibition of bone morphogenic proteins induces *n1-src* expression in ectodermal tissues

BMP inhibition by factors such as follistatin, chordin and noggin is one of the first steps in the development of the *X. tropicalis* primary nervous system. To determine whether BMP inhibition induces expression of *n1-src*, the animal cap assay was utilised. This assay exploits the pluripotent nature of the ectodermal cells dissected from *Xenopus* blastula embryos (Dale, Smith and Slack, 1985; Borchers and Pieler, 2010). Untreated cells adopt an epidermal fate, however exposure to relevant stimuli can induce the formation of different tissues, for example, noggin exposure induces neural tissue formation (Lamb *et al.*, 1993; Okabayashi and Asashima, 2003).

Animal cap explants were dissected from *X. laevis* embryos that were either untreated or injected with 50pg of *noggin* mRNA and were cultured to neurula stage 17/18. RT-PCR analysis on RNA from these samples revealed that BMP inhibition was sufficient to induce expression of *n1-src* to levels comparable with those seen in whole embryos (Figure 3.3).

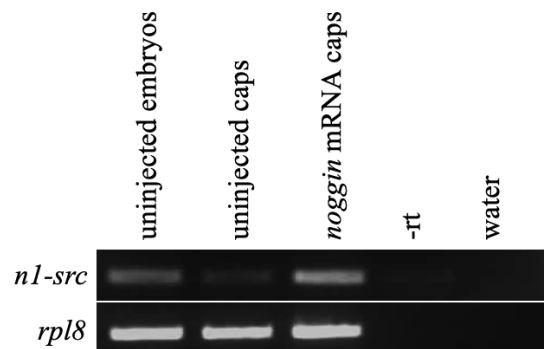


Figure 3.3. RT-PCR analysis for *n1-src* following BMP inhibition. Animal caps were dissected from blastula stage 8 *X. laevis* embryos that were uninjected or injected with 500pg *noggin* mRNA at the 2-cell stage. The caps were cultured to neurula stage 17-18 and RT-PCRs were carried out on RNA from these samples, alongside RNA from whole uninjected embryos. Noggin mediated inhibition of BMP signalling induces *n1-src* expression. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control.

N1-src is therefore expressed early in neuronal development in response to neural induction by BMP inhibitors.

3.2.2. Knockdown of *n1-src* causes a malformed tail phenotype and an aberrant touch response

To investigate *n1-Src* function in the developing *Xenopus* nervous system, antisense morpholino oligos (AMOs) were used to knockdown *n1-src* expression. AMOs are synthetic molecules that are capable of binding to DNA or RNA through complementary base pairing. They are particularly useful in the study of *n1-Src* as they can be designed against specific splice sites in pre-mRNA, binding ahead of the splicing machinery and thereby preventing the inclusion of the targeted exon in the final mature mRNA transcript (Schmajuk, Sierakowska and Kole, 1999; Draper, Morcos and Kimmel, 2001; Eisen and Smith, 2008). Fluorescently tagged AMOs were designed, by Gene Tools (<https://www.gene-tools.com/>) against the splice acceptor (AMO A) and splice donor (AMO D) sites of the *n1-src* microexon in the *src* pre-mRNA of *X. tropicalis* (Figure 3.4).

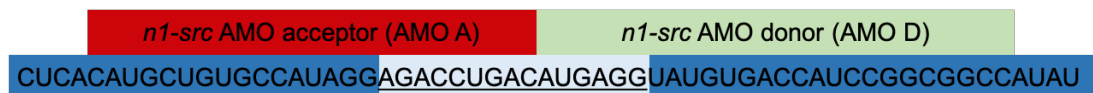


Figure 3.4. *Xenopus tropicalis* *n1-src* microexon skipping morpholinos. Exon skipping morpholinos, designed by Phil Lewis (Lewis et al. 2017), used in combination bind to the splice acceptor and splice donor site of *src* pre-mRNA, blocking the splicing machinery from including the micro exon. The splice acceptor morpholino is indicated in red and the splice donor morpholino in green. Exons three (left) and four (right) of *src* are in dark blue, and the *n1-src* microexon is light blue.

10ng AMO A+D (10ng each morpholino, 20ng total) was injected into *X. tropicalis* embryos at the one- to two-cell stages. Extraction of RNA from these embryos at neurula stage 14-15, followed by RT-PCR analysis shows that these morpholinos effectively knockdown *n1-src* without affecting *c-src* expression (Figure 3.5).

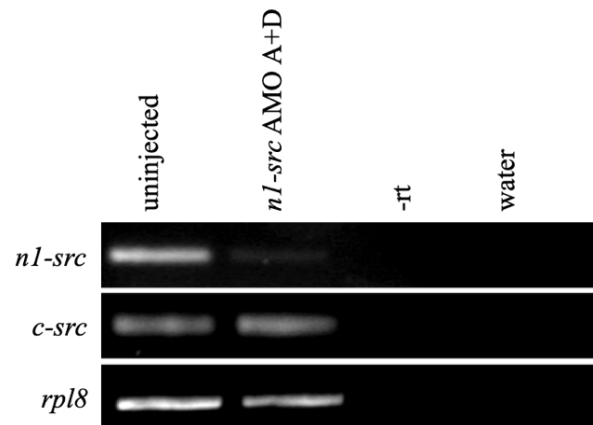


Figure 3.5. RT-PCR analysis showing effective knockdown of *n1-src* by splice blocking morpholinos. RT-PCR analysis of *c-src* and *n1-src* mRNA expression at Stage 16 in control uninjected embryos and embryos injected with a total of 10ng AMO A+D. Injection of the morpholinos leads to a knockdown of *n1-src* expression, without effecting expression of *c-src*. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control.

The culturing of embryos injected with the *n1-src* AMOs revealed a striking developmental phenotype. Injection of 10ng AMO A+D leads to embryos appearing less elongated during the early tailbud stages (~stage 26) than embryos that were uninjected or injected with 20ng of a standard fluorescent control morpholino (CMO) (Figure 3.6). This phenotype is observed in 89% of embryos (n=145). At the late tailbud stages (~stage 41-42) there is a clear and distinct phenotype in the *n1-src* knockdown embryos. These embryos exhibit malformed tails, which appear shorter and kinked in comparison to the long straight tails seen in uninjected or CMO injected embryos (89%, n=145) (Figure 3.6). *N1-src* knockdown embryos also exhibit a loss of retinal pigmentation.

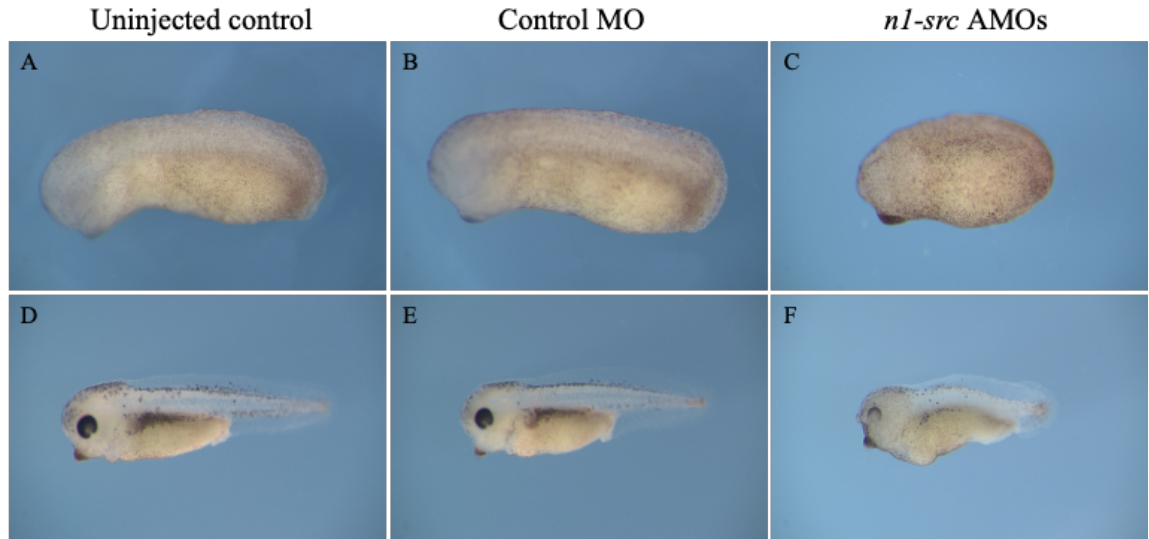


Figure 3.6. Representative phenotype of *n1-src* knockdown embryos. Stage 28 (**A-C**) and stage 41 (**D-F**) *X. tropicalis* embryos. Embryos are uninjected controls (**A and D**) or were bilaterally injected in the animal hemisphere at the 1-2 cell stages with 20ng control morpholino (**B and E**) or 10ng AMO A+D (**C and F**). Knockdown of *n1-src* leads to reduced elongation of embryos at stage 28. At stage 41, *n1-src* knockdown embryos have developed malformed tails that are shortened and/or kinked.

Further examination reveals that embryos exhibiting malformed tails also produce an aberrant touch response. Healthy *Xenopus* embryos, through a simple reflex arc, produce a characteristic movement in response to touch (Roberts, Li and Soffe, 2010). At the early tailbud stage the embryos are found lying on their flank and respond to the stimulus by twitching their posterior region multiple times from side to side but they do not right themselves or propel themselves forward. By the late tailbud stage this response has progressed, and upon sensing touch the embryos will right themselves and using fast, coordinated movements, they will rapidly dart away from the stimulus. Embryos injected with 20ng CMO produce these described responses at both the early tailbud and late tailbud stages in nearly 95% of cases, n=82 (Figure 3.7). In contrast, embryos injected with 10ng AMO A+D fail to show any form of response to touch at the early tailbud stage and by late tailbud stage, the embryos sense the stimulus, however, they are unable to generate a rapid and coordinated response (89% of cases, n=145). Instead of righting themselves and darting away, they remain on their flank and respond with a twitching of the tail that slowly propels them away from the stimulus (FIGURE 3.7/Supplementary videos 1-6). This data contributed to the Lewis *et al.* (2017) paper.

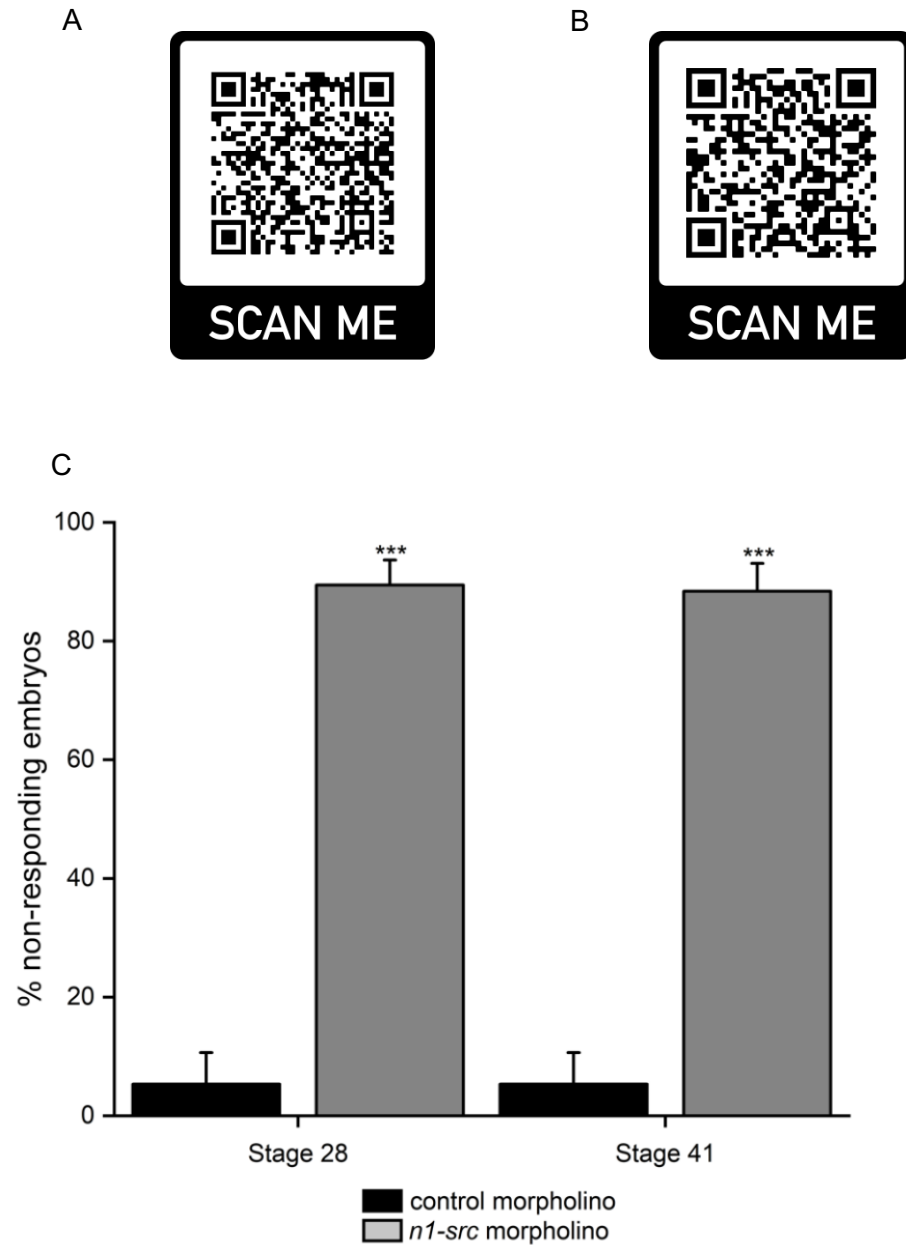


Figure 3.7. Knockdown of *n1-src* leads to aberrant touch responses in *X. tropicalis*. QR Codes for touch response videos from uninjected, CMO injected and *n1-src* morpholino injected embryos at **A)** early and **B)** late tail bud stages. Videos can also be found in the supplementary data. **C)** The touch response of embryos bilaterally injected with 20ng control morpholino or 10ng *n1-src* AMO A+D, tested at early tailbud stage 28 and then late tailbud stage 41. Data is plotted as mean $\pm$ SEM ($n = 3$ independent experiments, analysed by 1-way ANOVA with Tukey's post hoc test, *** $p < 0.001$, control MO vs *n1-Src* AMO).

3.2.3. *N1-src* knockdown inhibits the differentiation of primary neurons

Work in this thesis and Lewis *et al.* (2017) has shown that knockdown of *n1-src* results in embryos unable to respond correctly to an external touch stimulus. We hypothesise that this inability to respond is a result of primary neurons failing to differentiate (Lewis *et al.*, 2017). I have been building on this work, analysing whether *n1-Src* function is required for the differentiation of all three classes of primary neurons, or if it has a more restricted role.

X. tropicalis embryos were unilaterally injected in one-cell at the two-cell stage with 5ng AMO A+D or 10ng CMO. The uninjected hemisphere would then be used as an internal uninjected control. Embryos were fixed at stage 14 and *in situ* hybridisations were carried out to analyse the expression patterns of *n-tubulin* (*tubb2b*), which is expressed in differentiated neurons and can be used to visualise the primary neuron domains in the neural plate (Chitnis and Kintner, 1995).

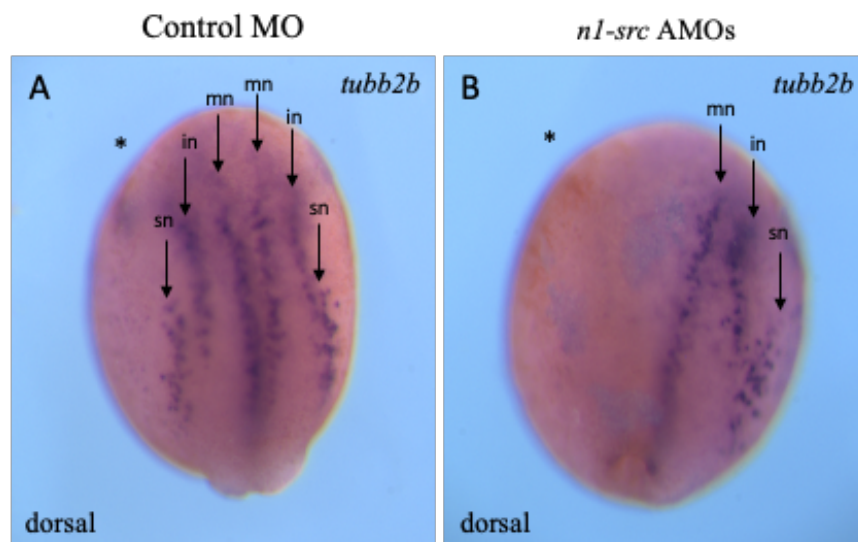


Figure 3.8. *N1-src* knockdown inhibits *tubb2b* expression. Dorsal view of *in situ* hybridisations, anterior to the top. Detecting *tubb2b* expression – a marker for differentiated neurons – in stage 14 *X. tropicalis* embryos unilaterally injected at the two-cell stage with either **(A)** 10ng CMO or **(B)** 5ng *n1-src* AMO A+D. Asterisk indicates the injected hemisphere. Knockdown of *n1-src* leads to reduced *tubb2b* expression and therefore a failure of primary neurons to differentiate. Mn=motor neurons, in=inter neurons, sn=sensory neurons.

There was no noticeable difference in *tubb2b* expression between the two hemispheres in 87% (n=16) of embryos unilaterally injected with the control morpholino (Figure 3.8). In contrast, only 9% (n=35) of embryos unilaterally injected with the *n1-src* AMOs showed no noticeable differences in *tubb2b* expression. The other 91% showed a marked decrease in expression in the injected side, with all three domains of primary neurons appearing affected (Figure 3.8).

Due to complexities with designing primers to analyse *tubb2b* expression by RT-PCR, another marker for differentiated primary neurons was chosen for examination. Like *tubb2b*, myelin transcription factor 1 (*myt1*) is expressed in the three columns of differentiated primary neurons (Bellefroid *et al.*, 1996). *In situ* hybridisations on embryos unilaterally injected with the *n1-src* AMOs show *myt1* expression is reduced or absent in the injected half of the embryos, compared uninjected control half, in 90% of samples (n=10) (Figure 3.9).

Expression of *myt1* following *n1-src* knockdown was further analysed by RT-PCR (Figure 3.9). Embryos were bilaterally injected at the one- and two-cell stages with 10ng AMO A+D or 20ng CMO. RNA was extracted at neurula stage 16 and the expression levels of *myt1* and *rpl8* were examined. Band intensity was quantified using ImageJ. Knockdown of *n1-src* leads to a significant reduction in *myt1* expression compared to control embryos. Expression of *n1-src* is therefore required for the differentiation of primary *Xenopus* neurons.

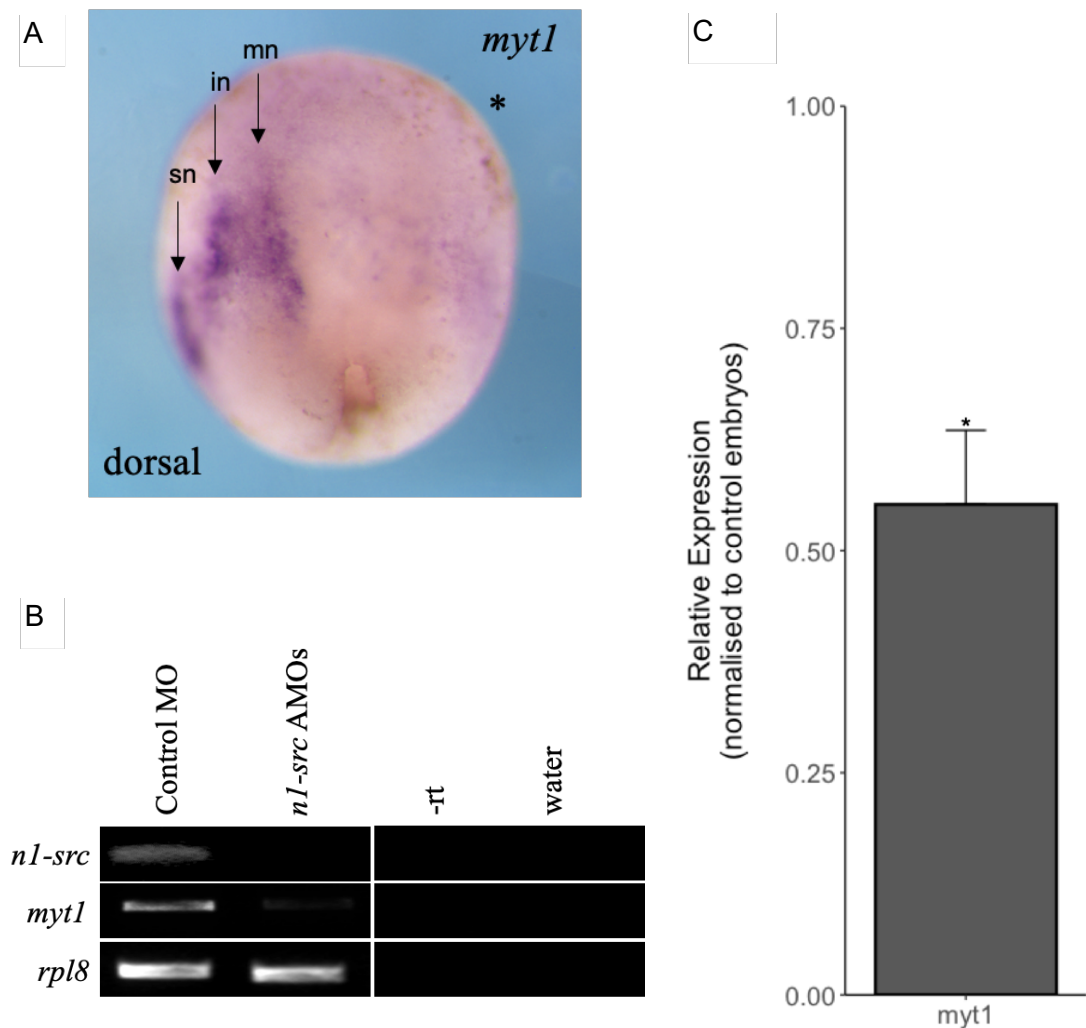


Figure 3.9. *n1-src* knockdown inhibits *myt1* expression. **A)** *in situ* hybridisations for *myt1* expression. Dorsal view of stage 14 *X. tropicalis* embryos, anterior to the top. Embryos were unilaterally injected at the two-cell stage with 5ng AMO A+D. Asterisk indicates the injected side. Knockdown of *n1-src* leads to a loss or reduction of *myt1* expression compared to the uninjected control hemisphere. mn=motor neurons, in=inter neurons, sn=sensory neurons. **B)** RT-PCR analysis of *n1-src* and *myt1* mRNA expression in neurula stage 16 embryos injected with 20ng CMO or 10ng AMO A+D. Knockdown of *n1-src* leads to a reduced expression of *myt1*. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. **C)** Quantification of *myt1* RT-PCR expression levels in *n1-src* knockdown embryos, normalised to control embryos (Control =1.0). Data is plotted as mean $\pm$ SEM (n = 3 independent experiments, analysed by t-test, *p < 0.05).

3.2.4. *N1-src* knockdown and regional specificity markers

The *tubb2b* and *myt1* *in situ* hybridisations show the loss of all three classes of primary neurons in the developing *Xenopus* embryo following *n1-src* knockdown. To confirm this, the expression levels of the regional specificity markers *nkx6.1*, *gsx2* and *msx1*, which mark the motor, inter and sensory neurons respectively (Illes, Winterbottom and Isaacs, 2009), were analysed by RT-PCR. These are homeobox genes involved in the development and patterning of the primary neuron columns.

RT-PCRs were carried out on RNA from embryos injected with 10ng AMO A+D or 20ng CMO, and gel pictures of a representative experiment are shown (Figure 3.10). Band intensities were quantified using ImageJ. *Msx1* and *nkx6.1* are significantly downregulated in *n1-src* knockdown embryos compared to control embryos, however, when assayed this way *gsx2* is not significantly downregulated. At the stage analysed, *nkx6.1* and *msx1* are expressed in the differentiated motor and sensory neurons. The significant downregulation in their expression further supports the role of *n1-Src* in neuronal differentiation.

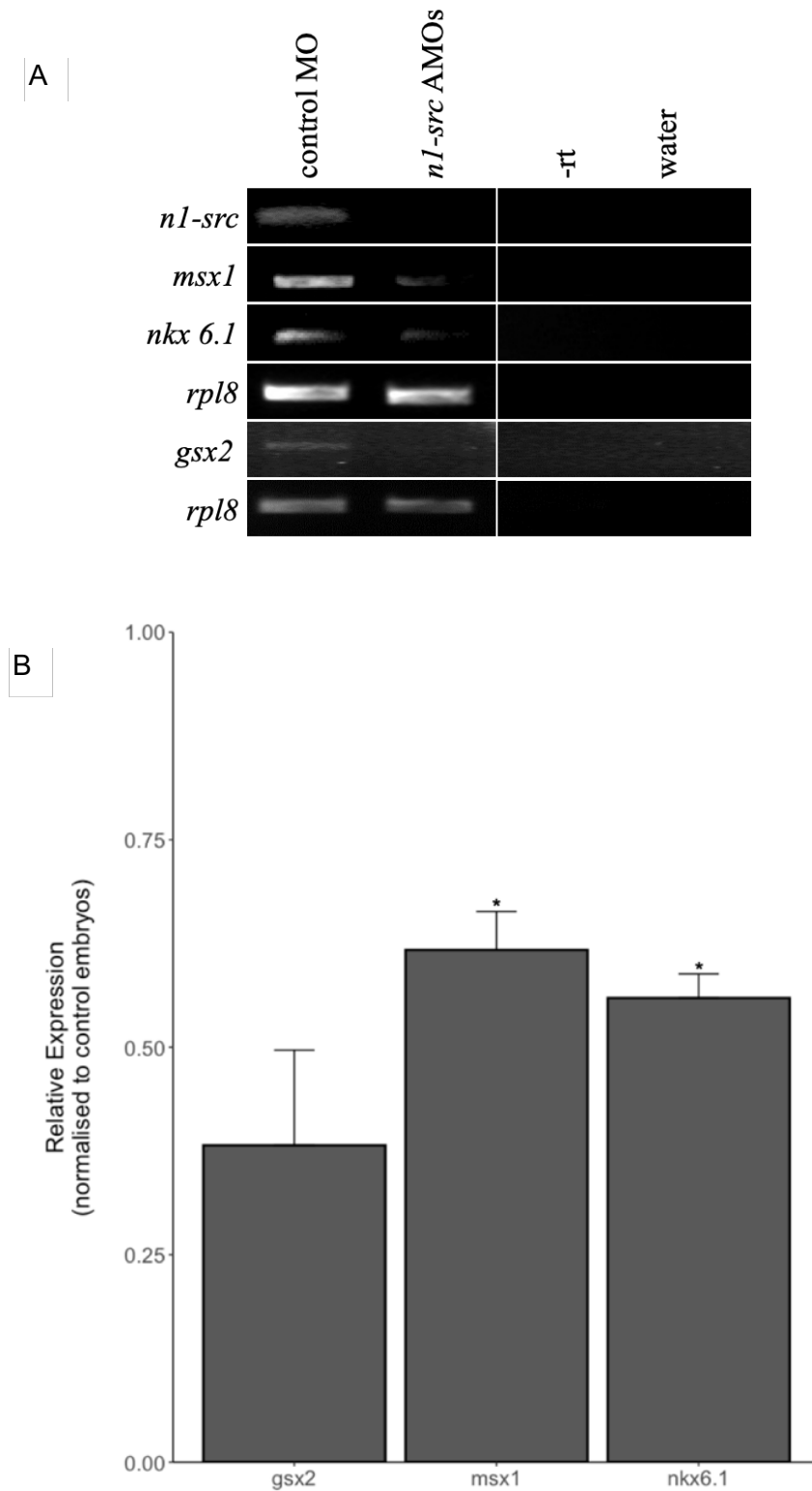


Figure 3.10. Effects of *n1-src* knockdown on expression of regional specificity markers. **A)** RT-PCR analysis of *msx1*, *nkx6.1* and *gsx2* expression in neurula stage 16 embryos injected with 20ng CMO or 10ng AMO A+D. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. **B)** Quantification of RT-PCRs examining *msx1*, *nkx6.1* and *gsx2* expression levels in *n1-src* knockdown embryos, normalised to control embryos (Control = 1.0). Data is plotted as mean $\pm$ SEM ($n = 3$ independent experiments, analysed by t-test, $*p < 0.05$).

3.2.5. N1-Src functions between the neural prepatter and proneural genes

The differentiation of neurons is the result of a cascade of transcriptional activation and repression that begins with the expression of neural inductive genes, followed by neural prepatter genes, then proneural genes and finally neurogenic genes (Mizuseki, Kishi, Matsui, *et al.*, 1998; Bylund *et al.*, 2003; Rogers *et al.*, 2009). To examine where n1-Src functions in this cascade, RNA was extracted at stage 14 from *X. tropicalis* embryos bilaterally injected with 10ng AMO A+D or 20ng CMO. RT-PCRs were carried out to examine the expression levels of the neural prepatter genes *sox2*, *sox3* and *irx1*, and the proneural gene *neurod1*. At stage 14, *sox2*, *sox3* and *irx1* are expressed widely throughout the neural plate, like *n1-src* (Schlosser and Ahrens, 2004). *Neurod1* is expressed downstream of these prepatter genes, and has an expression profile like *tubb2b*, with three columns of expressing cells either side of the neural midline (Cao, Zhao and Grunz, 2002).

Knockdown of *n1-src* does not significantly change the expression levels of the *sox2*, *sox3* or *irx1*, though there is a trend for these to increase in expression. However, there is a significant reduction in the expression of *neurod1* (Figure 3.11). The lack of changes in expression of neural prepatter genes indicates that n1-Src acts downstream of these in the neurogenic cascade, however it acts upstream of the proneural genes, as shown by the significant decrease in *neurod1* expression levels.

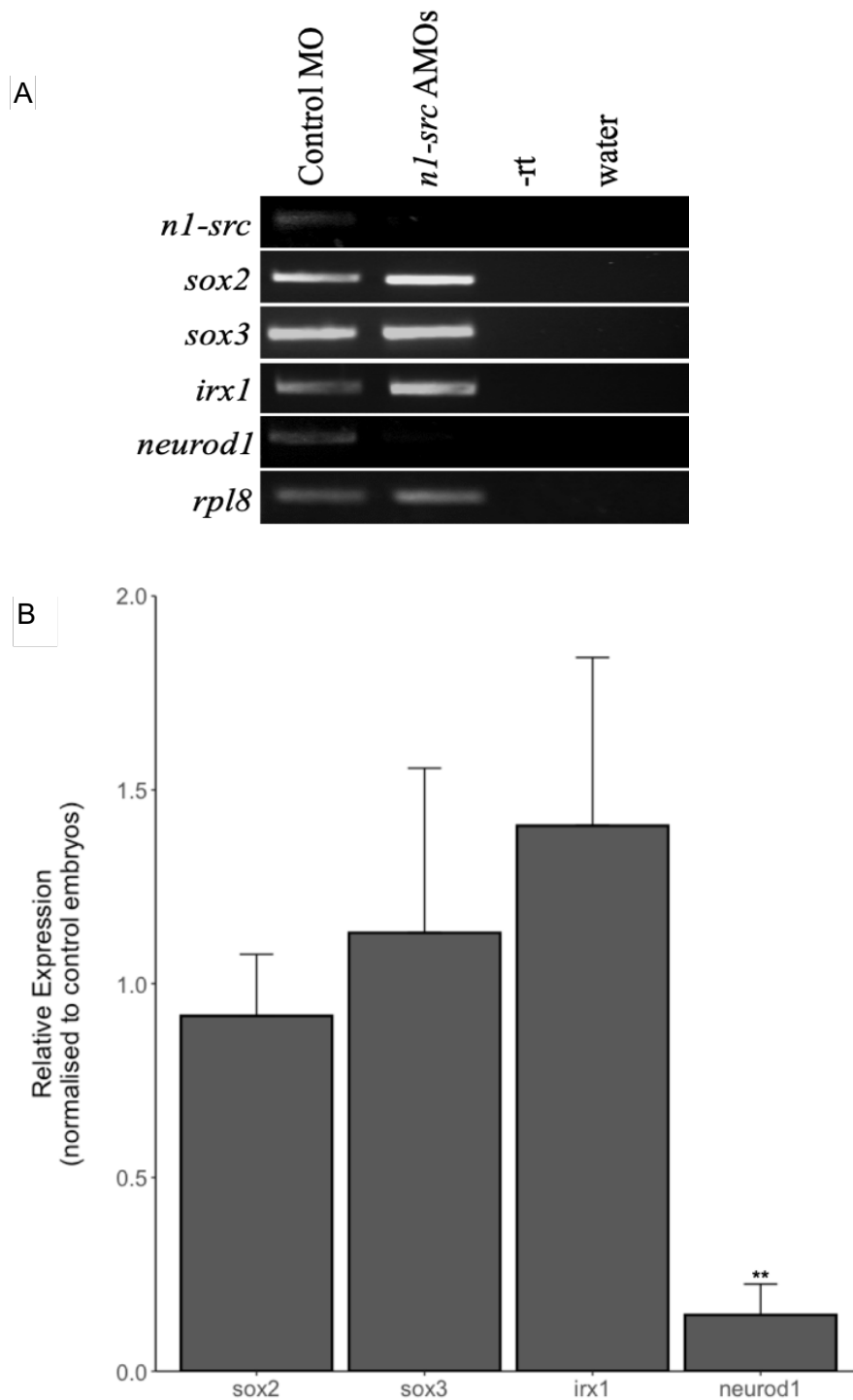
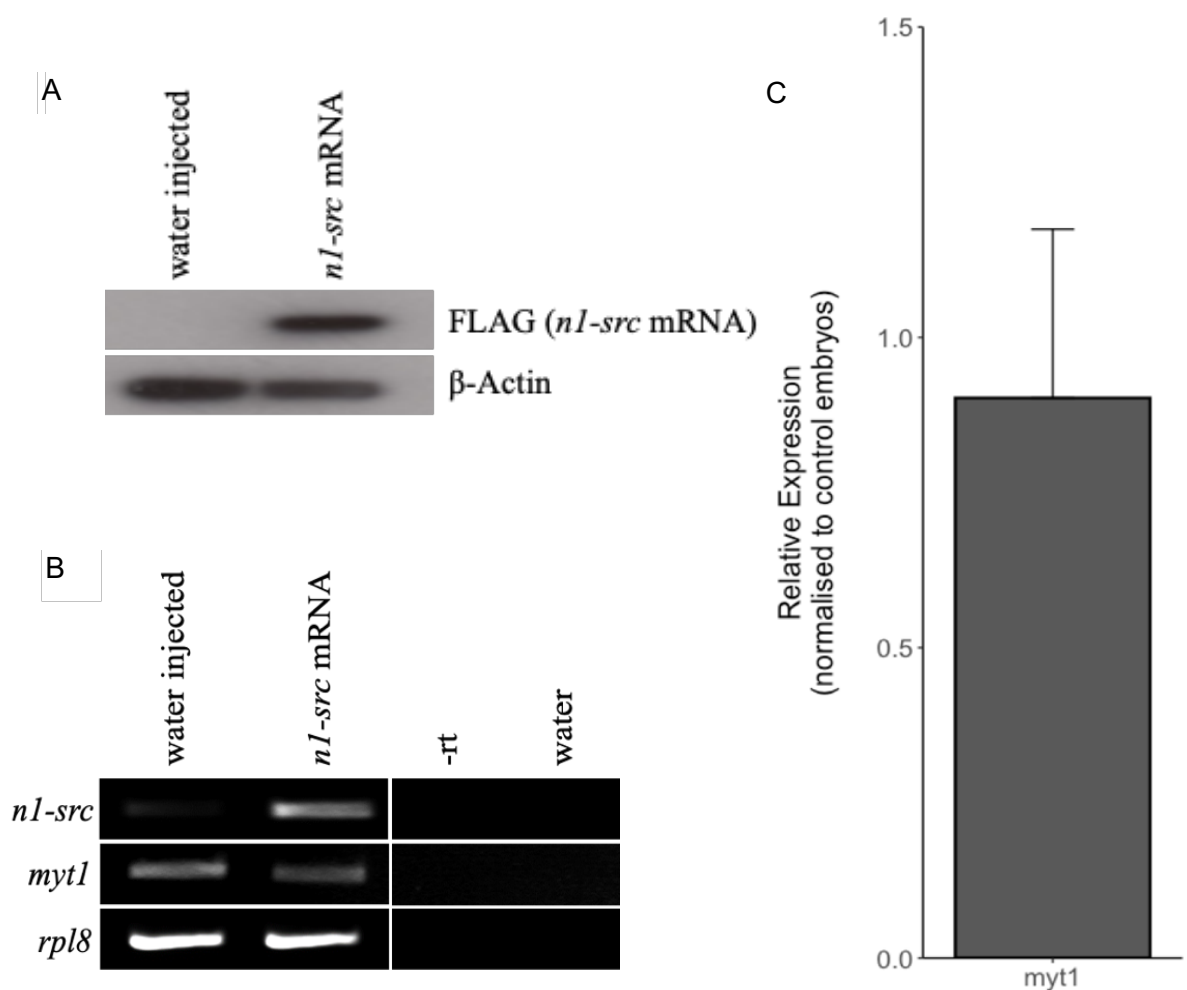


Figure 3.11. Effects of *n1-src* knockdown on expression of neurogenic pathway genes. A) RT-PCR analysis of *sox2*, *sox3*, *irx1* and *neurod1* expression in neurula stage 14 embryos injected with 20ng CMO or 10ng AMO A+D. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. Knockdown of *n1-src* leads to reduction in *neurod1* expression. **B)** Quantification of RT-PCRs examining *sox2*, *sox3*, *irx1* and *neurod1* expression levels in *n1-src* knockdown embryos, normalised to control embryos (Control = 1.0). Data is plotted as mean $\pm$ SEM (n = 3 independent experiments, analysed by t-test, **p < 0.01)

3.2.6. *N1-src* overexpression does not affect the expression of neuronal markers

I next investigated the effect that overexpressing synthetic FLAG-tagged *Xenopus n1-src* mRNA would have on the development of *X. tropicalis* embryos. *X. tropicalis* embryos were injected, at the one- and two-cell stages with 250pg of mRNA or the equivalent volume of water. To confirm translation of the FLAG-tagged mRNA, stage 11 embryos were analysed by western blotting. No FLAG signal is observed in the water injected samples, but a band is clearly present in the *n1-src* expressing embryos (Figure 3.12).

RT-PCRs were then carried out on RNA extracted from embryos at stage 16 to examine the relative expression levels of the regional specificity markers – *nkx6.1*, *gsx2* and *msx1* – and the differentiated neuron marker, *myt1*. Gel images of representative experiment are shown (Figure 3.12). Whilst *msx1*, *nkx6.1* and *myt1* were significantly downregulated following *n1-src* knockdown, the overexpression of *n1-src* causes no significant changes in the expression levels of any of the examined genes.



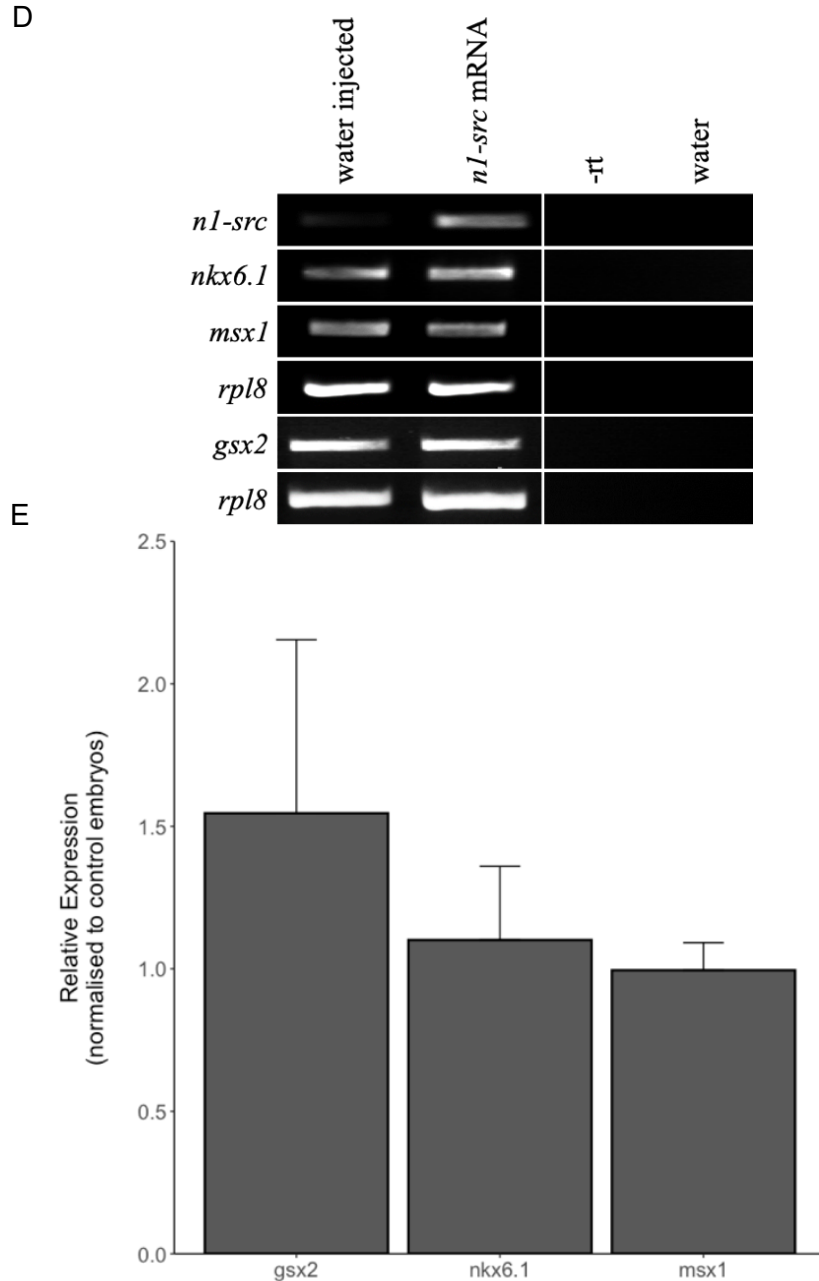
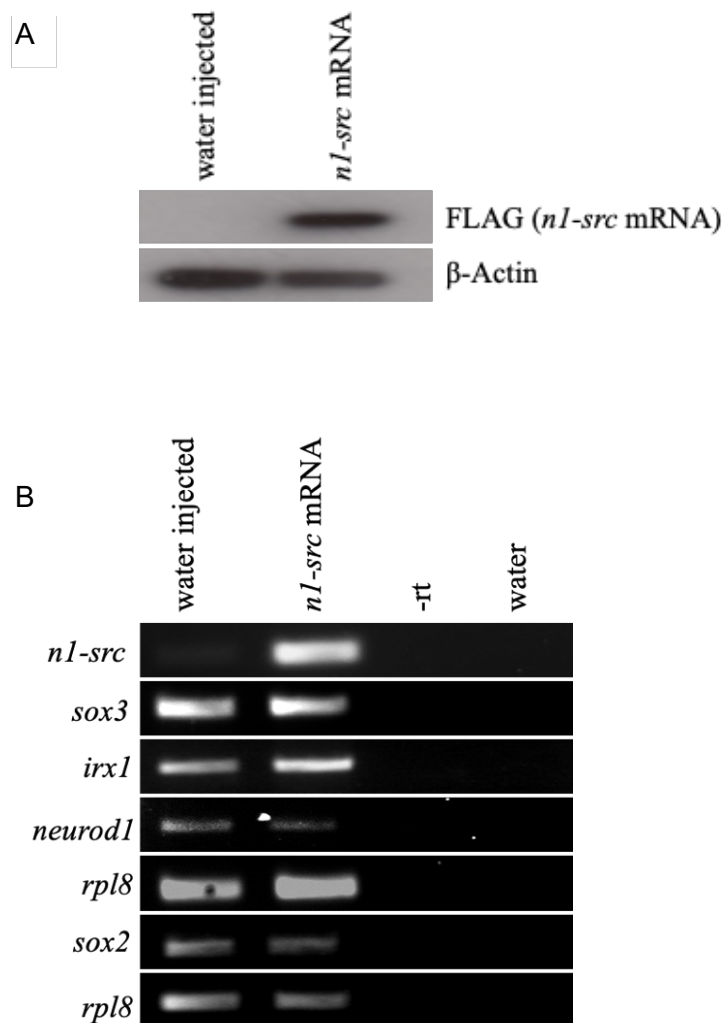


Figure 3.12. Effects of *n1-src* overexpression on expression of neuron markers. A) Western blot probing for FLAG epitope in stage 11 embryos injected with 250pg FLAG-tagged *n1-src* mRNA or the equivalent volume of water. **B)** RT-PCR analysis for differentiated neuron marker, *myt1*, in neurula stage 16 embryos injected with 20ng CMO or 20ng AMO A+D. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. **C)** Quantification of RT-PCRs examining *myt1* expression levels in *n1-src* knockdown embryos, normalised to control embryos (Control = 1.0). Data is plotted as mean $\pm$ SEM (n = 3 independent experiments, analysed by t-test). **D)** RT-PCR analysis for regional specificity markers *nkx6.1*, *msx1* and *gsx2* in neurula stage 16 embryos injected with 20ng CMO or 20ng AMO A+D. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. **E)** Quantification of RT-PCRs examining *nkx6.1*, *msx1* and *gsx2* expression levels in *n1-src* knockdown embryos, normalised to control embryos (Control = 1.0). Data is plotted as mean $\pm$ SEM (n = 3 independent experiments, analysed by t-test).

3.2.7. *N1-src* overexpression does not affect the expression of proneural genes

Embryos were bilaterally injected with 250pg FLAG-tagged *n1-src* mRNA or the equivalent volume of water. Western blot analysis on stage 11 embryos confirms that the mRNA is translated (Figure 3.13).

RT-PCRs were then carried out on stage 14 embryos to examine the expression levels of the neurogenic pathway genes – *sox2*, *sox3*, *irx1* and *neurod1* – in water injected or *n1-src* overexpressing embryos. Whilst *n1-src* knockdown significantly reduced the expression of *neurod1*, *n1-src* overexpression causes no significant change in the expression of any of the examined markers (Figure 3.13).



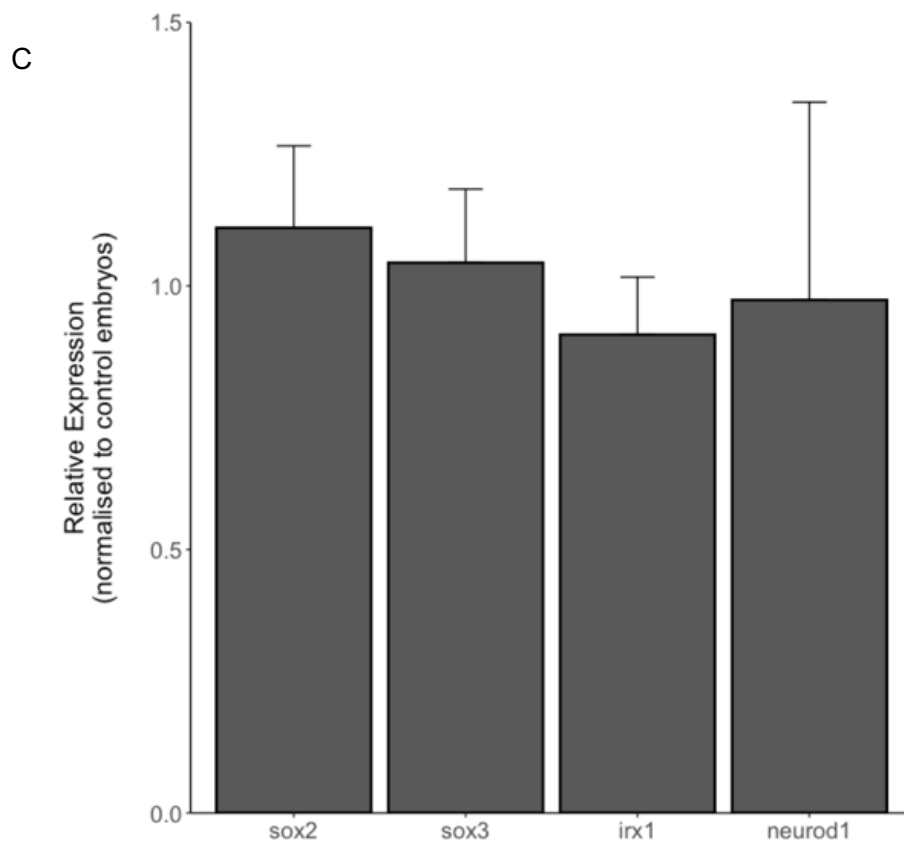


Figure 3.13. Effects of *n1-src* overexpression on expression of neurogenic pathway genes. **A)** Western blot probing for FLAG epitope in stage 11 embryos injected with 250pg FLAG-tagged *n1-src* mRNA or the equivalent volume of water. **B)** RT-PCR analysis for neurogenic pathway markers *sox2*, *sox3*, *irx1* and *neurod1* in neurula stage 14 embryos injected with 20ng CMO or 20ng AMO A+D. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. **C)** Quantification of RT-PCRs examining *sox2*, *sox3*, *irx1* and *neurod1* expression levels in *n1-src* knockdown embryos, normalised to control embryos (Control =1.0). Data is plotted as mean $\pm$ SEM ($n = 3$ independent experiments, analysed by t-test).

3.2.8. Overexpression of *n1-src* leads to posteriorisation of *Xenopus* embryos

Embryos were bilaterally injected at the one- and two-cell stages with varying concentrations of mRNA and allowed to develop to late tailbud stage 41. 83% of uninjected control embryos developed correctly (n=45). Meanwhile, overexpression of 125pg *n1-src* mRNA lead to developmental defects in 69% of embryos (n=49) (Figure 3.14). Embryos showed crano-facial defects, particularly issues with eye formation. Injection of 250pg leads to a more pronounced reduction in axial development and elongation in 90% of embryos (n=42).

To confirm that the injected mRNA is being translated, western blots were carried out on lysates from five embryos from each condition that had been flash frozen at neurula stage 14 (Figure 3.14).

Defects in the development of the anterior-posterior axis, and particularly in the development of the more anterior structures, is a phenotype also observed following the overexpression of *fgf4* in *Xenopus* embryos (Isaacs, Pownall and Slack, 1994).

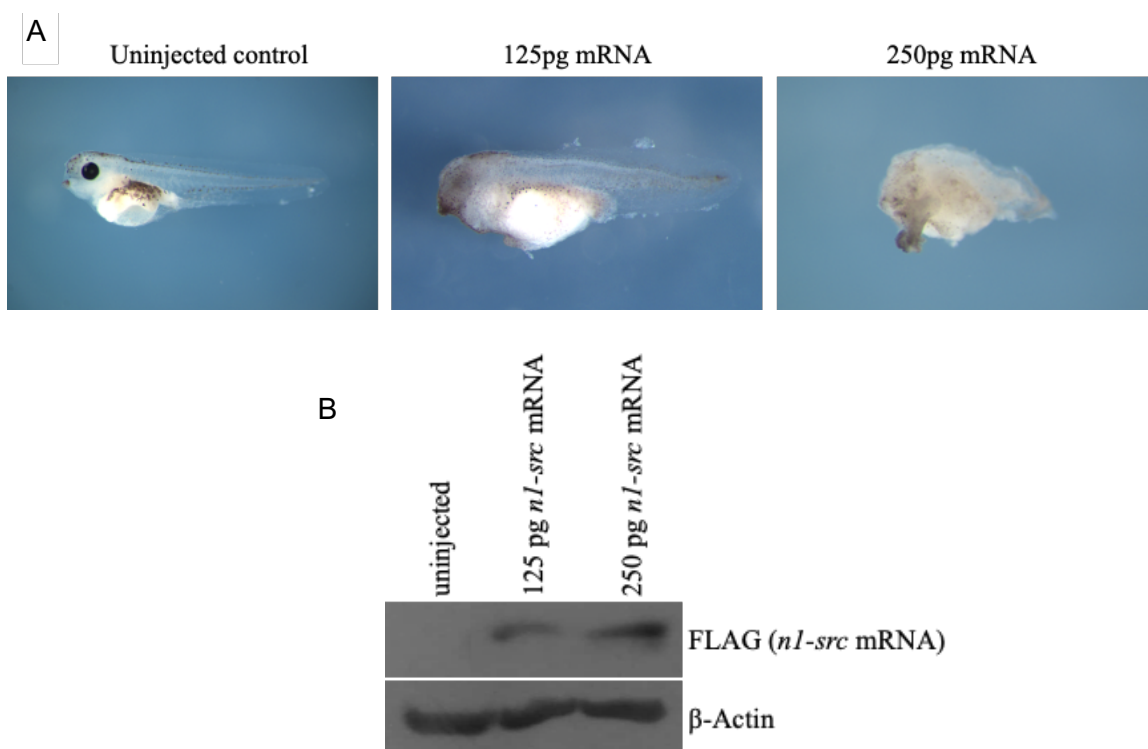


Figure 3.14. Effects of *n1-src* overexpression on *X. tropicalis* development. A) Representative phenotypes of uninjected control *X. tropicalis* embryos and embryos injected with increasing concentrations of synthetic FLAG-tagged *n1-src* mRNA. Embryos were injected at the one- to two-cell stage with 125 or 250pg of mRNA. Overexpression of *n1-src* leads to defects in development of the anterior-posterior axis. **B)** Western blot probing for FLAG epitope in stage 14 embryos injected with 125pg or 250pg FLAG-tagged *n1-src* mRNA, and uninjected embryos.

3.2.9. N1-Src regulates Erk phosphorylation in *Xenopus*

Based on the phenotype similarities in *n1-src* and *fgf4* overexpressing embryos, I decided to investigate whether n1-Src can interact with the Fgf regulated MAPK signalling pathway. Fgf signalling is required for patterning of the amphibian nervous system, particularly posterior patterning (Lamb and Harland, 1995; Holowacz and Sokol, 1999), whilst some studies have also implicated Fgf signalling in neural induction (Alvarez, Araujo and Nieto, 1998; Hongo, Kengaku and Okamoto, 1999; Delaune, Lemaire and Kodjabachian, 2005). Activation of the Fgf pathway leads to the phosphorylation of MAPK/Erk (Ornitz and Itoh, 2015), and the level of Erk phosphorylation was used to determine if n1-Src interacts with or regulates the Erk pathway

Western blotting was carried out for phosphorylated Erk on uninjected control *X. tropicalis* embryos and embryos injected with 10ng AMO A+D (Figure 3.15). The knockdown of *n1-src* expression results in a significant decrease in the amount of phosphorylated Erk.

Furthermore, whole mount immunohistochemistry was carried out on stage 14 *X. tropicalis* embryos injected with 5ng AMO A+D or 10ng CMO, to detect phosphorylated erk. At this stage phosphorylated Erk is detected in three domains; the midbrain-hindbrain boundary, the anterior neural ridge and the branchial arches (Figure 3.16). The level of phosphorylated Erk detected in these domains is reduced in the *n1-src* knockdown embryos compared to those injected with the control morpholino (100%, n=12).

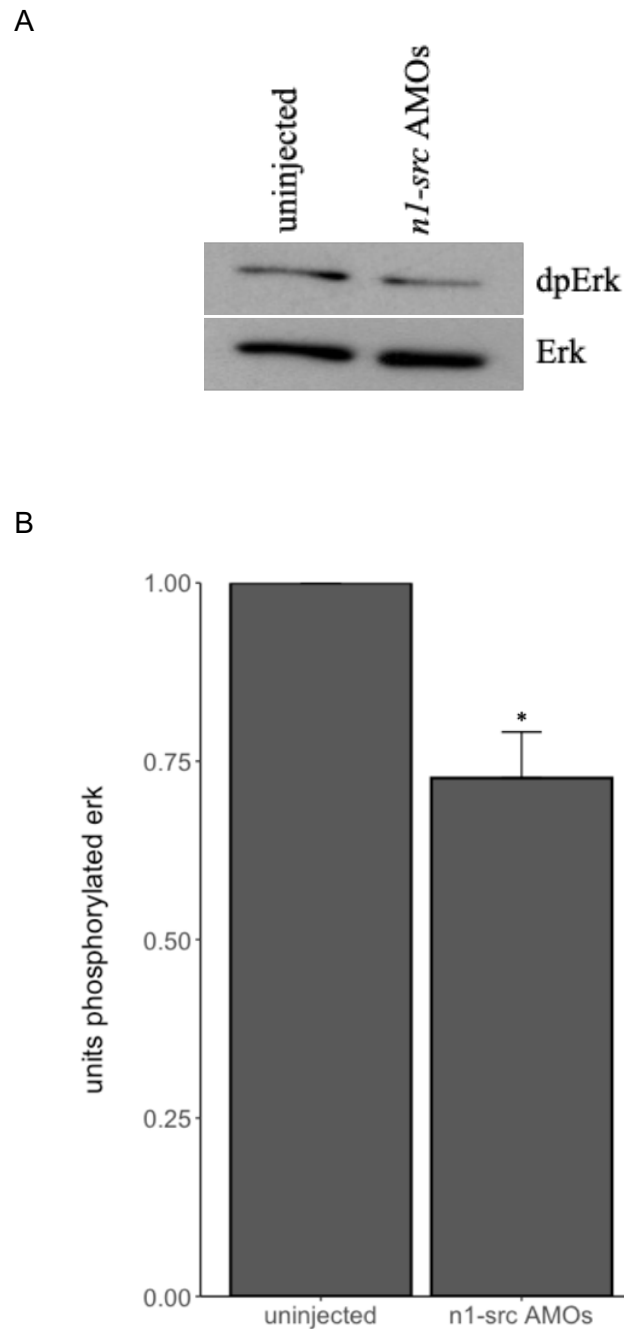


Figure 3.15. Knockdown of *n1-src* downregulates Erk phosphorylation **A)** Western blot on stage 14 *X. tropicalis* uninjected embryos and embryos injected with 10ng AMO A+D. Membranes were blotted for di-phospho-Erk and total Erk levels were used as a loading control. **B)** Quantification of western blots examining the levels of phosphorylated Erk compared to total Erk levels in uninjected and *n1-src* knockdown samples. Data is plotted as mean $\pm$ SEM ($n = 5$ independent experiments, analysed by t-test, * $p < 0.05$)

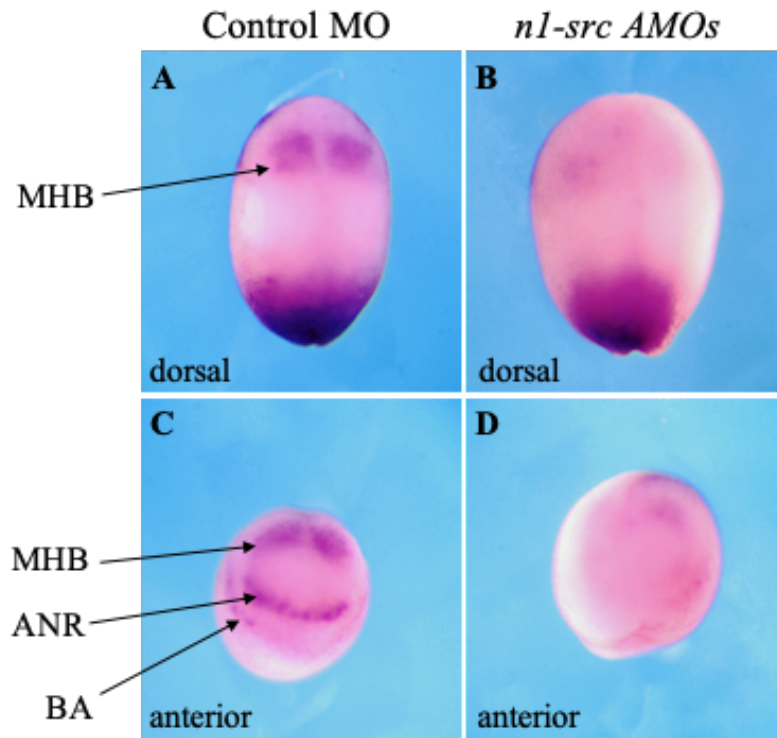


Figure 3.16. Knockdown of *n1-src* downregulates Erk phosphorylation in *X. tropicalis* embryos. Immunostaining of stage 14 neurula embryos with an antibody against phosphorylated Erk. **A-B)** Dorsal view of embryos, anterior to the top. **C-D)** Anterior view, dorsal to the top. Embryos were injected at the one-cell stage with 10ng CMO or 5ng AMO A+D. Embryos injected with *n1-src* morpholinos show reduced levels of phosphorylated Erk compared to controls. MHB=Midbrain-hindbrain border, ANR=Anterior neural ridge, BA=Branchial arches.

To test whether overexpressing *n1-src* leads to increased levels of Erk phosphorylation, the *X. laevis* animal cap assay was used. *X. laevis* embryos were injected at the two and four-cell stages with 500pg of FLAG-tagged *n1-src* mRNA or 5pg of *fgf4* mRNA. Animal caps were dissected from injected embryos and uninjected controls at blastula stage 8/9 and cultured to stage 12 when they collected and analysed by western blot. The overexpression of either *fgf4* or *n1-src* in *X. laevis* animal caps leads to the upregulation of Erk phosphorylation (Figure 3.17).

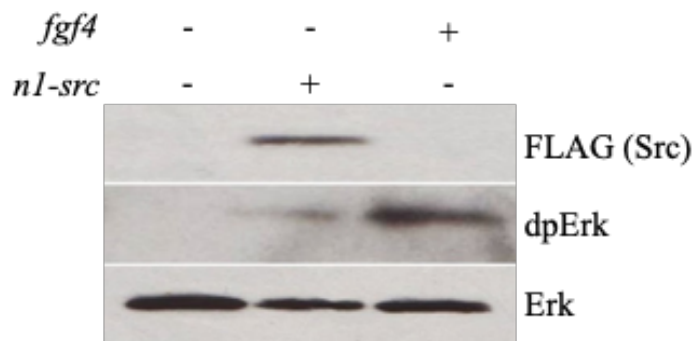


Figure 3.17. *N1-src* overexpression upregulates Erk phosphorylation in *X. laevis*. Western blots on stage 12 animal caps dissected from *X. laevis* embryos injected with 500pg FLAG-tagged *n1-src* mRNA or 5pg of *fgf4* mRNA, or from uninjected control embryos. Membranes were probed for the FLAG tagged n1-Src and di-phospho-Erk. Total Erk was probed as a loading control.

3.2.10. N1-Src can activate the mapk signalling pathway independently of Fgf ligands

I next wanted to determine whether n1-Src requires extracellular Fgf signalling to regulate Erk phosphorylation, or whether it activates the intracellular pathway leading to Erk activation. *X.laevis* embryos were injected at the two-cell stage with 2ng of *dn-fgfr1* mRNA, a dominant negative Fgf receptor that inhibits Fgf signalling, 1ng of FLAG-tagged *n1-src* mRNA or a combination of the two. Animal caps were dissected from uninjected embryos, and those injected with *n1-src* or *n1-src/dn-fgfr1* at stage 8/9 and cultured to stage 11. These caps were collected along with control uninjected embryos and *dn-fgfr1* injected embryos and levels of phosphorylated Erk were analysed by western blots (Figure 3.18).

Phosphorylated Erk levels are reduced in *dn-fgfr1* injected embryos relative to uninjected samples demonstrating that *dn-fgfr1* downregulates Fgf signalling. In the animal cap samples, overexpression of *n1-src* upregulates Erk phosphorylation when compared to caps from uninjected samples. This upregulation is still present in caps from embryos treated with both *n1-src* and *dn-fgfr1* mRNA, indicating that n1-Src activates the intracellular signalling pathway and this activation does not require extracellular Fgf signalling.

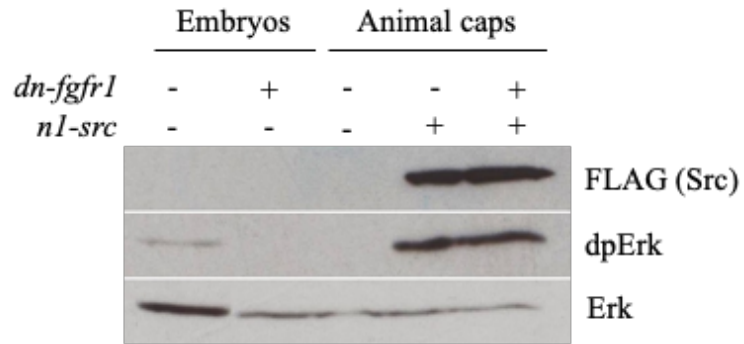


Figure 3.18. N1-src functions downstream of Fgf receptors to regulate Erk phosphorylation. *X. laevis* embryos were injected at the two-cell stage with 2ng dominant-negative Fgf receptor mRNA and/or 1ng *n1-src* mRNA. Western blot analysis was carried out at stage 11 on whole embryos and dissected animal caps, injected as indicated. Membranes were probed for the FLAG epitope - indicating translation of exogenous n1-Src - and for di-phospho-Erk. Total Erk was probed as a loading control. Inhibition of Fgf signalling does not prevent n1-Src from upregulating phosphorylation of Erk.

3.2.11. *N1-src* expression upregulates Erk phosphorylation in mammalian cells

The effects of expressing *n1-src* in mammalian cells was investigated to determine if *n1-Src* interacts with Erk signalling in a different system. A stable, inducible *n1-src* expressing cell line was utilised. This cell line expresses FLAG-tagged mammalian *n1-src* upon the addition of doxycycline (Lewis, 2014). Expression of *n1-src* was induced for a period, from three to 48 hours, and then cells were lysed and the level of phosphorylated Erk was examined via western blotting (Figure 3.19).

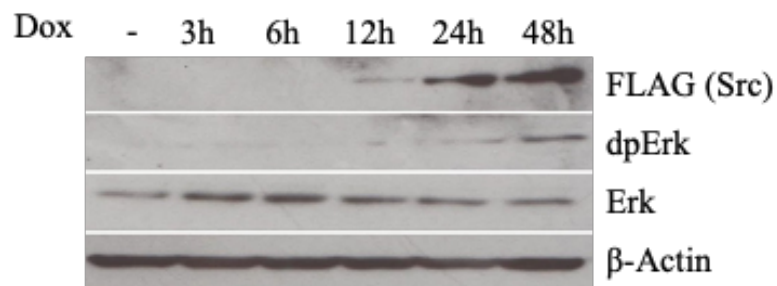


Figure 3.19. *N1-src* upregulates Erk phosphorylation. Inducible mammalian *n1-src* expressing HeLa cells were treated with doxycycline for up to 48 hours, to induce expression of *n1-src*. Cells were lysed in Laemmli SDS sample buffer and western blots were carried out probing for the FLAG epitope, marking translated *n1-Src* protein, and di-phospho-Erk. Total Erk levels and β -Actin were used as loading controls. An increase in *n1-src* expression is accompanied by an upregulation of Erk phosphorylation.

FLAG-tagged *n1-Src* is first detected after 12 hours of doxycycline treatment, and expression increases after 24 and 48 hours of treatment. The level of Erk phosphorylation increases in line with the increase of *n1-src* expression. The data indicates that *n1-Src* interacts with the MAPK pathway to upregulate Erk phosphorylation, both in mammalian cell culture and in *Xenopus* embryos.

3.3. Discussion

The work in this chapter has shown that n1-Src is essential for the differentiation of primary neurons in the vertebrate model organism, *X. tropicalis*. The use of splice blocking antisense morpholino oligos to block inclusion of the *n1-src* microexon leads to a loss of differentiated neurons at neurula stage 14 and an impaired touch response at the later stage 41. N1-Src activity appears to be required for proneural gene expression, but is acting downstream of neural prepatter genes in the neurogenic pathway. The use of *Xenopus* embryos and a mammalian cell line has revealed that n1-Src can regulate the intracellular Fgf/Erk signalling pathway, downstream of the Fgf receptor.

3.3.1. *N1-src* knockdown causes morphological defects

Knockdown of *n1-src* causes embryos to develop shorter and/or kinked tails. Outgrowth of the *Xenopus* tail bud commences around stage 27 but requires signalling between various regions as early as stage 13/14 (Beck and Slack, 2001). These regions have been identified in *X. laevis* as the most posterior 100 µm of the neural plate, the neural plate anterior to this, and the underlying mesodermal tissue (Tucker and Slack, 1995). As n1-Src is required for correct development within the neural plate, its loss in embryos could impact on the signalling between these regions and lead to defects in tail formation. Furthermore, correct elongation of the *Xenopus* tail involves convergent extension, a process that requires activity of the wnt/planar cell polarity pathway, whose disruption leads to posterior tail defects in stage 30 tail bud embryos (Goto and Keller, 2002). The neural plate is also a site of convergent extension (Tada and Heisenberg, 2012). It could therefore be that the posterior tail defects seen following *n1-src* knockdown is a result of deregulation of the signalling pathways involved in convergent extension. Posterior tail defects are also observed following inhibition of Fgf signalling by dominant-negative fgf receptors (Isaacs, Pownall and Slack, 1994). The authors postulated that this phenotype was a result of interference with extension movements in the developing embryo.

N1-src knockdown embryos also exhibit eye defects, with a lack of retinal pigmentation. Development of the eye begins in the anterior neural plate where a patch of cells – the “eye field” – forms (Fuhrmann, Zou and Levine, 2014). This cluster of neuroepithelial cells will give rise to the optic stalk, the retina and the retinal pigment epithelium. It could be that n1-Src regulates, not only the differentiation of primary neurons, but the differentiation of a multitude of different cells from the neuroepithelium, include those that will give rise to the retina and retinal pigment epithelium.

3.3.2. Knockdown of *n1-src* causes locomotive defects

Alongside visibly kinked and/or shortened tails, knockdown of *n1-src* causes sensory defects in *X. tropicalis* embryos with embryos unable to execute their characteristic dart response when stimulus is applied to their tails. The touch response and subsequent swimming of *Xenopus* embryos involves the coordinated activity of sensory, inter and motor neurons, therefore the lack of coordinated response is most likely due to inhibition of their differentiation at stage 14 (Roberts, Li and Soffe, 2010). By stage 41/42, embryos are able to generate a response, however it is aberrant and uncoordinated. Morpholinos function transiently, therefore the presence of an aberrant touch response at later stages could potentially be due to the return of *n1-src* expression, leading to the differentiation of primary neurons occurring much later in development and to the formation of incomplete neuronal circuits (Timme-Laragy, Karchner and Hahn, 2012; Ferguson, Schmitt and Lightfoot, 2013; Moulton, 2016). Indeed, preliminary RT-PCR analysis carried out on *n1-src* knockdown embryos at a variety of stages, detects the return of *n1-src* expression in embryos from stage 29 onwards (data not shown).

N1-src knockdown embryos may also be experiencing neuron myelination defects. Myelination begins around stage 42 in *Xenopus*, and therefore aberrant touch response seen could be due to oligodendrocytes failing to myelinate neurons correctly (Yoshida, 1997). Furthermore, motor neurons and oligodendrocytes share the same precursor cells, therefore incomplete neuron myelination is a possible defect (Park *et al.*, 2002). However, whilst it may be a potential defect, it can be ruled out as the sole or main source of the aberrant touch response due to the observed phenotype at stage 28.

3.3.3. N1-Src is required for differentiation of *X. tropicalis* primary neurons

Multiple studies, carried out both *in vivo* and *in vitro*, have linked the expression of *n1-src* with differentiation of cells. Increased levels of *n1-src* expression and kinase activity has been observed in differentiated neurons compared to undifferentiated precursors, and peaks in expression and activity in embryogenesis coincide with periods of organogenesis (Levy *et al.*, 1984; Scharf and Barnekow, 1984; Fults *et al.*, 1985; Cartwright *et al.*, 1987). Whilst many of these studies show that *n1-src* expression coincides with differentiation, they do not determine whether n1-Src is a driving force or its expression is a result of cellular differentiation.

Early work investigating *n1-src* in *Xenopus* development suggested that its expression was a result of neuronal differentiation. Expression was detected in stage 15 neural plate *X. laevis* embryos, but expression was absent from stage 11 gastrula embryos (Collett and Steele, 1992). Genes expressed as an early response to neural induction, like N-CAM, are first detected in gastrula stage 10-12 embryos, and by neurula stage 14, differentiated neurons can be detected in the neural plate. As *n1-src* was first detected at stage 15, it suggests that it is unlikely to function in early differentiation.

However, it was later seen that treatment of ectodermal animal caps with the PKC activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), a known neural inducer, also rapidly induces *n1-src* expression (Collett and Steele, 1993). Due to this rapid induction of *n1-src* expression it was suggested that n1-Src functions much earlier in neural development than previously thought and could be involved in early neural differentiation. This is supported by the observed expression of *n1-src* in stage 11-12 *X. tropicalis* embryos (Lewis *et al.*, 2017).

The results presented in this chapter confirm that *n1-src* is expressed in response to neural induction and its function is essential for early neuronal differentiation. Induction of neural tissue in *X. laevis* animal caps via BMP inhibition leads to *n1-src* expression, and knockdown of *n1-src* inhibits neuronal differentiation, as shown by *in situ* hybridisations for *tubb2b* in stage 14 *X. tropicalis* embryos. Interestingly, the inhibition of retinoic acid signalling in *Xenopus* embryos leads to a similar lack of differentiated primary neurons (Blumberg *et al.*, 1997). Retinoic acid drives differentiation of cultured neuroblastoma cell lines and is used as a treatment for neuroblastoma patients (Sidell, 1982; Reynolds *et al.*, 2003; Maris *et al.*, 2007). Overexpression of neuronal srcs can induce neuritogenesis of neuroblastoma cells *in vivo* (Lewis, 2014). The shared lack of differentiated neurons seen in *n1-src* knockdown and RA inhibited embryos, and the similar effects on neuroblastoma cells suggests a potential interaction between n1-Src and members of retinoic acid regulated pathways.

3.3.4. N1-Src's position in the neurogenic pathway

A hierarchical cascade of gene expression and repression follows neural induction, leading to differentiation of *Xenopus* primary neurons. Within this pathway are a cluster of proneural genes. These genes, like *neurod1*, are basic helix-loop-helix transcription factors that are necessary for neuronal differentiation in both vertebrates and invertebrates (Bertrand, Castro and Guillemot, 2002). Within *Xenopus*, *neurod1* is

expressed in the neural plate at stage 14 in the recently differentiated primary neurons and in later tail bud stages expression is detected transiently in central and peripheral nervous system neurons at the time of their terminal differentiation into mature neurons (Lee *et al.*, 1995). Furthermore, ectopic overexpression of *neurod1* in *Xenopus* embryos leads to ectopic neuron formation. The data presented here shows that *n1-src* functions ahead of *neurod1* in this neurogenic pathway, with knockdown of *n1-src* resulting in significantly reduced *neurod1* expression.

Upstream of the proneural genes are the neural prepatter genes, which includes members of the SOX gene family, *sox2* and *sox3*. These genes have widespread expression throughout the neural plate compared to the restricted *neurod1* expression (Piccolo *et al.*, 1997; Moore *et al.*, 2018). *Sox2* and *Sox3* function upstream of *n1-Src* in the neurogenic pathway as their expression levels are not significantly different between *n1-src* knockdown and control embryos.

Prior to the differentiation of primary neurons, the *Xenopus* neural plate consists of a proliferating progenitor population that must reach sufficient size for development of the full complement of neural tissues. These progenitor cells are marked by the expression of *sox2* and *sox3* (Pevny and Placzek, 2005). *Sox2* and *Sox3* maintain these progenitor cells in their proliferating state and inhibit their differentiation by counteracting the action of proneural genes, through the activation of transcriptional repressors like *her3* (Bylund *et al.*, 2003; Okuda *et al.*, 2010). As *n1-Src* is required for the differentiation of these progenitor cells, its location downstream, rather than upstream, of genes that inhibit differentiation of these cells is understandable.

There are three classes of primary neurons; motor, inter and sensory neurons. When assayed by *in situ* hybridisation, the knockdown of *n1-src* leads to the loss of all three classes. However, when using RT-PCRs to analyse expression of markers for these classes, expression of *gsx2*, an interneuron marker, is not significantly downregulated whilst sensory and motor neuron markers are significantly downregulated. This difference could be down to the semi quantitative nature of RT-PCRs, and qPCRs should be employed to accurately determine whether *gsx2* expression is significantly downregulated.

3.3.5. *N1-src* overexpression causes a loss of anterior structures

Overexpression of *n1-src*, like knockdown, causes dose-dependent defects in patterning of the anterior-posterior axis, with anterior structure failing to develop correctly. One of the most noticeable defects following overexpression of 125pg c-terminal FLAG-tagged *Xenopus* mRNA is in the development of the eye. Defects in eye development are seen following *n1-src* knockdown, but have also been previously seen when overexpressing constitutively active mammalian n1-src in *Xenopus* retinal precursor cells, which results in a significant reduction in the number of differentiated retinal cells (Worley, Cornel and Holt, 1997). It appears the activity levels of n1-Src is important for correct development of the *Xenopus* eye, with loss or gain in activity both impeding retinal cell differentiation.

The posteriorisation phenotype seen in *n1-src* overexpressing embryos is comparable to that which results from the treatment of *Xenopus* embryos with retinoic acid, a factor known to be involved in the differentiation of neurons and neuroblastomas (Sive *et al.*, 1990; Tonge and Andrews, 2010). Embryo posteriorisation also results from the overexpression of *fgf-4* during gastrula stages (Isaacs, Pownall and Slack, 1994). The anterior-posterior patterning defects caused by *fgf-4* overexpression are accompanied by an upregulation in expression of mesodermal markers like *brachyury*. It was postulated that Fgfs and brachyury are required to control mesodermal cell movements during gastrula stages and for specification of the anterior-posterior axis. The posteriorisation phenotype displayed following *n1-src* overexpression could therefore be a result of ectopic n1-Src driving the Fgf/Erk pathway in mesodermal tissues. Furthermore, the phenotypic similarities between *n1-src* overexpressing and retinoic acid treated embryos could point towards the two factors regulating a shared pathway in the differentiation of cultured neuroblastoma cells.

3.3.6. N1-Src and Fgf signalling

Fgf signalling via the Erk pathway plays a diverse role in early vertebrate development including neuroectoderm and mesoderm induction, anteroposterior patterning, organogenesis and somitogenesis (Dorey and Amaya, 2010). In the first step of the signalling pathway, Fgf ligands interact with their cell surface receptors, causing receptor dimerisation and cross-phosphorylation (Ornitz and Itoh, 2015). Adaptor proteins, like Frs2 then interact with receptors, and are phosphorylated and activated. Activated Frs2 recruits Grb2 and SOS, the latter of which activates Ras (Aronheim *et al.*, 1994; Kouhara *et al.*, 1997). Ras activates the Mapk signalling cascade, which results in the successive phosphorylation of Raf and Mek (Rosen *et al.*, 1994). At the end of this cascade is Erk,

which, once phosphorylated, translocates into the nucleus to phosphorylate target transcription factors to alter gene expression (Su and Karin, 1996).

SFKs, including c-Src, have already been shown to interact with various components of the mapk pathway (Sandilands *et al.*, 2007). C-Src is involved in the recruitment of Fgf receptors to the peripheral membrane, has been shown to phosphorylate and activate the Mapk pathway member, Raf, and also attenuates the activity of protein phosphatase 2A, which negatively regulates Erk activity (Williams, Roberts and Li, 1992; Stokoe and McCormick, 1997; Tran and Frost, 2003; Sandilands *et al.*, 2007; Hu *et al.*, 2009). Whilst activating Erk signalling, c-Src is also involved in its repression through the phosphorylation of sprouty on Tyr-55 (Li *et al.*, 2004). This phosphorylation event activates sprouty leading to it attenuating erk activity.

The posteriorisation phenotype caused by *fgf* or *n1-src* overexpression resembles that produced following the overexpression of *lalloo*, another SFK, in *Xenopus* embryos (Weinstein *et al.*, 1998). *Laloo*, identified later in other species as Hck, is involved in mesoderm induction and functions through interactions with the Mapk pathway (Weinstein *et al.*, 1998). In the *Xenopus* animal cap assay, *lalloo* overexpression induces mesoderm formation, whilst expression of a dominant-inhibitory *Laloo* protein blocks the ability of Fgf to induce mesoderm in animal caps. Further analysis investigating the nature of the interaction between *Laloo* and the Mapk pathway used a kinase-defective *Laloo* mutant (Kusakabe *et al.*, 2001). This mutant can inhibit mesoderm induction by Fgf2 in *Xenopus* animal caps, a result that can be rescued by co-expressing constitutively active Ras. This places *Laloo* downstream of Fgf receptors but upstream of Ras in the mapk pathway during mesoderm induction.

The interactions of c-Src and other SFKs with members of the Fgf/Mapk pathway, particularly in early developmental processes like mesoderm induction, and the similar anterior-posterior axis defects following Fgf-4, *Laloo* or *n1-Src* overexpression, suggests that *n1-Src* may also be capable of such interactions. The results presented in this chapter show that *n1-Src* can interact with this pathway, and does so downstream of the Fgf receptors.

With the hypothesis that ectopic overexpression of *n1-src* mRNA could be driving the Fgf/Erk pathway in mesodermal tissues, I investigated whether *n1-Src* could, like Fgf-4, induce mesodermal tissue in *Xenopus* animal cap explants. Indeed, overexpression of mammalian *n1-src* induces expression of mesodermal markers like *brachyury* in *Xenopus*

animal caps (data not shown), adding support to the theory that the posteriorisation phenotype is due to ectopic n1-Src upregulating the Fgf/Erk pathway. In normal *Xenopus* embryonic development however, this ability for n1-Src to induce mesodermal tissue is not important as mesoderm induction precedes *n1-src* expression.

I have shown that the manipulation of n1-Src levels in *Xenopus* embryos results in changes in levels of phosphorylated Erk. The morpholino mediated downregulation of *n1-src* expression results in a significant decrease in Erk phosphorylation, whilst *n1-src* overexpression, in mammalian cells and *Xenopus* embryos, upregulates Erk phosphorylation. Like other family members, n1-Src appears to do this downstream of the Fgf receptor as treatment with a dominant negative Fgf receptor is unable to prevent this n1-Src mediated upregulation of phosphorylation. However, I have not yet analysed where exactly within the downstream intracellular signalling pathway n1-Src does interact and this is an area of further research, through the systematic inhibition of individual signalling pathway members.

Alongside roles in mesoderm induction, Fgf signalling is required during neural development. BMP signalling inhibition can only induce the anterior subset of neural tissues (Lamb and Harland, 1995). Using animal cap assays, Lamb and Harland showed that Fgfs induce posterior neural tissue, and when combined with BMP inhibition, induces the anterior, intermediate and posterior neural tissues. Dissociation of animal caps eliminates BMP activity, and dissociated caps from uninjected embryos express the forebrain marker *otx2* (Weinstein and Hemmati-Brivanlou, 2001). However, dissociated cells that express ectopic *lalloo*, express high levels of midbrain-hindbrain marker, *engrailed-2*, with reduced expression of *otx2*, showing *Lalloo*, like Fgfs, can posteriorise anterior neuroectoderm. With Fgfs being required for the development of the full complement of neural tissues, and ectopic expression of other SFK members replicating the ability of Fgfs to induce posterior neural tissues, it could be that n1-Src interacts with Fgf regulated pathways, such as the Mapk signalling pathway, during *Xenopus* neural development.

3.3.7. Conclusion

Expression of *n1-src* has repeatedly been associated with differentiated neurons and embryogenesis (Levy *et al.*, 1984; Scharf and Barnekow, 1984; Fults *et al.*, 1985; Cartwright *et al.*, 1987; Collett and Steele, 1992). In this chapter I have shown that knockdown of *n1-src* expression in *Xenopus tropicalis* embryos prevents the

differentiation of primary neurons. The inhibition of *n1-src* splicing also affects patterning of the anterior-posterior axis, with defects in posterior structures. Overexpression of *n1-src* mRNA results in a posteriorisation phenotype with defects in the development of anterior structures. Work in this chapter has progressed the understanding about the relationship between n1-Src and other genes in the differentiation pathway. I have shown that n1-Src acts between the neural prepatter genes, such as *sox2* and *sox3*, and the proneural genes like *neurod1*. Whilst I have shown that *n1-src* has a key role in regulating the differentiation of *Xenopus* primary neurons, rescue experiments still need to be conducted to confirm the specificity of the *n1-src* splice blocking morpholinos and further validate these results.

The phenotypes observed in this chapter reflect those seen when manipulating the activity of retinoic acid and Fgf signalling, suggesting that n1-Src could be a factor through which both pathways act in neuronal differentiation. During *Xenopus* development, retinoic acid and Fgf signalling interact at multiple different points to regulate the development of the anterior-posterior axis (Shiotsugu *et al.*, 2004). This chapter has advanced the understanding of n1-Src and its relationship with these signalling pathways, and shown that n1-Src can influence the activity of the Fgf regulated mitogen activated kinase pathway, acting downstream of the Fgf receptor. More work however is needed to identify the exact members of this pathway n1-Src interacts with, and the relationship between n1-Src and retinoic acid.

4. Transcriptomic analysis of *n1-src* knockdown in *X. tropicalis* development

4.1. Introduction

To understand development and disease, it is essential to discover the transcripts that are present at a particular developmental stage, in a particular biological condition, in a particular tissue or even in a particular cell (Wang, Gerstein and Snyder, 2009). Two of the main techniques used in transcriptomic analysis are DNA microarrays and RNA sequencing (RNA-Seq) (Lowe *et al.*, 2017). Whilst each approach has its pros and cons, the main advantage of RNA-Seq is that it allows the analysis of all the transcripts present, whilst microarrays are used to investigate the expression of a biased, predetermined set of genes (Wildsmith and Elcock, 2001; Zhao *et al.*, 2014). RNA-Seq can also be used to identify novel transcripts and splice events (Wilhelm and Landry, 2009).

Illumina next-generation RNA sequencing was utilised to examine the changes to the *X. tropicalis* transcriptome as a result of *n1-src* knockdown. The RNA present in all organisms can be split into two groups: coding and non-coding, with coding RNA being messenger RNA (mRNA) - transcribed from a gene - that codes for a protein, and non-coding RNA including transfer RNA and ribosomal RNA that do not encode proteins (Palazzo and Lee, 2015; Li and Liu, 2019). Mature, fully processed mRNA has a poly(A) tail that is important for its stability and translation (Colgan and Manley, 1997). The poly(A) tail can be used to isolate mature coding mRNA transcripts from the total RNA pool (Zhao *et al.*, 2018). A complementary DNA (cDNA) library can be produced from these isolated mRNA transcripts, adapters are ligated to the ends of the cDNA fragments and the cDNA library is then PCR amplified and sequenced (Kukurba and Montgomery, 2015).

Work in chapter three of this thesis has shown in that *n1-Src* functions in regulating the differentiation of primary neurons in *X. tropicalis* embryos. Inhibiting splicing of the *n1-src* microexon via splice blocking antisense morpholino oligos results in a failure of primary neurons to differentiate (Lewis *et al.*, 2017). In this chapter I investigate whether the differential gene expression changes following *n1-src* knockdown support these observations and the role of *n1-Src* in the developing amphibian nervous system.

The differentiation of neuroblastoma cells has also been linked with *n1-Src*, with the more differentiated cell lines having higher *n1-src* expression levels, and higher expression levels of *n1-src* relative to *c-src* expression is associated with a positive prognosis in patients (Bjelfman *et al.*, 1990; Matsunaga *et al.*, 1991; Matsunaga *et al.*, 1993). Neuroblastoma is a childhood developmental tumour that arises in cells originally derived

from the neural crest (Ciccarone *et al.*, 1989; Brodeur, 2003). *N1-src* expression and activity could therefore potentially contribute to development of the neural crest, and the use of next-generation sequencing will test this hypothesis by looking for changes in gene expression that indicate a role in crest development.

4.1.1. Aims of this chapter

By using Illumina next-generation RNA sequencing, I aim in this chapter to:

- Establish the transcripts and their associated biological/developmental processes that are significantly upregulated or downregulated after *n1-src* knockdown.
- Analyse whether *n1-src* knockdown leads to alterations in expression of genes involved in neuronal differentiation.
- Establish whether *n1-src* knockdown effects development of the neural crest in *X. tropicalis*.

4.2. Results

4.2.1. Sample generation and read alignment

Sibling *X. tropicalis* embryos were bilaterally injected at the one- and two-cell stages with 10ng of *n1-src* AMO A+D (20ng total) or with 20ng of control morpholino. Embryos were cultured to neurula stage 16 and flash frozen on dry ice. Stage 16 was chosen for the RNA-Seq experiment due to a previous RT-PCR time series. This time series had shown a neuronal marker, with expression levels at stage 14 that were similar in *n1-src* knockdown embryos and control embryos. However, expression of this marker was downregulated in knockdown embryos compared to controls when examined from stage 16 onwards. Stage 16 was therefore selected to try and avoid missing any potential *n1-Src* targets. After RNA extraction, RT-PCRs were carried out to confirm *n1-src* knockdown in the experimental samples (Figure 4.1). *N1-src* expression is downregulated in experimental samples compared to controls in all four repeats.

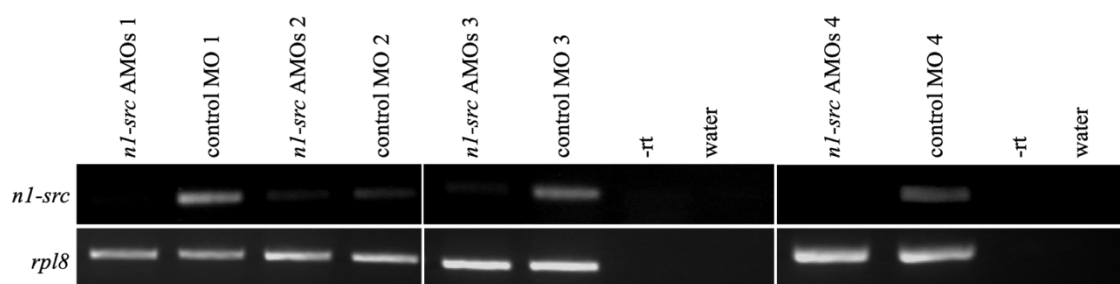


Figure 4.1. RT-PCR analysis for *n1-src* knockdown. *Xenopus tropicalis* embryos were bilaterally injected, at the one- or two-cell stages, with 10g of *n1-src* AMO A+D (20ng total) or 20ng of control morpholino, and cultured to stage 16. RT-PCR was carried out on RNA extracted from these samples to confirm injection of the splice blocking morpholino downregulates *n1-src* expression. -rt, No reverse transcriptase control; water, no template control.

One of the RNA-Seq samples failed to pass quality control and was removed from the experiment, leaving an experiment with three biological repeats. RNA-Seq library preparation was carried out by the staff at the Bioscience Technology Facility at the University of York and samples were run on a single lane of an Illumina HiSeq 3000 sequencer at the University of Leeds Next Generation Sequencing Facility, resulting in 35-50 million reads per sample. Initial results were obtained by aligning the raw reads to the NCBI v9.1 *X. tropicalis* reference transcriptome (<https://www.ncbi.nlm.nih.gov/genome/?term=xenopus%20tropicalis>), with Salmon (<http://salmon.readthedocs.io>).

This produces an estimated read count for each transcript. The R package, Sleuth (<http://pachterlab.github.io/sleuth/>), was then used to analyse for differential expression between the control and experimental samples. The raw reads were normalised as transcripts per million (TPM) values.

The first step in analysing these data was to confirm that the *n1-src* AMOs had knocked down *n1-src* expression. For all three samples, the expression of *n1-src* is reduced in the knockdown samples compared to controls (Table 4.1). Furthermore, earlier in this thesis *tubb2b* expression was shown to be significantly downregulated following *n1-src* knockdown. In all three RNA-Seq knockdown repeats, *tubb2b* expression is downregulated compared to control samples.

Table 4.1. Transcripts per million values for *n1-src* and *tubb2b*. RNA-Seq samples were run on a single lane of an Illumina HiSeq 3000 sequencer. Transcript expression values were normalised as transcripts per million (TPM). In all three samples, the TPM values for *n1-src* and *tubb2b* are reduced in the knockdown samples compared to controls, indicating successful knockdown of *n1-src*.

Transcript	CMO 1	<i>n1-src</i> AMOS 1	CMO 2	<i>n1-src</i> AMOS 2	CMO 3	<i>n1-src</i> AMOS 3
<i>n1-src</i>	6.60	0	4.09	1.53	4.27	0
<i>tubb2b</i>	71.33	39.66	71.21	47.97	81.39	40.74

The *X. tropicalis* reference transcriptome contains many transcript models that are predicted by automated pipelines and do not necessarily have any supporting experimental data. In the case of *src*, eight different transcripts were identified, including one corresponding to *n1-src*, one corresponding to *c-src*, and six other predicted transcripts, many of which had no TPM values (Table 4.2).

Table 4.2. Transcripts per million values for *src* transcripts. Salmon and sleuth analysis on the three biological repeats identified eight *src* transcripts. One corresponding to *c-src*, and the TPM values show no real difference between the control and experimental samples. One transcript corresponds to *n1-src*, with TPM values reduced in the experiment sample compared to the relevant control. The six other transcripts were all predicted transcripts, which may not actually be present in the data

Transcript	CMO 1	<i>n1-src</i> AMOS 1	CMO 2	<i>n1-src</i> AMOS 2	CMO 3	<i>n1-src</i> AMOS 3
<i>src</i>	0	0	0.121	0	0	0
<i>src</i>	0	0	0	0	0	0
<i>n1-src</i>	6.60	0	4.09	1.53	4.27	0
<i>src</i>	0	0	0	0	0	0
<i>c-src</i>	8.53	12.01	16.05	12.52	28.88	26.26
<i>src</i>	0	0	0.41	0	0.25	0.12
<i>src</i>	2.20	1.71	2.95	0	2.97	1.60
<i>src</i>	1.47	2.94	1.81	3.60	8.97	6.46

To clear up the *src* transcript data and identify only the real transcripts actually present in the data set, a de novo transcript assembly was attempted by the Bioscience Technology Facility, building a transcriptome from the sequenced reads. The sequencing reads were aligned to the *X. tropicalis* reference genome by HISAT2 (<http://www.ccb.jhu.edu/software/hisat2>) and StringTie (<https://ccb.jhu.edu/software/stringtie/index.shtml>) was run on these alignments to generate a novel transcriptome, reflecting the transcripts present in my data set at this particular developmental stage. The reads were then aligned to this novel transcriptome and the Salmon and Sleuth packages were run again. This generated a clearer *src* picture with only two different transcripts identified, one with and one without the neuronal microexon (Table 4.3). However, the Q-Value shows that *n1-src* is not significantly downregulated, at the level of $Q < 0.05$, in the experimental samples.

The Q-Value is an adjusted P-value used to counter the problem with multiple testing and false discovery rates (FDR) (Storey and Tibshirani, 2003). Using a normal P-Value of 0.05 means there is a 5% chance that a result is a false positive. When multiple statistical tests are carried out a large number of false positives will be recorded. The P-Value implies that 5% of all the tests will result in a false positive, for example, in an experiment looking at differential expression of 1000 transcripts, then chance alone would lead to 50 false positives (5%) being recorded. An FDR adjusted P-Value, the Q-Value, of 0.05

means that 5% of only the significant tests will be false positives and thus fewer false positives are recorded.

Table 4.3. Transcripts per million values for *c-src* and *n1-src* using a de novo transcriptome. TPM values for *n1-src* are reduced in knockdown samples compared to their relevant controls, whilst *c-src* TPM values are similar between controls and knockdowns. Despite the differences in TPM values, *n1-src* is not significantly downregulated at the level of $Q < 0.05$.

Transcript	CMO 1	<i>n1-src</i> AMOS 1	CMO 2	<i>n1-src</i> AMOS 2	CMO 3	<i>n1-src</i> AMOS 3	Q-Value
<i>c-src</i>	10.85	15.92	17.89	16.12	40.60	34.46	0.99992
<i>n1-src</i>	8.29	1.05	7.62	1.56	7.77	1.15	0.18797

Salmon and Sleuth, the tools used to analyse abundance and differential expression, estimate the abundance by generating bootstrap samples of the reads. Salmon resamples the reads randomly and recalculates the abundances, and then Sleuth uses the range of abundances generated to estimate the variance in the data and calculate the statistical significance based on this variance. Examination of the TPM plots show that for *c-src*, the TPM values (the central line on the boxplots) are similar between the experimental samples and their relative controls, and there is very little variance in the data – as shown by the box and whiskers (Figure 4.2). In contrast, for *n1-src*, the TPM values are much lower in the experimental samples compared to their relative controls, however, the variance calculated is quite large. Therefore, despite the TPM plots showing that the *n1-src* transcripts, in all three repeats, are less abundant in the experimental samples compared to the controls, the large variance means this difference is not significant, at the level of $Q < 0.05$.

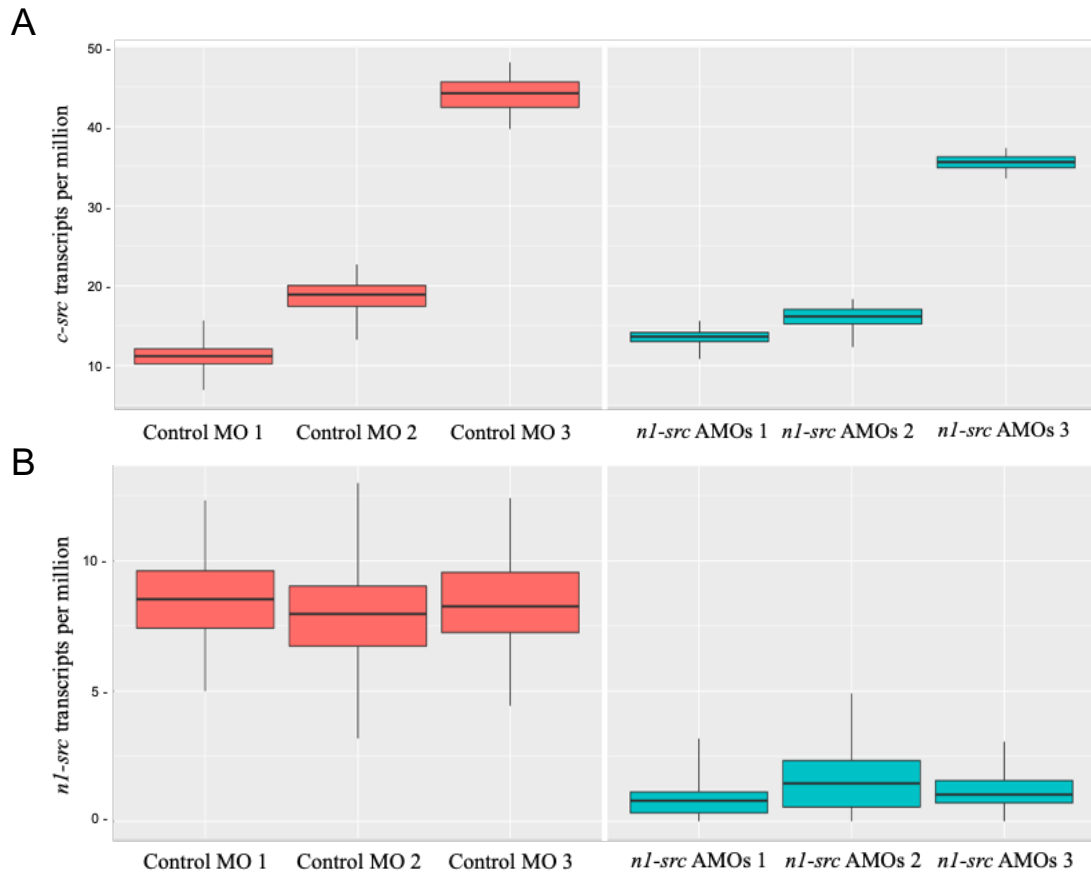


Figure 4.2. Transcripts per million plots for *c-src* and *n1-src* reads. Sleuth produced transcript per million values for (A) *c-src*, and (B) *n1-src*, in the three control samples (Control MO 1, 2 and 3) (red) and three *n1-src* knockdown experimental samples (*n1-src* AMOs 1, 2 and 3) (green/blue). The central line on the boxplots is the abundance calculated from the reads in the data set. The box and whiskers show the variance calculated by Sleuth. The calculated variance is minimal for *c-src*, both in the control and knockdown samples, and similar abundances were calculated for the experimental samples and their relevant controls. The transcripts per million values for *n1-src* appears reduced in the knockdown samples compared to the controls, however, in both conditions there is a large variance as indicated by the box and whiskers.

The *n1-src* RT-PCR (Figure 4.1) and the TPM values and plots for *n1-src* (Table 4.3, Figure 4.2) are evidence that, despite the lack of statistical significance, *n1-src* expression was knocked down by the morpholino treatment. This is further supported by examining the read alignments with IGV (<http://software.broadinstitute.org/software/igv/download>) (Figure 4.3). Reads map to the *n1-src* microexon and exon 4 of *src*, however, there is also a number of reads mapping to intron regions around the 15-base pair *n1-src* microexon. The absence of intronic mapping from other *src* introns suggests this is not due to genomic DNA contamination. Despite the mapping of reads to these flanking introns, distinct read pileups for the *n1-src* microexon can be seen in control samples.

These distinct pileups are missing from experimental samples indicating exclusion of the *n1-src* microexon in transcripts from samples treated with the *n1-src* splice blocking morpholinos.

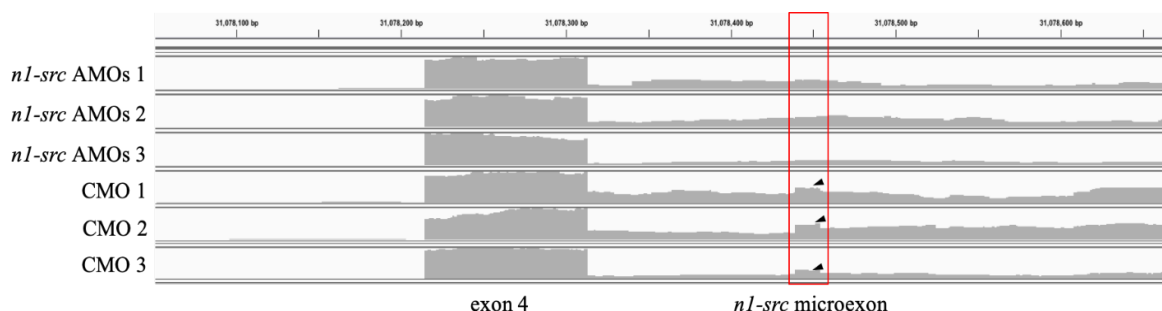


Figure 4.3. Mapping of the RNA-Seq reads to the *n1-src* microexon. When reads are mapped to *n1-src*, reads not only map to the *n1-src* microexon (red box), they also map to the introns either side of it. Reads fail to map to the intron on the other side of exon 4, indicating no genomic contamination is present. A clear build-up of reads can be seen in the control morpholino injected samples (arrow heads), that is absent from the *n1-src* knockdown samples suggesting knockdown of the microexon was successful. Reads mapping to specific introns, rather than all introns, is an indication of potential splicing failures.

The combination of the above figures and tables provides evidence that the *n1-src* knockdown was successful and that further analysis of the dataset can be conducted.

4.2.2. Differential expression resulting from *n1-src* knockdown

Analysis of the *de novo* dataset identified 698 transcripts upregulated 1.15-fold following *n1-src* knockdown, and 867 transcripts to be downregulated the same amount, using a Q-Value of <0.05. The Q-Value of 0.05 allows for 5% false positives, meaning that 35 of the 698 upregulated and 43 of the 867 downregulated transcripts might be false positives. A volcano plot was generated to give an overview of the transcript expression data, mapping Log₂ (Effect size) vs -Log₁₀ (Q-Value) (Figure 4.4). Each dot represents an individual transcript, with the horizontal black line representing a Q-Value of 0.05. For those genes below the line, in black, there is no significant differential expression. All those above the line show a Q-Value<0.05 however, those in cyan show fold changes, either up or down, of less than 1.15. Those in red represent genes with a fold change greater than 1.15, with a Q-Value <0.05.

A small effect size of 1.15 was chosen due to the nature of the experiment. The RNA-Seq experiment was carried out on RNA extracted from the entire *Xenopus* embryo, whilst *n1-src* is only expressed in a specific region – the neural plate. Many genes are expressed throughout the embryo, both in the neural plate and in other tissues. Knockdown of *n1-src* in the cells of the neural plate could significantly affect the expression of genes within these cells, however, as their expression in other tissues will be unaffected, the effect sizes would be greatly diluted.

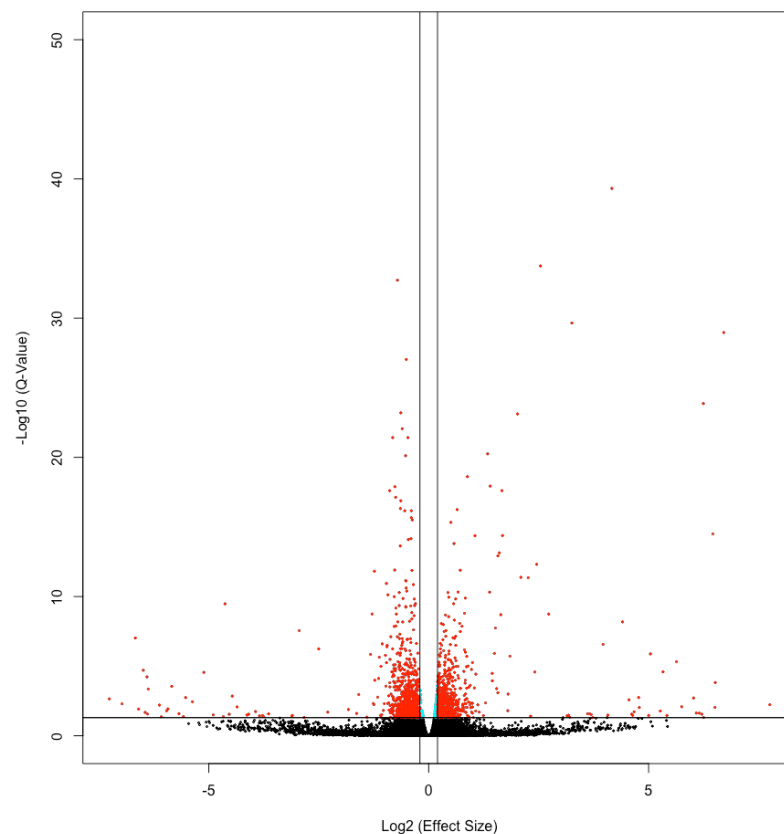


Figure 4.4. Transcript volcano plot. Mapping the Log2 (Effect size) vs $-\text{Log}_{10}$ (Q-Value) of the RNA-Seq transcripts. Black dots represent those with a Q-Value > 0.05 . Cyan dots represent transcripts with a Q-Value < 0.05 but a fold change of less than 1.15. Red dots represent transcripts with both a Q-Value < 0.05 , and a fold change greater than 1.15. 698 transcripts are significantly upregulated and 901 are significantly downregulated.

4.2.3. *N1-src* knockdown downregulates expression of neuronal differentiation genes

Two transcripts identified by RNA-Seq analysis to be significantly downregulated in *X. tropicalis* embryos following *n1-src* knockdown are *neurod1* and *tubb2b* (Figure 4.5). This supports and validates the previous findings in this thesis that expression of both *tubb2b* and *neurod1* is downregulated following *n1-src* knockdown (section 3.2.3). The significant downregulation of these transcripts also provides evidence that *n1-src* expression was knocked-down in the RNA-Seq samples, even if the calculated Q-Value for *n1-src* is >0.05.

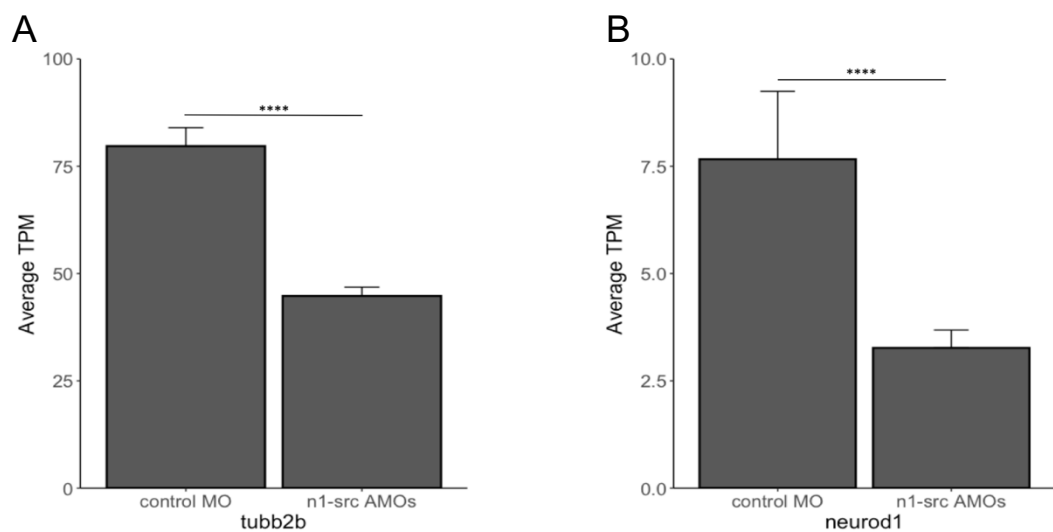


Figure 4.5. Average TPM plots for *tubb2b* and *neurod1*. RNA-Seq analysis reveals significant downregulation of *tubb2b* and *neurod1* expression in *n1-src* knockdown embryos, compared to control embryos. Data is plotted as mean $\pm$ SEM (n = 3 independent experiments, analysed by t-test, $P < 0.0001$).

4.2.4. Gene ontology analysis of downregulated transcripts

To obtain an overview of the biological processes that the downregulated transcripts function in, and determine if this supports the results from earlier in this thesis, the significantly downregulated transcripts were uploaded onto the Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System to identify statistically significant over-represented biological processes (<http://pantherdb.org/>) (Mi *et al.*, 2021). When analysing the transcript list for over-represented processes, the *M. musculus* gene database was used instead of the *X. tropicalis* database due to better annotation. PANTHER successfully mapped 719 of the transcripts.

The biological processes over-represented in this transcript set were examined by significance level. Considering all levels of the gene ontology term hierarchy, 560 out of 15,822 biological processes are significantly over-represented within the downregulated transcript set ($Q < 0.05$). The full list of over-represented biological processes can be found in the file “Supplementary File 1 – Panther Downregulated”.

The 20 most significantly over-represented processes are broad developmental processes and include nervous system development and cell differentiation (Figure 4.6). The over-representation of these processes within the significantly downregulated transcripts, complements the results shown earlier in this thesis, and supports the role for n1-Src in development of the vertebrate nervous system and the differentiation of primary neurons in *Xenopus* embryos. PANTHER classified 168 downregulated transcripts as being associated with nervous system development and 239 with cell differentiation. Those transcripts associated with nervous system development are listed in table 4.4 whilst transcripts associated with the other 19 processes are listed in the file “Supplementary File 1 – Panther Downregulated”.

Two of the biological processes significantly over-represented in the downregulated transcripts set are cytoskeletal organisation and cell projection organisation (Figure 4.6). This supports previous studies showing n1-Src regulates neuronal morphology through cytoskeletal modifications (Worley, Cornel and Holt, 1997; Kotani *et al.*, 2007; Lewis *et al.*, 2017). Another biological process over-represented is locomotion, supporting the touch response phenotype observed in *X. tropicalis* n1-src knockdown embryos, which fail to exhibit the characteristic dart response to touch stimulus (Lewis *et al.*, 2017). Overall, the top 20 significantly over-represented biological processes within the downregulated transcripts list supports a role for n1-Src in development of the *Xenopus* nervous system.

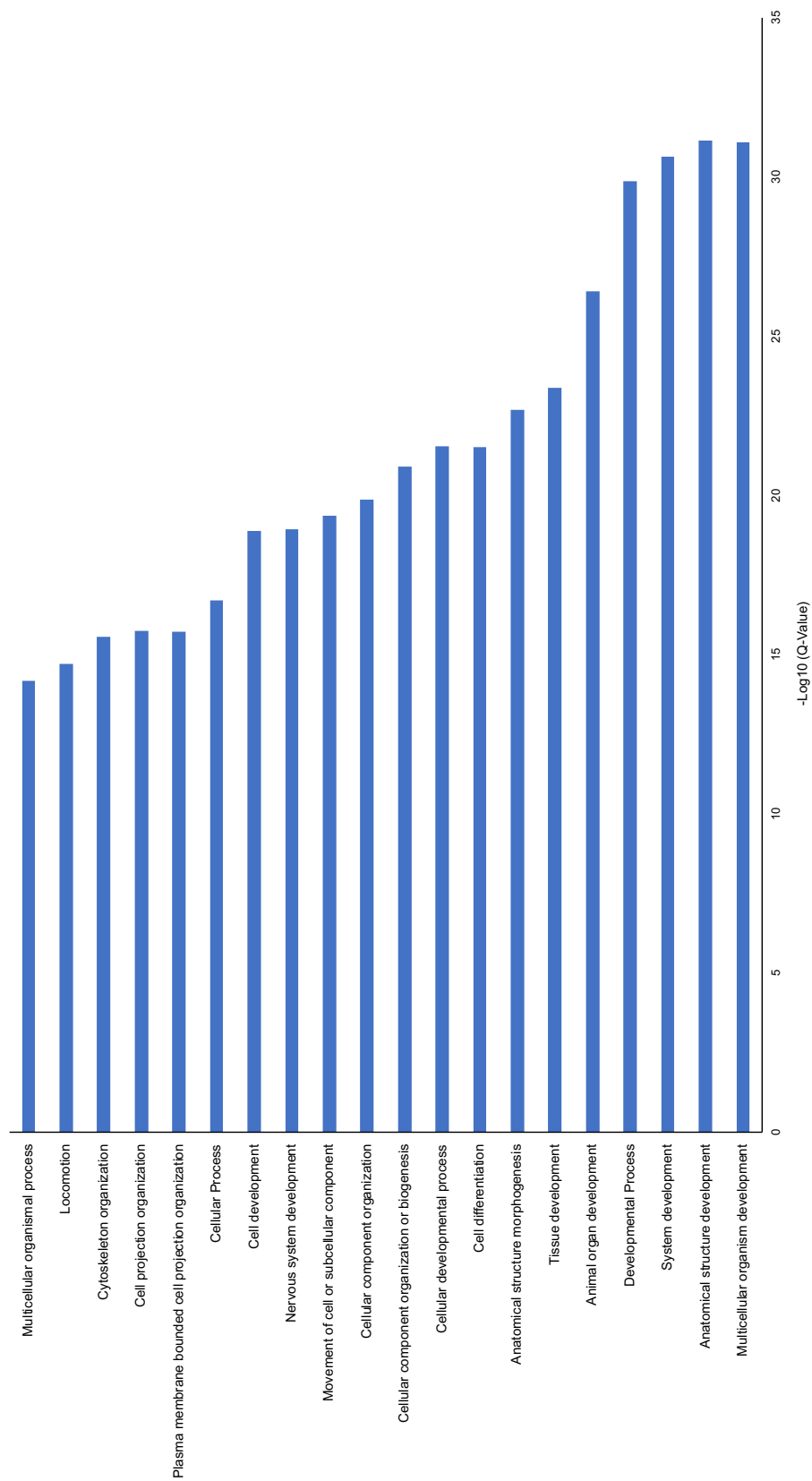


Figure 4.6. Top 20 biological processes, based on significance, overrepresented within the significantly downregulated transcripts. Significantly downregulated transcripts were uploaded onto the PANTHER Classification system to identify significantly overrepresented biological processes. Within the top 20 processes, based on significance, are many associated with development, including cell differentiation and nervous system development

Table 4.4. Significantly downregulated transcripts involved in nervous system development. The average TPM values in the control and experimental samples for the significantly downregulated transcripts that PANTHER classifies as functioning in nervous system development.

Transcript	CMO Average TPM	<i>n1-src</i> AMOS Average TPM	Q-Value	Log2 (Effect size)
adarb1	8.3275	5.2657	0.0356	-0.4224
adcy6	3.3441	2.2350	0.0330	-0.3768
adgrg1	3.7087	2.2657	0.0141	-0.4913
adnp	34.7734	26.3251	0.0005	-0.2902
agrn	26.9788	20.8172	0.0500	-0.2462
agtpbp1	3.5341	1.9818	0.0019	-0.5340
ak8	7.5271	5.4029	0.0489	-0.3962
anks1a	7.7937	5.4010	0.0000	-0.3663
anxa1	57.4020	42.5866	0.0004	-0.2784
apbb2	3.8237	2.6765	0.0063	-0.3835
apcdd1	11.1476	8.2653	0.0343	-0.3112
apoe	39.2357	29.8712	0.0211	-0.2981
armc4	10.0621	6.4991	0.0020	-0.4463
arx	7.7486	5.3593	0.0188	-0.3723
atat1	6.7499	4.1109	0.0003	-0.4905
axl	7.5316	5.8646	0.0245	-0.2625
bhlhe22	7.8533	4.7590	0.0009	-0.5103
bmp5	7.0646	4.9205	0.0354	-0.3491
carm1	48.7609	28.1742	0.0485	-0.5888
cav1	16.2922	7.2241	0.0000	-0.7981
ccdc141	4.2584	2.2103	0.0000	-0.6655
ccdc85c	7.5993	5.4534	0.0074	-0.3331
ccdc88a	3.1860	1.6216	0.0000	-0.6657
cd44	4.0565	2.2699	0.0001	-0.6090
cep68	16.9755	10.3966	0.0002	-0.5060
cfap43	2.4225	1.4737	0.0367	-0.4991
cfl1	358.9387	276.4134	0.0036	-0.2401
chac1	6.5582	4.5694	0.0480	-0.3641
cit	1.0057	0.0000	0.0257	-5.6791
cldn1	48.7420	27.5414	0.0000	-0.5449
clic5	22.8753	14.3081	0.0270	-0.4728
cluap1	13.8575	10.2647	0.0167	-0.3129
col2a1	128.9771	76.1018	0.0000	-0.5447
crb2	2.8188	1.0850	0.0002	-0.8860
crmp1	5.9044	3.5570	0.0312	-0.5551
dag1	62.8619	44.7799	0.0000	-0.3476
dagla	5.1720	3.7157	0.0003	-0.3532
dcdc2	4.1807	2.2020	0.0203	-0.5706
deaf1	3.8878	2.8592	0.0472	-0.3181
dhx30	19.1202	15.0468	0.0195	-0.2792
dlg5	2.0465	0.0000	0.0004	-6.3754

Transcript	CMO Average TPM	<i>n1</i> -src AMOS Average TPM	Q-Value	Log2 (Effect size)
dlg5	2.0465	0.0000	0.0004	-6.3754
dmrta2	15.8280	10.3984	0.0000	-0.4270
dpysl3	103.7142	83.0347	0.0001	-0.2445
dst	1.4780	0.9129	0.0062	-0.5112
efhc2	8.6281	4.3781	0.0035	-0.5942
elavl3	1.2440	0.0000	0.0000	-5.1140
emx1	38.4917	29.0097	0.0019	-0.2931
en2	11.4026	6.3376	0.0032	-0.5369
enah	4.3672	3.1465	0.0180	-0.3507
epha4	46.5875	36.3526	0.0056	-0.2631
ephb1	15.0772	9.6340	0.0137	-0.4379
erbb2	18.2725	12.1255	0.0111	-0.4594
fa2h	26.1440	16.8466	0.0174	-0.4051
fat4	1.9842	0.9149	0.0054	-0.7494
fgf14	2.9805	1.5139	0.0001	-0.6202
fgf2	14.9052	9.5432	0.0000	-0.3934
flna	52.6169	24.2256	0.0010	-0.8576
flrt3	34.6023	26.6459	0.0019	-0.2397
fmn1	3.2376	2.0409	0.0112	-0.4437
fry	9.7346	6.4834	0.0003	-0.4247
fubp1	159.7192	113.4324	0.0000	-0.3323
fzd5	4.6802	3.5130	0.0442	-0.2754
gli3	15.1072	11.5363	0.0206	-0.2838
gprc5b	3.8380	1.9263	0.0049	-0.6846
hapln3	24.3562	17.3920	0.0399	-0.2979
hdac9	7.3510	5.2125	0.0174	-0.3254
hook3	18.7254	14.7216	0.0219	-0.2312
hoxa3	3.8089	2.8854	0.0486	-0.2820
hoxb8	3.3267	1.9389	0.0233	-0.5332
hoxd10	2.2809	1.2166	0.0296	-0.6399
hydin	1.7126	0.8860	0.0018	-0.6432
ift140	8.1393	5.9755	0.0075	-0.3501
ift57	5.0092	3.7024	0.0437	-0.2666
igf1r	3.4849	2.7280	0.0294	-0.2466
igsf9	2.8108	1.6722	0.0380	-0.5294
itga3	31.7023	24.4390	0.0001	-0.2792
itpka	3.8758	2.7272	0.0325	-0.3303
jam3	24.1680	16.1541	0.0000	-0.3846
kdm4c	5.8584	4.4248	0.0352	-0.2574
kif26a	21.8011	12.9704	0.0000	-0.5215
lama1	2.8387	0.3030	0.0202	-2.2937
lifr	10.1690	5.6451	0.0042	-0.5735
map1a	2.6273	1.5170	0.0108	-0.5363
map3k7	7.1899	4.1197	0.0418	-0.4752
map4k4	2.5074	1.3143	0.0018	-0.6518
mapt	2.8707	1.3547	0.0199	-0.7245

Transcript	CMO Average TPM	<i>n1-src</i> AMOS Average TPM	Q-Value	Log2 (Effect size)
marcks	75.6890	53.1080	0.0105	-0.3095
mef2c	6.7812	3.4763	0.0117	-0.5388
meis1	42.3657	32.0671	0.0000	-0.2849
mnx1	13.2449	9.4816	0.0195	-0.3575
mtr	3.7523	2.0402	0.0062	-0.6446
mycn	38.1157	24.7153	0.0000	-0.4306
mypn	5.8078	2.7034	0.0000	-0.7836
myt1	4.3993	2.4786	0.0190	-0.6163
ndrg1	38.9581	30.7775	0.0032	-0.2588
ndrg2	38.2143	26.5499	0.0289	-0.3740
nes	13.0258	10.3659	0.0344	-0.2168
neurod1	7.6623	3.2683	0.0000	-0.8204
neurod4	17.5722	13.1877	0.0122	-0.2755
nexn	82.4625	52.9788	0.0000	-0.4271
notch2	1.2515	0.6654	0.0453	-0.7465
nr2f1	8.4506	6.4986	0.0472	-0.3126
nrp1	14.2603	10.6719	0.0194	-0.2833
nrp2	17.8714	12.7006	0.0036	-0.3203
ntn1	26.0811	17.0783	0.0003	-0.4493
ntn3	10.0403	7.3946	0.0041	-0.3304
numb	2.5600	1.4874	0.0012	-0.5219
ogdh	44.1753	35.9119	0.0185	-0.2340
otx2	22.2920	17.7037	0.0363	-0.2755
palld	9.2484	5.4879	0.0000	-0.5389
pax8	27.1550	17.8998	0.0290	-0.4404
phip	4.6515	3.0560	0.0222	-0.3886
plag1	2.5719	1.1805	0.0019	-0.7116
pmp22	131.3686	103.0133	0.0000	-0.2716
ppp1r9b	2.0002	0.9705	0.0003	-0.7561
prtg	21.8375	15.4909	0.0007	-0.3292
psmg1	1251.1426	996.0901	0.0017	-0.2160
ptch1	10.3944	8.4921	0.0413	-0.2053
rere	1.4003	0.0000	0.0003	-5.8437
rgs2	11.4961	6.8956	0.0006	-0.5322
rps7	581.3708	465.1507	0.0124	-0.2385
rtn4	45.8850	29.5668	0.0004	-0.4459
sall2	40.0892	30.3906	0.0011	-0.2720
sema3a	26.3886	18.2814	0.0000	-0.3368
sema3b	2.3017	1.1493	0.0098	-0.6628
sema3f	7.5417	4.8856	0.0160	-0.4746
sema6a	21.4450	13.2607	0.0010	-0.4452
sema6b	4.7013	3.3743	0.0325	-0.3355
serpinf1	18.3446	8.9160	0.0003	-0.7323
sfrp2	66.5320	46.4696	0.0000	-0.3466
sin3a	2.7333	1.2997	0.0185	-0.7239
slc1a3	7.7588	2.2188	0.0000	-1.2345
slc23a2	6.2133	0.0000	0.0051	-6.9726

Transcript	CMO Average TPM	<i>n1</i>-src AMOS Average TPM	Q-Value	Log2 (Effect size)
slc39a12	4.8786	3.0173	0.0263	-0.5019
slit1	2.3218	1.5351	0.0385	-0.4119
slit2	16.8284	13.0870	0.0010	-0.2781
slit3	3.1195	1.6178	0.0000	-0.6764
sox8	28.3566	20.5818	0.0009	-0.3110
spag6	42.0566	30.8293	0.0195	-0.3328
spef2	4.3287	3.0786	0.0311	-0.3409
stk24	6.4022	4.8731	0.0079	-0.2785
stk4	7.7108	4.6312	0.0012	-0.5092
stmnd1	24.9285	15.1724	0.0004	-0.4894
sulf1	3.6126	1.8618	0.0017	-0.6750
sun1	4.1873	0.0000	0.0270	-6.3954
svil	14.4046	11.0416	0.0055	-0.2984
tacc1	7.4180	4.4885	0.0165	-0.5138
tbx1	31.4036	24.9179	0.0033	-0.2285
thbs4	56.0303	31.8894	0.0000	-0.6020
tlx3	4.7169	3.0406	0.0342	-0.4211
top2b	11.3349	8.3266	0.0483	-0.2919
traf3ip1	6.8418	4.7746	0.0078	-0.3854
tubb2b	79.6980	44.7764	0.0000	-0.5970
tubb3	14.4443	7.1640	0.0000	-0.7135
tulp3	21.2294	14.6529	0.0007	-0.4095
twist1	189.4767	148.1582	0.0000	-0.2657
tyro3	19.8556	13.8130	0.0046	-0.3575
ugt8	10.2217	6.8794	0.0244	-0.3397
vcan	7.2340	4.5611	0.0000	-0.4447
vim	124.8554	95.1537	0.0014	-0.3001
vwc2l.2	5.0940	2.1135	0.0003	-0.8823
wnt4	12.8119	9.0921	0.0086	-0.3403
xylt1	3.8949	2.5396	0.0012	-0.3807
zbtb16	18.1329	12.9448	0.0000	-0.3094
zc4h2	43.2723	33.4414	0.0264	-0.2936
zfp36l1	12.4791	8.7317	0.0060	-0.3352
znf423	6.7439	4.6729	0.0195	-0.3498

The significantly over-represented biological processes in the downregulated transcript set were also ordered by their fold enrichment, rather than significance level. Examination of the top 20 (out of 560) processes with the greatest total fold enrichment also identifies processes involved in neuron differentiation and axonal guidance (Figure 4.7). Processes related to development and migration of the neural crest are also seen in the top 20 enriched processes (Figure 4.7).

In contrast to the top 20 most significantly enriched processes, which are broad developmental processes with hundreds of associated transcripts (Table 4.4. and Supplementary File 1 – Panther Downregulated”), the top 20 process with the greatest fold enrichment are more specific processes that have a small cluster of associated transcripts (Tables 4.5 and 4.6).

The majority of these biological process share a combination just seven transcripts, *nrp1*, *nrp2*, *sema3a*, *sema3f*, *slit1*, *slit2* and *slit 3* (Tables 4.5 and 4.6). These transcripts are involved in neural development, and development of the neural crest. Overall, the top 20 overrepresented processes based on fold enrichment display a role for n1-Src in development of the nervous system and in neural crest development.

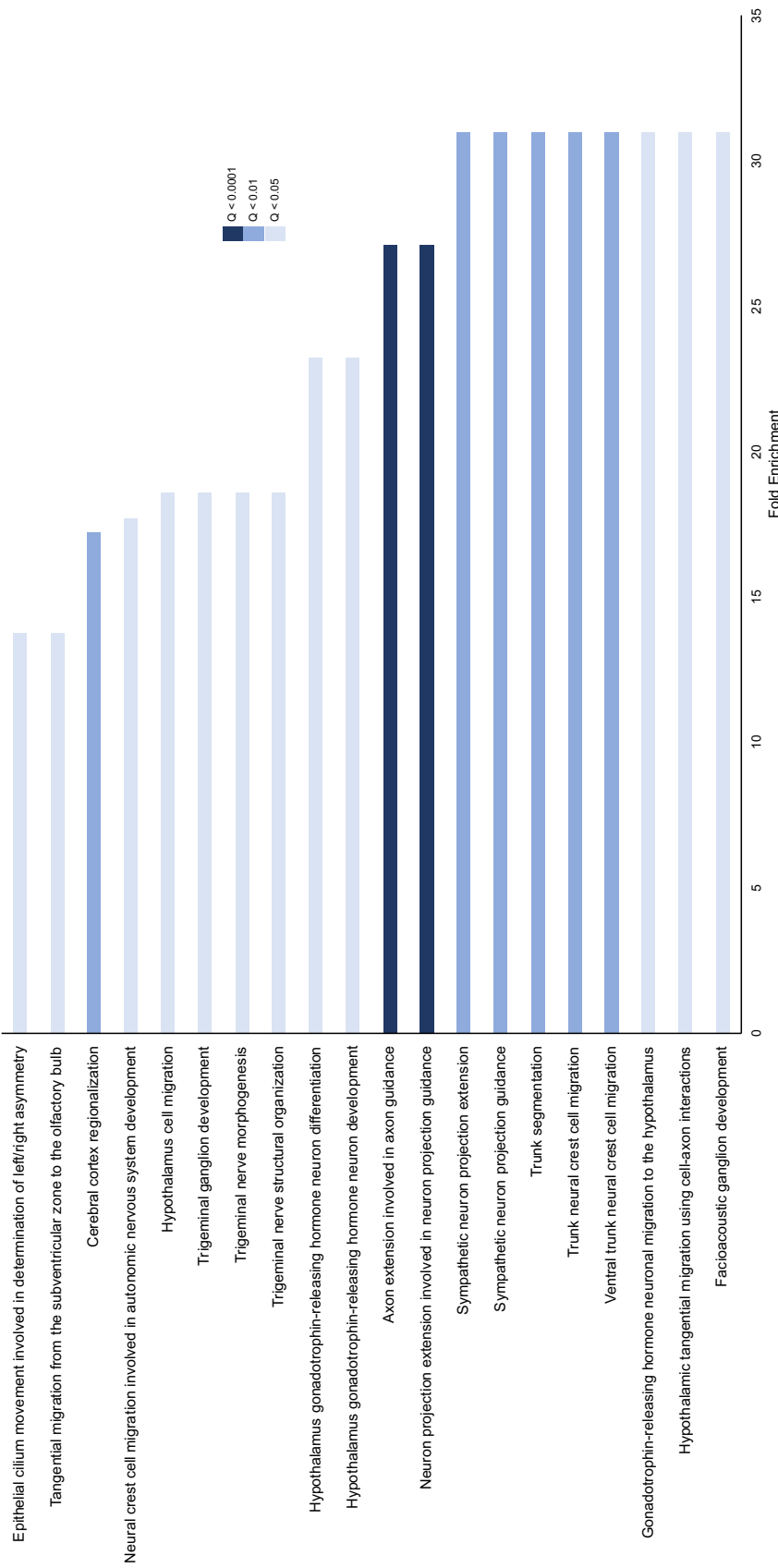


Figure 4.7. Top 20 biological processes, based on fold enrichment, overrepresented within the significantly downregulated transcripts. Significantly downregulated transcripts were uploaded onto the PANTHER Classification system to identify significantly overrepresented biological processes. Within the top 20 processes, based on fold enrichment, are process associated with neuronal development. Three of the top 20 processes are related to development/migration of the neural crest.

Table 4.5. Top 20 biological processes, based on fold enrichment, overrepresented within the significantly downregulated transcripts. The significantly downregulated transcripts were uploaded onto the PANTHER Class faction system to identify significantly overrepresented processes. The table contains the top 20 biological processes, based on fold enrichment, and the transcripts that PANTHER has attributed to each process.

Biological process	Transcripts
Epithelial cilium movement involved in determination of left/right asymmetry	ccdc151, ccdc40, lrcc6, rfx3
Tangential migration from the subventricular zone to the olfactory bulb	arx, ogdh, slit1, slit2
Cerebral cortex regionalization	adgrg1, bhlhe33, dmrta2, emx1, nr2f1
Neural crest cell migration involved in autonomic nervous system development	nrp1, nrp2, sema3a, sema3f
Hypothalamus cell migration	nrp1, nrp2, sema3a
Trigeminal ganglion development	nrp1, nrp2, sema3a
Trigeminal nerve morphogenesis	nrp1, sema3a, sema3f
Trigeminal nerve structural organization	nrp1, sema3a, sema3f
Hypothalamus gonadotrophin-releasing hormone neuron differentiation	nrp1, nrp2, sema3a
Hypothalamus gonadotrophin-releasing hormone neuron development	nrp1, nrp2, sema3a
Axon extension involved in axon guidance	nrp1, nrp2, sema3a, sema3f, slit1, slit2, slit3
Neuron projection extension involved in neuron projection guidance	nrp1, nrp2, sema3a, sema3f, slit1, slit2, slit3
Sympathetic neuron projection extension	nrp1, nrp2, sema3a, sema3f
Sympathetic neuron projection guidance	nrp1, nrp2, sema3a, sema3f
Trunk segmentation	nrp1, nrp2, sema3a, sema3f
Trunk neural crest cell migration	nrp1, nrp2, sema3a, sema3f
Ventral trunk neural crest cell migration	nrp1, nrp2, sema3a, sema3f
Gonadotrophin-releasing hormone neuronal migration to the hypothalamus	nrp1, nrp2, sema3a
Hypothalamic tangential migration using cell-axon interactions	nrp1, nrp2, sema3a
Facioacoustic ganglion development	nrp1, nrp2, sema3a

Table 4.6. Significantly downregulated transcripts involved in the top 20 overrepresented biological processes (based on fold enrichment). The average TPM values in the control and experimental samples for the significantly downregulated transcripts, that PANTHER classifies as functioning in top 20 enriched biological processes.

Transcript	CMO Average TPM	<i>n1-src</i> AMOS Average TPM	Q-Value	Log2 (Effect size)
adgrg1	3.7087	2.2657	0.0141	-0.4913
arx	7.7486	5.3593	0.0188	-0.3723
bhlhe22	7.8533	4.7590	0.0009	-0.5103
ccdc151	8.5565	5.2411	0.0122	-0.5755
ccdc40	6.6097	4.2358	0.0146	-0.4314
dmrta2	15.8280	10.3984	0.0000	-0.4270
emx1	38.4917	29.0097	0.0019	-0.2931
lrrc6	11.8033	8.6526	0.0247	-0.3243
nr2f1	8.4506	6.4986	0.0472	-0.3126
nrp1	14.2603	10.6719	0.0194	-0.2833
nrp2	17.8714	12.7006	0.0036	-0.3203
ogdh	44.1753	35.9119	0.0185	-0.2340
rfx3	5.5308	3.4693	0.0002	-0.4259
sema3a	26.3886	18.2814	0.0000	-0.3368
sema3f	7.5417	4.8856	0.0160	-0.4746
slit1	2.3218	1.5351	0.0385	-0.4119
slit2	16.8284	13.0870	0.0010	-0.2781
slit3	3.1195	1.6178	0.0000	-0.6764

The file “Supplementary File 1 – Panther Downregulated” contains the full list of 560 biological processes significantly overrepresented within the significantly downregulated transcript list. In addition to those seen in the top 20 enriched processes, three more neural crest processes are contained within the significantly overrepresented processes (Table 4.7). These three processes are “neural crest development”, “neural crest differentiation” and “neural crest migration”, and the transcripts involved include some of those seen previously in table 4.6, plus additional transcripts such as *twist1* and *sox8* (Table 4.8). These results indicate a role for n1-Src in regulating development of the neural crest.

Table 4.7. Biological processes related to neural crest development that are overrepresented within the significantly downregulated transcript list. Three overrepresented biological processes within the downregulated transcript list, outside of the top 20, are involved in development of the neural crest.

Biological process	Fold enrichment	Q-Value
Neural crest development	4.53	0.0002
Neural crest cell differentiation	4.47	0.0013
Neural crest cell migration	6.52	0.0001

Table 4.8. Significantly downregulated transcripts involved in the three additional neural crest processes. The average TPM values in the control and experimental samples for the significantly downregulated transcripts, that PANTHER classifies as functioning in neural crest cell development, neural crest cell differentiation and neural crest cell migration.

Transcript	CMO Average TPM	<i>n1-src</i> AMOS Average TPM	Q-Value	Log2 (Effect size)
<i>anxa6</i>	4.4574	2.0513	0.0000	-0.8401
<i>cfl1</i>	358.9387	276.4134	0.0036	-0.2401
<i>mef2c</i>	6.7812	3.4763	0.0117	-0.5388
<i>nrp1</i>	14.2603	10.6719	0.0194	-0.2833
<i>nrp2</i>	17.8714	12.7006	0.0036	-0.3203
<i>sema3a</i>	26.3886	18.2814	0.0000	-0.3368
<i>sema3b</i>	2.3017	1.1493	0.0098	-0.6628
<i>sema3f</i>	7.5417	4.8856	0.0160	-0.4746
<i>sema6a</i>	21.4450	13.2607	0.0010	-0.4452
<i>sema6b</i>	4.7013	3.3743	0.0325	-0.3355
<i>sox8</i>	28.3566	20.5818	0.0009	-0.3110
<i>tbx1</i>	31.4036	24.9179	0.0033	-0.2285
<i>twist1</i>	189.4767	148.1582	0.0000	-0.2657

The analysis of the RNA-Seq data so far in this chapter has consistently shown that downregulated transcripts are involved in neuronal development and development of the nervous system. This supports the results shown earlier in this thesis, where the differentiation of primary neurons was inhibited/impaired in *n1-src* knockdown embryos. Knockdown of *n1-src* also leads to the downregulated expression of transcripts involved in the development and migration of the neural crest.

4.2.5. Gene ontology analysis of upregulated transcripts

Using the previously described criteria, 698 transcripts were significantly upregulated in response to *n1-src* knockdown. To determine the processes they function in, the transcripts were uploaded onto PANTHER to identify statistically over-represented biological processes. Once again, the *M. musculus* gene database was used instead of *X. tropicalis* due to better annotation. PANTHER successfully mapped 488 of the transcripts.

The over-represented biological processes were ranked by their significance level. 239 of the 15,822 biological processes are significantly over-represented within the transcript set ($Q < 0.05$). The full list of over-represented biological processes can be found in the file “Supplementary File 2 – Panther Upregulated Top 20 Significant”.

The 20 most significantly over-represented processes are a variety of metabolic process, including RNA metabolism (Figure 4.8). One of the most significantly enriched processes is RNA splicing, and 50 transcripts were attributed to this, listed in Table 4.9.

Similarly, when the 239 significantly enriched biological processes are ranked by their fold enrichment, within the top 20 processes are many involved in the regulation of mRNA processing and splicing (Figure 4.9). Of the top 20 processes, seven are not related to mRNA processing or splicing and instead are processes such as “Response to amino acid starvation” and “Salivary gland development”. The transcripts associated with these seven biological processes are listed in the file “Supplementary File 3 – Panther Upregulated Top 20 Fold Enrichment”

The 13 other most enriched processes within the significantly upregulated transcript set are all involved in mRNA processing and splicing. They consist of broad processes such as “Regulation of RNA splicing” and “Regulation of mRNA processing”, and more specific biological processes such as “mRNA cis splicing, via spliceosome” (Figure 4.9). These 13 processes use a combination of just 38 different transcripts, which are listed in table 4.10.

The gene ontology analysis therefore suggests that *n1-Src* is involved in the transcriptional downregulation of a variety of mRNA splicing and processing factors.

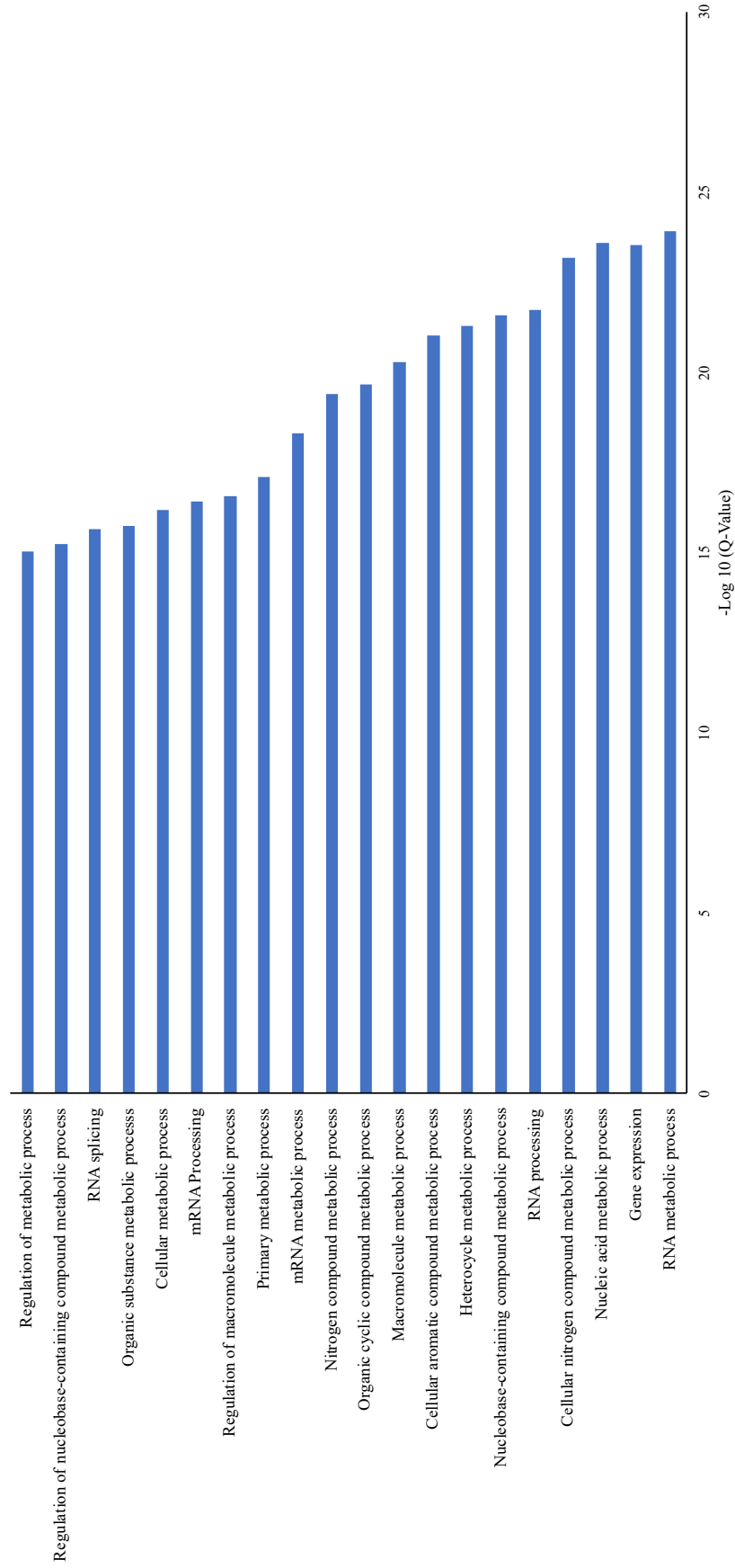


Figure 4.8. Top 20 biological processes, based on significance, overrepresented within the significantly upregulated transcripts. Significantly upregulated transcripts were uploaded onto the PANTHER Classification system to identify significantly overrepresented biological processes. The top 20 processes, based on significance, are mainly metabolic processes, but also includes RNA processing and splicing.

Table 4.9. Significantly upregulated transcripts involved in RNA splicing. The average TPM values in the control and experimental samples for the significantly upregulated transcripts that PANTHER classifies as functioning in nervous RNA splicing.

Transcript	CMO Average TPM	<i>n1-src</i> AMOS Average TPM	Q-Value	Log2 (Effect size)
bud31	131.3784	170.1970	0.0001	0.2619
cwf19l2	12.4693	28.4499	0.0000	0.8649
ddx17	16.7350	37.5728	0.0059	0.9388
dhx16	35.2107	44.4619	0.0013	0.2153
esrp1	29.6593	40.1991	0.0011	0.3034
fus	28.1596	42.4817	0.0021	0.4066
fus	913.2221	1173.4076	0.0001	0.2201
gpkow	94.5637	120.2213	0.0339	0.2177
hnnpa1	3.0780	259.1352	0.0000	4.4161
hnnpa2b1	1.9841	9.8999	0.0000	1.6067
hnmpc	289.8084	416.5489	0.0081	0.3368
hnmpc	141.1719	196.0513	0.0001	0.3210
ivns1abp	0.0000	1.5829	0.0344	5.0007
larp7	45.7530	69.2293	0.0009	0.4227
ncbp1	2.8793	5.1656	0.0001	0.6050
ncbp2	151.0123	195.6142	0.0001	0.2374
phf5a	684.5935	870.3421	0.0001	0.2280
pnn	415.7131	522.1271	0.0159	0.2046
prkip1	69.6770	97.0348	0.0000	0.3516
prpf19	8.4014	19.1504	0.0353	0.8796
prpf3	5.3976	22.4925	0.0000	1.3848
prpf38b	15.4984	23.1854	0.0430	0.4312
prpf40a	11.5822	20.7767	0.0052	0.5648
qki	16.0522	31.0389	0.0000	0.6451
rbm28	64.7339	80.0832	0.0012	0.2211
rbm39	25.6661	46.1503	0.0059	0.5569
rbm39	115.7960	157.1882	0.0026	0.3135
rbm4b	49.8741	70.5700	0.0000	0.3635
rp9	16.5427	21.1516	0.0426	0.2944
sfswap	38.7690	53.7428	0.0210	0.3395
snrnp48	5.9153	9.2738	0.0275	0.4786
snrnp70	44.8710	64.6813	0.0004	0.3527
snrnp70	272.8312	344.7260	0.0080	0.2242
srpk1	8.7630	19.0158	0.0000	0.8132
srrm1	45.1183	68.9909	0.0000	0.4488
srsf1	374.1892	481.4584	0.0000	0.2599
srsf2	283.0298	422.5561	0.0305	0.3515
srsf4	24.4887	32.6747	0.0027	0.2811
srsf5	917.7341	1232.5249	0.0118	0.2907
srsf9	587.4531	744.8385	0.0000	0.2358
thoc2	53.9586	68.4840	0.0001	0.2408

Transcript	CMO Average TPM	<i>n1-src</i> AMOS Average TPM	Q-Value	Log2 (Effect size)
tra2a	5.0937	67.7917	0.0000	2.5427
tra2a	3.0745	36.6516	0.0000	2.4542
tra2a	21.0326	150.8036	0.0000	2.0197
ythdc1	58.7398	75.2323	0.0035	0.2372
zc3h13	78.5514	125.9254	0.0290	0.5265
zc3h13	71.1427	111.5759	0.0090	0.4694
zcrb1	0.7327	3.0409	0.0001	1.4468
zmat5	84.0253	102.2273	0.0012	0.2251
zrsr2	25.6804	33.5500	0.0288	0.2297

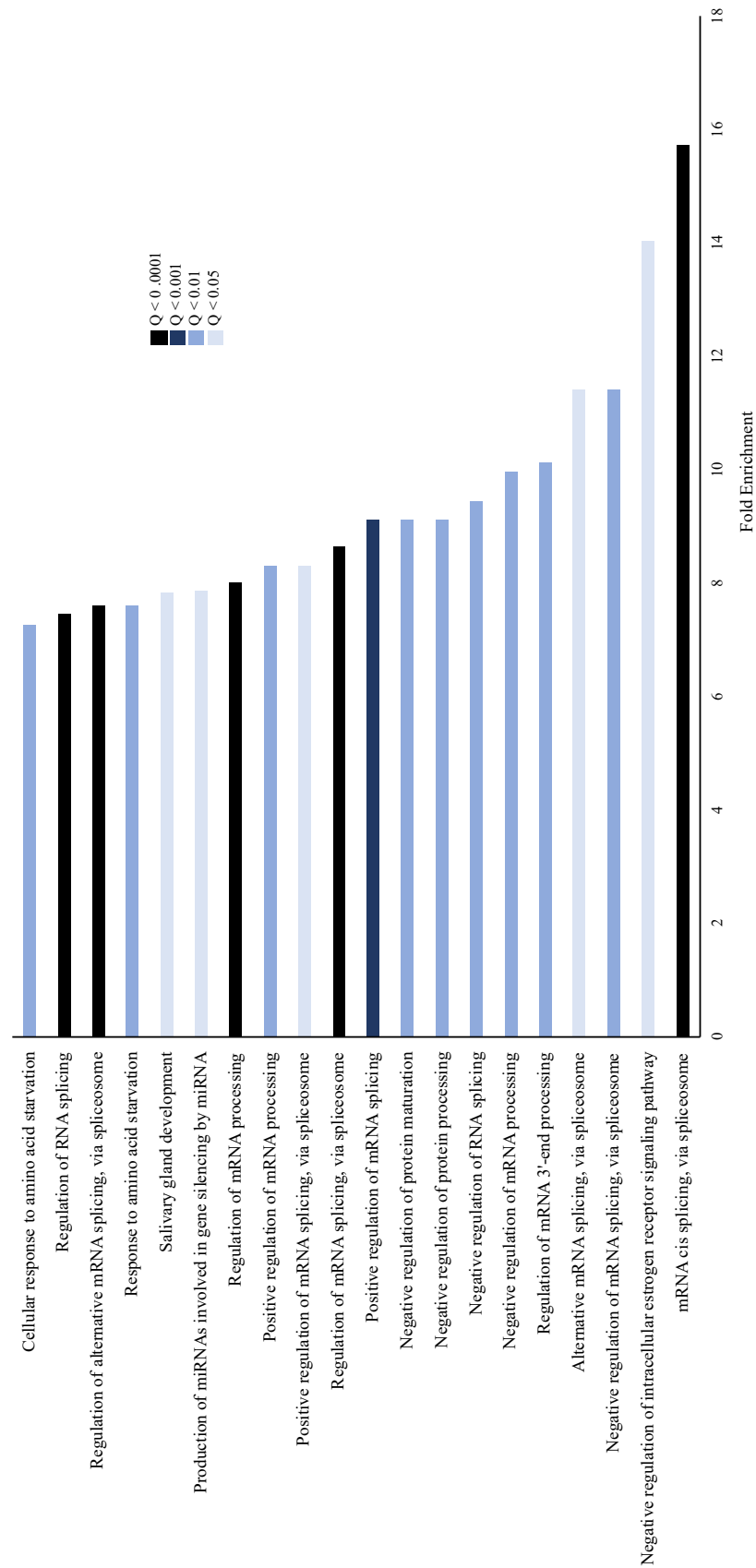


Figure 4.9. Top 20 biological processes, based on fold enrichment, overrepresented within the significantly upregulated transcripts. Significantly upregulated transcripts were uploaded onto the PANTHER Classification system to identify significantly overrepresented biological processes. Within the top 20 processes, based on fold enrichment, are processes associated the regulation of RNA processing and mRNA splicing.

Table 4.10. Significantly upregulated transcripts involved in biological processes associated with RNA processing and mRNA splicing. The average TPM values in the control and experimental samples for the 38 significantly upregulated transcripts that PANTHER classifies as functioning in the 13 processes associated RNA processing and splicing.

Transcript	CMO Average TPM	<i>n1-src</i> AMOS Average TPM	Q-Value	Log2 (Effect size)
ahnak	0.0429	15.4079	0.0000	5.3289
ahnak	2.5847	10.6116	0.0000	1.3992
ahnak	0.4583	1.0662	0.0419	0.9141
clk1	17.5450	24.7030	0.0397	0.3068
clk3	26.6912	36.1765	0.0028	0.2812
cpsf6	22.8233	29.4569	0.0155	0.2815
dazap1	22.5299	35.2990	0.0113	0.4441
ddx17	16.7350	37.5728	0.0059	0.9388
dyrk1a.2	16.0162	25.6618	0.0097	0.4803
esrp1	29.6593	40.1991	0.0011	0.3034
hnmpa2b1	1.9841	9.8999	0.0000	1.6067
hnrnp1	3.1855	4.9156	0.0423	0.4653
khdrbs3	6.8157	11.4844	0.0014	0.5098
larp7	45.7530	69.2293	0.0009	0.4227
ncbp1	2.8793	5.1656	0.0001	0.6050
ncbp2	151.0123	195.6142	0.0001	0.2374
paf1	159.3003	206.6072	0.0001	0.2265
prpf19	8.4014	19.1504	0.0353	0.8796
prpf40a	11.5822	20.7767	0.0052	0.5648
qki	16.0522	31.0389	0.0000	0.6451
rbm39	25.6661	46.1503	0.0059	0.5569
rbm39	115.7960	157.1882	0.0026	0.3135
rbm4b	49.8741	70.5700	0.0000	0.3635
rnf40	1.0355	17.1392	0.0000	2.7286
sfswap	38.7690	53.7428	0.0210	0.3395
snrnp70	44.8710	64.6813	0.0004	0.3527
snrnp70	272.8312	344.7260	0.0080	0.2242
srpk1	8.7630	19.0158	0.0000	0.8132
srsf1	374.1892	481.4584	0.0000	0.2599
srsf2	283.0298	422.5561	0.0305	0.3515
srsf4	24.4887	32.6747	0.0027	0.2811
srsf5	917.7341	1232.5249	0.0118	0.2907
srsf9	587.4531	744.8385	0.0000	0.2358
tra2a	5.0937	67.7917	0.0000	2.5427
tra2a	3.0745	36.6516	0.0000	2.4542
tra2a	21.0326	150.8036	0.0000	2.0197
ythdc1	58.7398	75.2323	0.0035	0.2372
zfp64	12.0426	18.3937	0.0073	0.4227

4.2.6. Identifying enriched gene sets in the RNA-Seq data

Gene Set Enrichment Analysis (GSEA) (<https://www.gsea-msigdb.org/gsea/index.jsp>) was also utilised to examine the effects of interfering with *n1-src* expression (Mootha et al., 2003; Subramanian et al., 2005). Unlike the previously used gene ontology analysis, GSEA is used to analyse the entire transcript set produced from the RNA-Seq experiment, rather than just those with statistically significant changes in expression (Yoon, Kim and Nam, 2016).

GSEA examines two different biological states, in this case control and knockdown transcript data, to determine if there are significant differences between pre-defined sets of genes in the two states. These pre-defined gene sets are provided by the Molecular Signature Database (Broad Institute), which organises the gene sets into seven collections (Liberzon *et al.*, 2015). The analysis here uses collection five, where genes are grouped according to gene ontology categories.

GSEA ranks the genes based on each gene's differential expression with respect to the two phenotypes, and then analyses where members of each gene set are distributed in the ranked list. If genes from a gene set are enriched in one of the two conditions they will be overrepresented at the top or bottom of the ranked list, whereas genes from sets that are not enriched will be spread uniformly throughout the list. GSEA normally uses a FDR of 0.25 rather than 0.05 or 0.1 due to the lack of coherence in most expression datasets and the relatively small number of gene sets being analysed, which means more stringent FDR cut offs would potentially overlook significant results. However, this assumes that the phenotype permutation is used when assessing the statistical significance of the enrichment score. The use of this permutation type yielded no results from my data so the gene set permutation was used instead. This is a less stringent permutation and therefore a more stringent FDR of 0.05 was used.

In this data set, genes that are distributed towards the top of the ranked list are those whose expression is enriched in the control samples compared to the knockdown. The GSEA will identify gene sets, to which these genes belong, as being enriched in the control samples, meaning that these sets are downregulated in the experimental samples. Genes distributed towards the bottom of the ranked list have expression enriched in the knockdown samples, and GSEA will identify gene sets that they contribute to as being enriched in the experimental rather than control samples, and give them a negative enrichment score.

GSEA revealed 10 gene sets significantly enriched ($Q < 0.05$) in the control samples (Table 4.11) and within these enriched gene sets are multiple related to development of the nervous system (Figure 4.10). The presence of these processes in here means that they are downregulated in the experimental samples and this supports a role for *n1-Src* in development of the nervous system and in cytoskeletal remodelling. No gene sets were significantly enriched in the *n1-src* knockdown samples ($Q < 0.05$).

Table 4.11. Gene sets significantly enriched in the control samples. The 10 gene sets that gene set enrichment analysis identifies as being significantly enriched within the control samples, therefore downregulated in the experimental samples. Amongst the most enriched processes are several related to development of the nervous system.

Gene set	Normalised enrichment score	Q-Value
Cell projection	2.38	0.006
Neuron part	2.36	0.003
Intracellular vesicle	2.33	0.004
Neuron projection	2.09	0.013
Intrinsic component of plasma membrane	2.08	0.012
Cell projection part	1.97	0.021
Cell junction	1.92	0.023
Synapse part	1.87	0.027
Synapse	1.83	0.030
Organelle inner membrane	1.73	0.045

Overall, the GSEA results show an enrichment of gene sets in the control samples that are involved in development of the nervous system, meaning they are downregulated in the *n1-src* knockdown samples, which supports the gene ontology analysis.

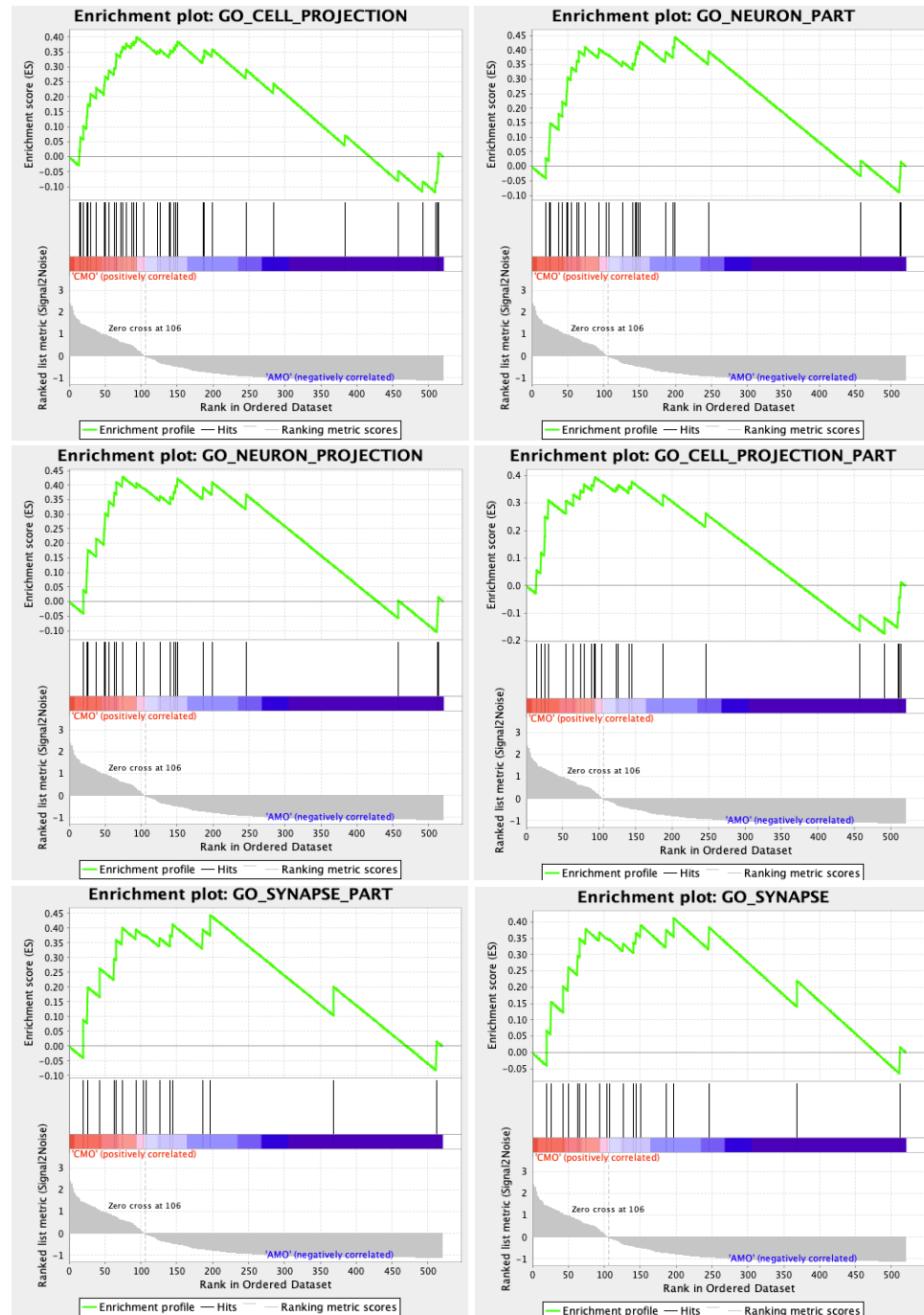


Figure 4.10. Gene set enrichment analysis revealed neuronal gene sets enriched in the control samples. The gene sets significantly enriched in the control samples that are related to development of the nervous system and cell projections. The top portion of each plot shows the running enrichment score for each gene set, and the score at the peak of the plot is the enrichment score for each gene set. The middle portion shows the position of members of the gene set within the ranked list of genes. The bottom portion of each plot shows the ranking metric, which measures a gene's correlation with a phenotype. A positive value indicates a correlation with the control embryos.

4.3. Discussion

N1-Src is known to be required for primary neurogenesis in the developing *Xenopus* embryo and function in neurite outgrowth and axonogenesis (Lewis *et al.*, 2017; Worley, Cornel and Holt, 1997). Transcriptomic analysis was carried out on *n1-src* knockdown *X. tropicalis* embryos to discover the pathways that n1-Src regulates in development.

4.3.1. *N1-src* knockdown lacked statistical significance

The first noticeable observation from the preliminary analysis of the transcriptome data from control and *n1-src* knockdown embryos, was the lack of statistically significant downregulation of *n1-src* expression. Despite the transcript per million data and the mapping of the *src* reads showing the *n1-src* microexon being knocked down, a statistically significant result could not be produced. There are several factors thought to be affecting this gene and the lack of significance.

Firstly, accurate quantifications of abundances for *src* transcripts are difficult to obtain due to the length of neuronal microexon being targeted. The exon is 15 base pairs long, the *src* transcripts are around ~2800 base pairs long, whilst the raw Illumina reads are 151 base pairs long. Therefore, most of the reads do not span the target exon, and it is impossible to tell if a read comes from a transcript containing that exon unless the actual exon is included. If the read comes from the start, or end, of the transcript then it is indistinguishable from those that come from transcripts that exclude the exon RNA-Seq packages try to account for this by "sharing" reads that map to multiple transcripts, but for two transcripts with such small differences, definite abundances cannot be obtained.

Another factor potentially contributing is the number of reads mapping to the intron regions around the 15-base pair microexon of *src* (Figure 4.3). When looking at the other *src* exons, very little intronic mapping is seen, suggesting this is not down to genomic DNA contamination, which would have caused intronic mapping throughout. The presence of reads in introns has also been linked to alternative splicing (Ameur *et al.*, 2011) and in the case of the *src* microexon, it is possible that for many transcripts there have been alternative splicing failures resulting in read build ups in the surrounding introns. Examination of the raw alignments with IGV shows that despite the mapping of reads to introns, distinct read pileups for the microexon can be seen in control samples. These distinct pileups are missing from experimental samples, but reads are still mapping across that exon, possibly contributing to transcript abundances.

Salmon and Sleuth, the abundance and differential expression tools estimate abundance variance by generating bootstrap samples of the reads. Salmon will repeatedly randomly resample reads and recalculate abundances. This range of abundances is then used by Sleuth to estimate the variance in the data and calculate the statistical significance based off this. The above-mentioned factors mean that the variance for the *src* transcripts is too large to be statistically significant, even though the differences in transcript abundance is quite large.

4.3.2. N1-Src and nervous system development

Previous data revealed a role for *n1-Src* in neuronal development, with primary neurogenesis being inhibited in *X. tropicalis* embryos following *n1-src* knockdown (Lewis *et al.*, 2017). The use of RNA-Seq and subsequent downstream analysis on *n1-src* knockdown and control embryos has offered support to those results presented previously in this thesis and in Lewis *et al* (2017). Gene ontology and gene set enrichment analysis have both shown that transcripts downregulated in response to *n1-src* knockdown function in processes relating to nervous system development. Within these transcripts are *tubb2b* and *neurod1* that were identified earlier in this thesis, by *in situ* hybridisation and RT-PCR analysis respectively, to be significantly downregulated in response to *n1-src* knockdown. *Tubb2b* is a neural specific beta-tubulin which is expressed in the developing central nervous system and is an early differentiation marker for primary neurons (Oschwald, Richter and Grunz, 1991). *Neurod1* is a proneural basic helix-loop-helix transcription factor transiently expressed in development and is involved in terminal differentiation of neurons (Gao *et al.*, 2009). Their downregulation indicates *n1-Src* acts upstream to regulate neuronal differentiation.

Another downregulated transcript that is involved in neuronal differentiation is *numb*. In *X. tropicalis* development, primary neurons differentiate from cells in the neural plate, in three columns either side of the midline (Brewster, Lee and Altaba, 1998). The three columns are established by the process of notch and delta-mediated lateral inhibition (Chitnis *et al.*, 1995). In *Drosophila*, sensory organ precursor (SOP) cells give rise to four cells after two rounds of cell division, the neuron, it's sheath cell and two nonneuronal support cells, and this process also utilises lateral inhibition (Rhyu, Jan and Jan, 1994). Disruption of *numb* expression in *Drosophila* SOP cells affects their cell lineage, with reduced numbers of neuronal cells but an increase in the related nonneuronal support cells, without changes overall cell number. The significantly downregulated expression of

numb following *n1-src* knockdown supports a role for n1-Src in regulating neuronal differentiation.

In addition to regulating neuron differentiation, n1-Src has been implicated in regulating neuronal morphology, in mammals and *Xenopus*, through cytoskeletal modifications, leading to changes in neurite outgrowth and axonogenesis (Worley, Cornel and Holt, 1997; Kotani *et al.*, 2007). Neurite-like processes can be induced in cultured COS7 cells in response to *n1-src* expression (Keenan *et al.*, 2017), whilst inhibition of n1-Src activity with a novel n1-Src SH3 peptide ligand, or knockdown with an *n1-src* specific shRNA reduces neurite formation in cultured rodent cerebellar granule neurons. The gene ontology and gene set enrichment analysis carried out on the *n1-src* knockdown transcriptomic data supports these results, with processes like “Axon extension involved in axon guidance” and “Neuron projection extension involved in neuron projection guidance” being overrepresented within the significantly downregulated transcript list.

The downregulated transcripts that are involved in these processes includes two semaphorin transcripts, *sema3a* and *sema3f*, and three slit transcripts. Slit proteins are involved in axon guidance, where they act as a ligand for the repulsive guidance receptor, roundabout, and one of their main functions is to prevent longitudinal axons from crossing the midline of the nervous system (Brose *et al.*, 1999). Semaphorins make up a large family of proteins, that can be either secreted or membrane bound, which also act as signalling ligands to control cell movement during the development of various systems, including nervous, cardiovascular, reproductive and musculoskeletal (Dickson, 2002; Alto and Terman, 2017). To drive the attraction and repulsion of neuronal cell axons and dendrites, semaphorins bind to several different families of proteins through their N-terminal Sema domain (Yazdani and Terman, 2006). Semaphorin receptors include members of the Plexin family and the Neuropilin family, specifically, neuropilin-1 (Nrp1) and neuropilin-1 (Nrp2), transcripts of which are also significantly downregulated following *n1-src* knockdown.

Worley *et al* (1997) demonstrated a role for n1-Src in regulating axon outgrowth in *Xenopus* retinal growth cones. *Sema3a* is also involved in retinal development and can cause the collapse, branching or turning *Xenopus* retinal growth cones (Campbell *et al.*, 2001). Experiments on stage 35/36 *Xenopus* retinal growth cone explants showed a 76% collapse rate in response to *Sema3a*. When cones are presented a gradient of *Sema3a* rather than a uniform amount, collapse is seen along with repulsion of cones away from the semaphorin gradient.

In summary, the transcriptomic analysis of *n1-src* knockdown embryos in this chapter supports previous results, both in this thesis and our published paper (Lewis *et al.*, 2017) that n1-Src is required for the neuronal development in the developing *X. tropicalis* embryo.

4.3.3. N1-Src and neural crest development

Neuronal Srcs have been implicated with neuroblastoma prognosis. Neuroblastoma is childhood cancer that arises from precursor cells in the sympathetic nervous system, which is itself derived from the neural crest (Ciccarone *et al.*, 1989; Brodeur, 2003). Examination of neuroblastoma cell lines, each at a different stage of differentiation, revealed that the further differentiated the line was, the higher the Src kinase activity level (Mellström *et al.*, 1987). Furthermore, only neuroblastoma cell lines with the ability to differentiate express *n1-src*, and higher expression levels of *n1-src* relative to *c-src* in neuroblastoma cell lines is associated with a more favourable prognosis (Bjelfman *et al.*, 1990; Matsunaga *et al.*, 1993). The expression of *n1-src* within the neural plate of *Xenopus*, along with the development of the neural crest at the border of the neural plate in vertebrates (Milet and Monsoro-Burq, 2012; Lewis *et al.*, 2017) and the association between neural crest derived neuroblastomas and n1-Src, together lead to the hypothesis that n1-src could be involved in regulating neural crest development.

PANTHER gene ontology analysis of the significantly downregulated transcripts, to examine processes that are overrepresented within the transcript list, revealed that some of the overrepresented biological processes with the greatest fold enrichments were involved in neural crest migration (Figure 4.7). Despite being the processes with the greatest fold enrichment, only four significantly differentially expressed transcripts were identified by PANTHER as being involved in these processes; *sema3a*, *sema3f*, *nrp1* and *nrp2* (Table 4.5). Alongside the neural crest processes that appeared in the top 20 significant processes based on enrichment, a further three neural crest development processes were seen outside the top 20 (Table 4.7). The four previously mentioned transcripts are associated with these three processes, along with additional transcripts like *sema6a*, *sema6b*, *sox8* and *twist1*.

Twist1 has multiple roles in neural crest development, including roles in mammals in directing crest cell migration and controlling differentiation once they have reached their

terminal location within the embryo (Soo *et al.*, 2002). Sox8 meanwhile has been shown in *Xenopus* to function in the earliest stages of neural crest induction (O'Donnell *et al.*, 2006).

In ovo downregulation of *sema6a* in chicks revealed a key role in nervous system development, leading to *Sema6a* being described as a gate keeper between the central and peripheral nervous system, that also plays a role in controlling migration of neural crest cells (Mauti *et al.*, 2007). Double stranded RNAs were used for *in ovo* RNA interference, and the RNAi mediated downregulation of *sema6a* resulted in the streaming of motor neurons out of the ventral spinal cord. The boundary region where the central nervous system and the peripheral nervous system meet is marked by a group of neural crest derived cells, termed the boundary cap cells (Gresset *et al.*, 2015). Boundary cap cells prevent the emigration of motor neurons out of the ventral spinal cord (Vermeren *et al.*, 2003). In the *sema6a* RNAi experiments, clustering of these boundary cap cells was impeded, thus removing this prevention, and motor neurons were seen emigrating out of the spinal cord. Analysis of the migratory neural crest cells that give rise to the boundary cap found *sema6a* expression in the migrating cells (Mauti *et al.*, 2007).

Semaphorin receptors, the neuropilins, are also involved in helping guide the migrating neural crest. In mice, *sema3a* expression is seen in the posterior dermamyotome of each somite and in the cranial neural tube (Sheperd *et al.*, 1996). During development, the neural crest migrates from the neural tube through the anterior half of each somite (He *et al.*, 2002). Crest cells therefore appear to selectively migrate through regions where *sema3a* expression is absent. Indeed, *sema3a* expression is detected in regions bordering trunk neural crest migration pathways, and the migrating cells of the neural crest express *nrp1* (Eickholt *et al.*, 1999). Furthermore, attenuation of *Sema3a* signalling allows the migration of neural crest cells away from their normal pathways, into adjacent crest-free regions (Osborne *et al.*, 2005). Guiding of neural crest cells by semaphorins is also seen in Zebrafish (Yu and Moens, 2005). During migration of the cranial neural crest, cells move into the pharyngeal arches as three primary streams. Positioned between these streams are crest-free zones that express *sema3f*, whilst *nrp2* expression is seen in the migrating crest cells. The knockdown of *n1-src* in *X. tropicalis* embryos therefore potentially affects migration of the neural crest through downregulated expression of semaphorin family members and their receptors.

On top of roles in nerve and neural crest development, neuropilins and class 3 semaphorins are implicated in several different cancers. One proposition is that

semaphorins act as tumour suppressors, and studies have linked semaphorins and neuropilins with favourable outcomes in various cancers, including neuroblastomas (McArdle *et al.*, 2004. Nair *et al.*, 2007). Studies with neuroblastoma cell lines and tumours found that those with lowest expression of *sema3b* had a more unfavourable histopathology, and the more favourable and benign tumours were those with high expression levels.

Other studies researching Nrp1 and downstream cytosolic protein collapsin response mediator protein-1 (Crmp1) support a role for class 3 Semaphorins as tumour suppressors. Within high grade prostate cancer cells, *crmp1* expression is significantly downregulated to facilitate the epithelial to mesenchymal transition needed for cancer cells to metastasise (Cai *et al.*, 2017). Recently, low levels of *nrp1* expression has been shown to be associated with shorter patient survival times and, like Crmp1, reduced levels of Nrp1 in neuroblastoma-derived SK-N-AS cells leads to elevated cell migration (Ishizuka *et al.*, 2018).

This points toward n1-Src, alongside having a role in neuronal development, potentially functioning in the development of the neural crest, with several transcripts involved in crest development, and associated with crest derived neuroblastomas, downregulated in response to *n1-src* knockdown. More work is required however to investigate n1-src and potential neural crest regulation (See Chapter 6).

4.3.4. N1-Src and alternative splicing

N1-src knockdown resulted in a 1.15-fold upregulated expression of 698 transcripts, at a significance level of $Q < 0.05$. Gene ontology analysis of these transcripts, via PANTHER to identify overrepresented processes revealed enrichment in RNA processing and mRNA splicing.

The transcriptomic approach to investigate *n1-src* and its regulated pathways was part of a three-pronged approach, along with pulldowns and a phosphoproteomic screens carried out in the Evans laboratory to identify n1-Src interactants and targets (West, 2019). A GST-n1-SH3 pulldown using P1 rat brain lysates was used to identify protein that interact with SH3 domain of n1-Src. P1 rat brain lysates were also used in the phosphoproteomic screen to identify proteins phosphorylated by n1-Src. In the phosphoproteomic screen the rat brain lysates were treated with a universal kinase

inhibitor and then HIS-tagged n1-Src was added before incubation with ATP for three hours.

The proteins identified by the GST-n1-SH3 pulldown was enriched for the gene ontology terms “mRNA processing” and “RNA splicing” (West, 2019). In the phosphoproteomic analysis, STRING was used to generate protein-protein interaction networks and to identify functional enrichments within the n1-Src dataset, one of identified functional clusters was “Poly(A) RNA binding” (West, 2019).

Each of the three approaches therefore identifies clusters of genes or proteins involved in RNA processing and splicing, suggesting n1-Src is phosphorylating, interacting with, and transcriptionally regulating splicing factors to control alternative splicing.

Alternative splicing is a regulatory method that, through the inclusion or exclusion of exons, allows multiple protein isoforms to be encoded from a single gene, and is important in tissue specific transcript expression (Noh *et al.*, 2006). The alternative splicing of pre-mRNAs is vital in neural development and the control of neuronal gene expression (Li, Lee and Black, 2007). An example of this is *numb*, a gene involved in cell fate determination, which has multiple different alternatively splice isoforms. Alternatively spliced isoforms that contain a proline rich region are expressed in undifferentiated progenitors and promote proliferation, whilst differentiation is promoted by numb isoforms that lack the exon (Li, Lee and Black, 2007). Another example is *src* itself, with the *n1* microexon being alternatively spliced into mature mRNA transcripts in neural tissues.

The combination of n1-Src being required for differentiation of primary neurons in *Xenopus tropicalis*, the differential expression of transcripts and phosphorylation of proteins involved in mRNA splicing, and the key role that alternative splicing has in neuronal development, suggests that n1-src could potentially control neuronal differentiation through the regulating expression and activity of splicing factors. The potential effects of n1-src knockdown on mRNA splicing, and the genes whose splicing is affected will be analysed in chapter 5 of this thesis.

4.3.4 Conclusion

The analysis in this chapter on the transcriptomic data from *n1-src* knockdown embryos supports the data in chapter 3 of this thesis. Knockdown of *n1-src* leads to the significant downregulation of transcripts involved in neuronal development. The data also indicates

potential roles for n1-Src in regulating alternative splicing and neural crest development, which will be further analysed in chapters 5 and 6 of this thesis respectively.

5. N1-Src regulates alternative splicing in *X. tropicalis*

5.1. Introduction

The transcriptomic analysis carried out in this thesis showed that knockdown of *n1-src* leads to the significant upregulation of a variety of mRNA slicing and processing transcripts (Chapter 4). N1-Src SH3 domain pulldowns and phosphoproteomic analysis carried out in the Evans laboratory also identified RNA processing factors that either interact with, or are phosphorylated by, n1-Src (West, 2019).

5.1.1. Alternative splicing

The number of proteins found in eukaryotes greatly outweighs the number of genes, with humans having more than 90,000 different proteins but only ~25000 protein-coding genes (Wang *et al.*, 2015). This vast difference in number is attributed to alternative splicing. First proposed in 1978 by Walter Gilbert, alternative splicing is a process that generates a diverse range of mature mRNAs from a single pre-mRNA through the use of different combinations of 5' and 3' splice sites. More recently, studies using high throughput RNA sequencing of 15 human tissues and cell lines show that 92-94% of multi-exon genes undergo alternative splicing (Wang *et al.*, 2008).

In multiexon genes, the majority of exons are constitutive and therefore found in all isoforms of the mature mRNA. Those that are alternatively skipped or retained are referred to as cassette exons (Figure 5.1) (Gurskaya *et al.*, 2012). In some cases, two or more cassette exons can be mutually exclusive, meaning that inclusion of one leads to the exclusion of the other, and vice versa. Along with the inclusion or exclusion of cassette exons, alternative splicing can also lead to the lengthening or shortening of exons through the use of different 5' and 3' splice sites (Figure 5.1) (Black, 2003). Another method of alternative splicing is the retention of introns in particular isoforms of the mature mRNA, whilst there is exclusion of those introns from other isoforms (Figure 5.1)

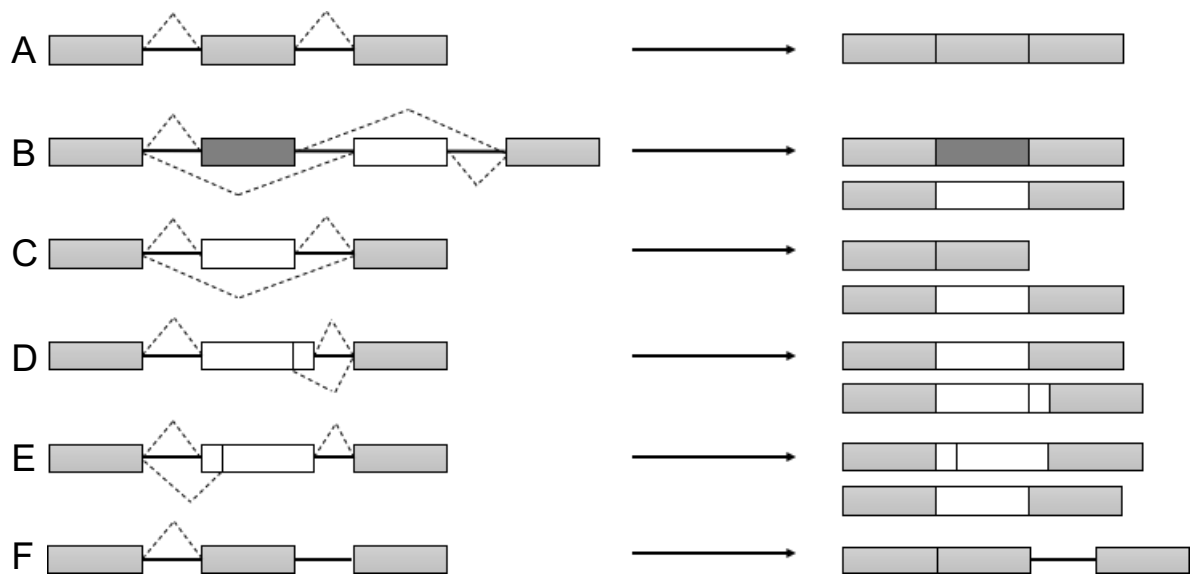


Figure 5.1. Schematic showing the most common forms of alternative splicing events. A) Constitutive splicing. **B)** Mutually exclusive exons. **C)** Alternative cassette exon. **D)** Alternative 5' splice site. **E)** Alternative 3' splice site. **F)** Intron retention.

No matter which method, or methods, of alternative splicing a pre-mRNA undergoes, splice sites are the key factors that direct exon inclusion or exclusion. The junction between a 5' exon and a 3' intron is the 5' splice site, or donor site, and the junction at the other end of the intron is the 3' splice site, or acceptor site (Black, 2003). Both splice sites are marked by short conserved sequences. The 5' splice site contains an essential GU dinucleotide at the beginning of the intron, located within a slightly less conserved nine nucleotide consensus sequence (Figure 5.2) (Cieply and Carstens, 2015). The 3' splice site shows more conservation, with three conserved regions: the branch point which contains an essential adenine nucleotide; a downstream polypyrimidine tract and then a terminal AG dinucleotide at the end of the intron (Black, 2003).

Prior to the splicing reaction, the spliceosome must be formed around the splice sites. The spliceosome is a large ribonucleoprotein made up of five small nuclear ribonucleoproteins (snRNPs) - termed U1-, U2-, U4-, U5- and U6-snRNP - and more than 70 additional proteins (Will and Lührmann, 1997). In eukaryotes there are two spliceosomes, the U12-dependent spliceosome which splices a few rare exons, and the much more abundant U2-dependent spliceosome that is responsible for the majority of

splicing (Will and Lührmann, 2011). In fact the U2-dependent spliceosome is responsible for splicing more than 99% of all introns (Turunen *et al.*, 2013).

Splicing by the U2-dependent spliceosome is a multistep process that begins with binding of the U1-snRNP at the 5' splice site (Black, 2003). This is followed by the recognition of the 3'-splice site by two non-ribonucleoproteins, SF1 and U2AF (Kent, Ritchie and MacMillan, 2005). SF1 binds to the branch point whilst U2AF interacts with the polypyrimidine tract and the AG dinucleotide. At this point in assembly the complex is called the early complex (E complex) (Rino *et al.*, 2008). SF1 is then displaced from the branch point by the U2-snRNP, in an ATP-dependent manner, leading to the formation of the A complex (Wahl, Will and Lührmann, 2009). Subsequently the U4/U5/U6 tri-snRNP binds to the A complex to form the pre-catalytic B complex. A series of structural rearrangements then follows which result in the removal of the U1- and U4-snRNPs and the binding of the U6-snRNP to the 5'-splice site, forming the activated spliceosome complex (Sawa and Abelson, 1992). The U12-dependent spliceosome contains 5 snRNPs, U5, U11, U12, U4atac, and U6atac (Turunen *et al.*, 2013). The U11, U12, U4atac, and U6atac correspond to the U1, U2, U4 and U6-snRNPs respectively.

The pre-mRNA is then cleaved by two transesterification reactions (Figure 5.2). First, the guanine residue in at the 5'-splice site undergoes a nucleophilic attack by the hydroxyl group on the branch site adenosine (Will and Lührmann, 2011). This forms an intron-3' exon lariat intermediate, called the C1 complex, and the cleaved 5' exon. There is then a second transesterification reaction in which the exposed hydroxyl group at the 3' end of the cleaved exon attacks the guanine residue at the 3' splice site (Horowitz, 2012). The adjoining exons are then covalently bonded together and the lariat with the snRNPs attached is released.

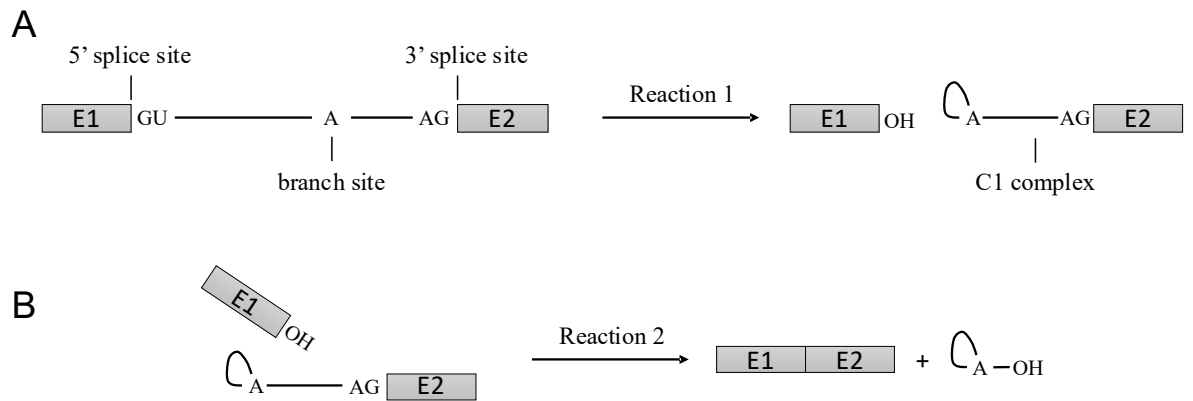


Figure 5.2. Schematic showing the two transesterification reactions in pre-mRNA splicing. A) In the first reaction, the hydroxyl group on the branch site adenosine carries out a nucleophilic attack on guanine residue at the 5' splice site. This forms the cleaved 5' exon and the C1 complex, which is an intron-3' exon intermediate product. **B)** The now exposed hydroxyl group at the 3' end of the cleaved exon attacks the guanine residue at the 3' splice site. This results in the two exons covalently bonding together, and the release of the intron lariat.

The splicing of pre-mRNAs is facilitated by a large number of non-snRNP factors, which includes SR family proteins like SF2/ASF, and SR-related proteins such as U2AF and Tra2 (Blencowe, 2000). These proteins contain RNA recognition motifs through which they bind to pre-mRNA, generally on exon splicing enhancer (ESEs) sequences to promote exon splicing (Zhou and Fu, 2013). In contrast to ESEs, exon splicing silencers (ESS) are sequences within exons involved in the inhibition of exon inclusion (Wang and Burge, 2008). These ESSs are often bound proteins like hnRNP A1, which can function by blocking interactions between the snRNPs, displacing bound snRNPs, or binding to either side of the exon and “loop out” the exon to prevent its inclusion (del Gatto-Konczak *et al.*, 1999; Graveley, 2009).

5.1.2. Alternative splicing in the nervous system

Alternative splicing is incredibly important in many eukaryotic tissues, but appears especially prominent in the nervous system, where it contributes to each step of nervous system development, from the differentiation of the initial neuronal progenitor cells to the complete formation of the adult brain (Su, D and Tarn, 2018). Indeed, the brain has the

highest proportion of alternatively spliced genes in the body, with more than 40% experiencing some form of alternative splicing (Yeo *et al.*, 2004; Mazin *et al.*, 2013). As alternative splicing has such an important role in nervous system, it is tightly regulated, however, aberrant alternative splicing does occur and it is a major contributor to neurological diseases (Soto *et al.*, 2019).

One of the first steps in neuronal differentiation involves a switch in usage between two alternative-splicing proteins, polypyrimidine tract-binding protein (PTB or PTBP1) and its paralog, neural-PTB (nPTB or PTBP2) (Boutz *et al.*, 2007). These two proteins show mutually exclusive expression patterns, with PTB protein present only in non-neuronal lineages, and nPTB restricted to neurons. PTB functions as splicing repressor, binding to conserved intronic elements and preventing spliceosome assembly (Hu *et al.*, 2018). One target of splicing repression by PTB is exon 10 of *nPTB* and the skipping of this exon generates a mature mRNA that is sensitive to nonsense mediated decay (Makeyev *et al.*, 2007). Within neural progenitor cells, a neuron-specific microRNA, miR-124, directly targets and downregulates PTB, leading to the correct splicing of nPTB and the translation of functional protein (Makeyev *et al.*, 2007) (Figure 5.3).

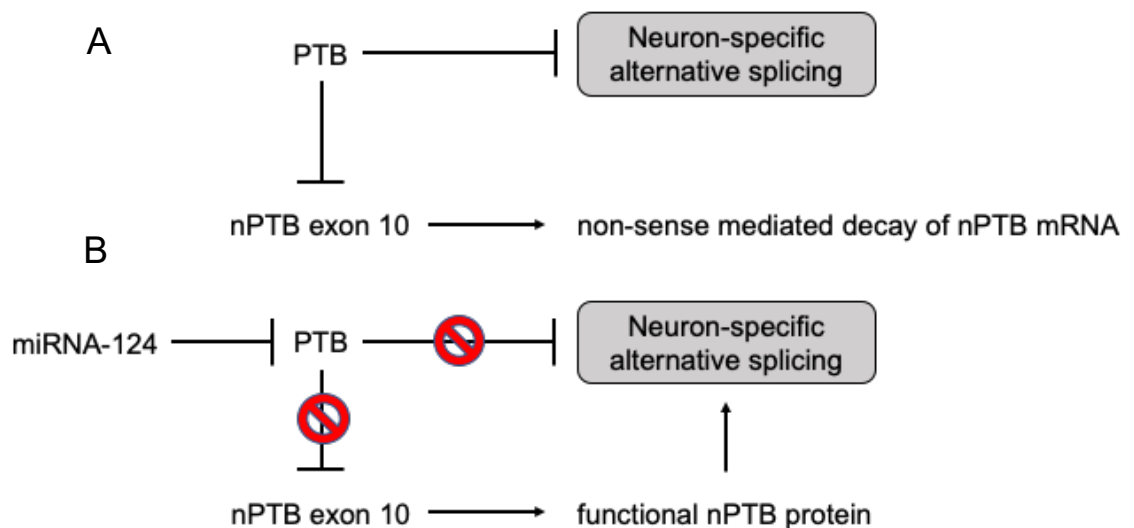


Figure 5.3. Schematic depicting the relationship between PTB and nPTB in neuronal development. **A)** Within non-neuronal cells, PTB inhibits the inclusion of exon 10 in the mature nPTB mRNA transcripts, leading to the mRNA being a target for nonsense mediated decay. PTB also inhibits neuron-specific alternative splicing. **B)** In neuronal cells, a neuron-specific microRNA, miR-124, inhibits PTB, leading to the inclusion of exon10 in the nPTB transcripts and the translation of a functional protein. nPTB then helps direct a neuron-specific pattern of alternative splicing.

Microarray experiments examining splicing changes between undifferentiated p19 mouse embryonal carcinoma cells, and those differentiated into neurons, found 202 changes in exons, and it was estimated that around 25% of those changes were dependent on the switch from PTB to nPTB (Boutz *et al.*, 2007). One such target of PTB and nPTB activity is the *n1* microexon of *src*, with PTB binding to conserved CUCUCU elements in regions flanking *n1* exon, preventing its inclusion in non-neural cells (Chan and Black, 1997).

5.1.3. Protein phosphorylation and alternative splicing

The post-translational phosphorylation of splicing factors plays an important role in regulating alternative splicing. *In vitro* experiments, in HeLa cell nuclear extracts, found that treatment with a serine/threonine phosphatase prevented the formation of the early spliceosome complex and the stable binding of U2 and U4/U5/U6 tri-snRNPs to the pre-mRNA (Mermoud, Cohen and Lamond, 1994). Further support for a role for phosphorylation in the regulation of splicing comes from experiments that have revealed that the phosphorylation of the SR family protein, SF2/ASF/SRSF1, enhances its binding to the U1-snRNP whilst reduces its non-specific RNA binding (Xiao and Manley, 1997). Meanwhile the U1-snRNP itself is phosphorylated, specifically on serine residues in the U1-70K protein (Woppmann *et al.*, 1990).

Protein phosphorylation is therefore required for the correct association of various splicing factors. In addition to this, phosphorylation of splicing factors also regulates their localisation within the cell. For example, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) can be serine phosphorylated, a modification that reduces its binding to transportin-1, a factor involved in shuttling hnRNPA1 between the cytosol and nucleus (Allemand *et al.*, 2005). As such, phosphorylation of hnRNPA1 sequesters the protein in the cytoplasm, thereby reducing its activity.

Whilst the majority of phosphorylation events that the spliceosome proteins and other splicing related proteins undergo are on serine and threonine residues, there is some evidence that tyrosine phosphorylation may also have a role in splicing regulation (Neel *et al.*, 1995; Shi, Reddy and Manley, 2006). Neel *et al.* used a *TNF β* reporter construct in NIH 3T3 cells alongside an expression vector for *c-src*. A set period of time after transfection, they examined the maturation of *TNF β* mRNA and found that transfection of *c-src* lead to alterations in the processing of *TNF β* , with transcripts present that contained retained introns. More recently, work in the Evans lab has shown that c-Src and n1-Src are both capable of interacting with and phosphorylating RNA processing factors (West,

2019). A GST-n1-SH3 pulldown using P1 rat brain lysates identified RNA processing factors that interact with SH3 domain of both c-Src and n1-Src, whilst phosphoproteomic screens using P1 rat brain lysates identified RNA processing protein that are phosphorylated by n1-Src. Based on its interaction, phosphorylation and transcriptional regulation of splicing factors, and the key role alternative splicing plays in nervous system development, n1-Src could regulate alternative splicing to define the neural transcriptome.

5.1.4. Aims of this chapter

In this chapter I aim to:

- Determine if there are any changes in alternative splicing during primary neurogenesis as a result of *n1-src* knockdown in *X. tropicalis* embryos. The Illumina RNA-Seq dataset from Chapter 4 will be analysed using splice aware software to identify any differential splice events.
- Verify that there are *in vivo* transcripts containing alternative splice isoforms using Nanopore long read RNA-Seq and RT-PCR analysis.
- Attempt to obtain mechanistic insight by searching for enrichment of RNA binding protein motifs associated with the splicing changes that occur following *n1-src* knockdown.

5.2. Results

5.2.1. DEXSeq analysis of the Illumina RNA-Seq data

Transcriptomic analysis on *n1-src* knockdown embryos revealed transcripts related to mRNA processing and alternative splicing are significantly upregulated (Chapter 4). As splicing factors appear to be transcriptionally regulated by n1-Src, DEXSeq analysis was carried out, in conjunction with the bioinformatics team in the Bioscience Technology Facility at the University of York, to investigate the changes in splicing and exon usage that occur in *X. tropicalis* embryos as a result of *n1-src* knockdown. This analysis was carried out using the same RNA-Seq dataset used in the differential expression chapter in this thesis. Poly(A) tailed mRNA had been sequenced from embryos injected with either 10ng of each *n1-src* splice blocking morpholino (20ng total) or 20ng of control morpholino.

DEXSeq tests for changes in the relative usage of exons caused by the experimental conditions (McLeay and Bailey, 2010). The first step in DEXSeq analysis aligns the sequencing reads to the *X. tropicalis* reference genome (v9.1 NCBI) with HISAT (<https://ccb.jhu.edu/software/hisat/index.shtml>). The transcript data for each gene then gets collapsed into “exon counting bins” (Figure 5.4). These exon counting bins are made up of exon units rather than complete exons. For example, in Figure 5.4 transcripts A-C contain 2 or 3 exons each. Meanwhile transcript D contains the same 3 exons as transcript C plus a retained intron. However, when collapsed down, the exon counting bin contains 5 exon units.

These exon units reflect the underlying intron-exon boundaries across all of the transcripts for the gene. Transcript reads are then aligned to the exon counting bin and the number of transcript reads that overlap with each of the exon units is counted. Whilst DEXSeq can identify differentially expressed exons/junction between experimental conditions, it is unable to properly identify the type of splicing event occurring (McLeay and Bailey, 2010).

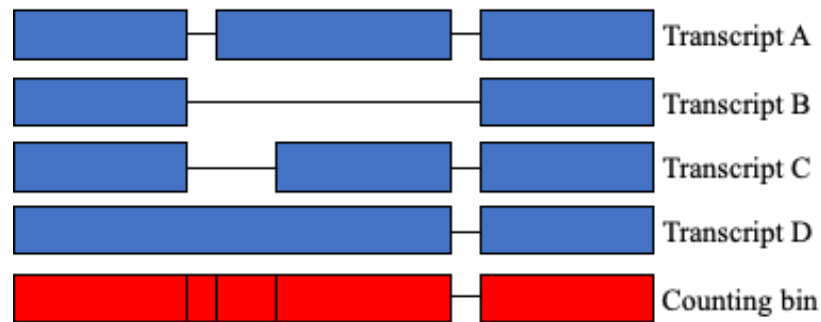


Figure 5.4. DEXSeq generates counting bins to examine differential exon usage.

To examine differential exon usage, DEXSeq generates exon counting bins, which are made up of exon units rather than complete exons. Here, transcripts A-D are aligned to the genome. The transcripts are then collapsed down to form a single exon bin. Whilst the transcripts only contain 2 or 3 exons, the counting bin is made up of 5 exon units, due to the different lengths of exon 2, and the retained intron in transcript D. The transcript reads are then aligned to the exon counting bin and the number of transcript reads that overlap with each of the exon units is counted.

DEXSeq analysis on the *n1-src* knockdown transcriptomic data identified 147 genes that show differential exon usage between the two conditions. Following the DEXSeq analysis, I uploaded the identified genes onto the Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System to identify statistically significant over-represented biological processes. The *M. musculus* gene database was used instead of the *X. tropicalis* database due to better annotation. PANTHER successfully mapped 139 of the genes.

Considering all levels of the gene ontology term hierarchy, 41 out of 15822 biological processes are significantly over-represented within the gene list ($Q < 0.05$). The full list of over-represented biological processes is listed in the “Supplementary File 4 – DEXSeq Panther Analysis”.

The over-represented processes were ordered by fold enrichment and within the top 20 are processes related to RNA processing and metabolism (Figure 5.5). This suggests that in addition to regulating the transcription of RNA processing and splicing factors (Chapter 4), *n1-Src* also regulates their splicing.

Of the 147 genes showing differential exon usage, PANTHER attributed 16 to function in RNA processing. These are listed in table 5.1, along with their role in *M. musculus* according to UniProt (<https://www.uniprot.org/>). Within these 16 genes are two of particular interest, *hnmpa1* and *tra2a*. This interest is due to their prominence in the differential expression analysis of the *n1-src* knockdown transcriptomic data. *Hnmpa1* and *tra2a* transcripts showed some of the largest and most significant increases in expression following *n1-src* knockdown (Table 4.9).

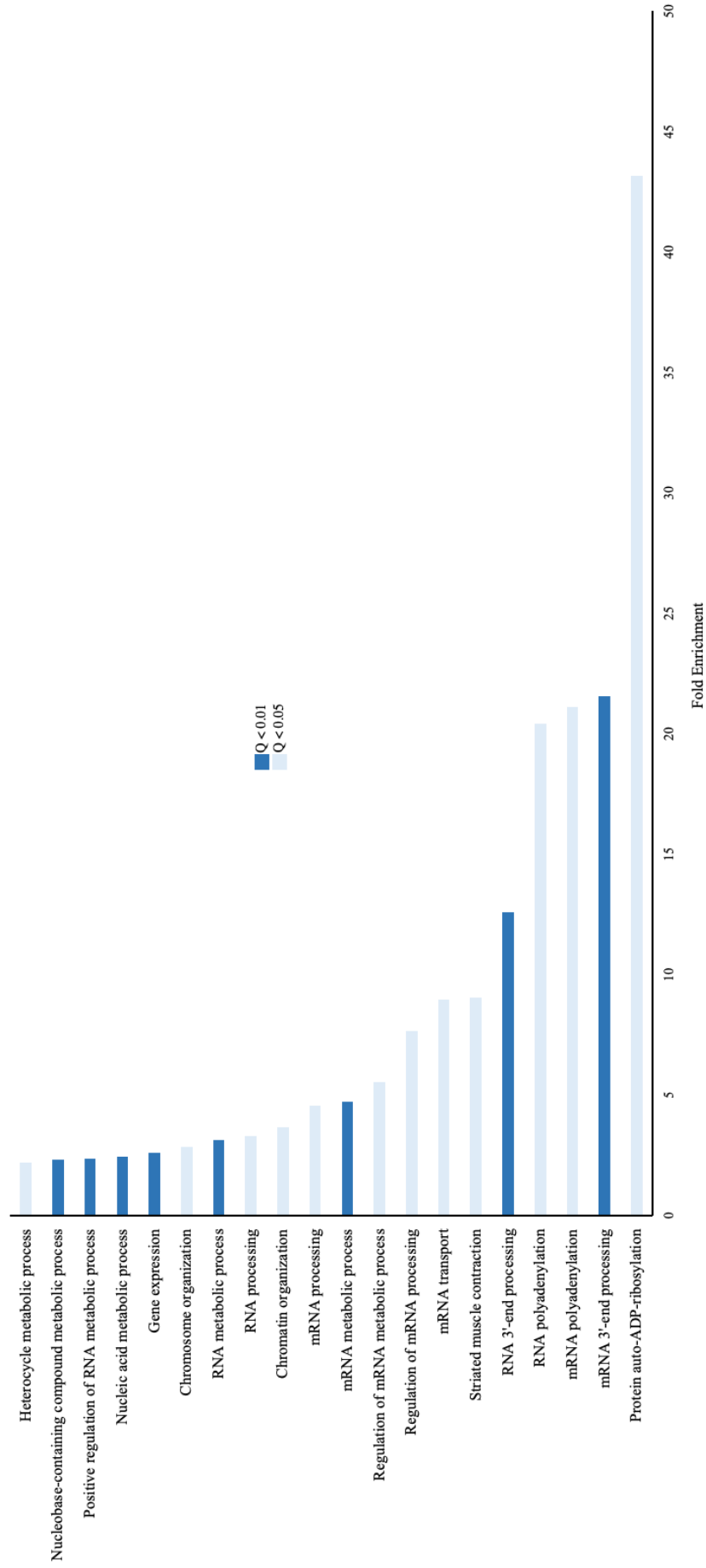


Figure 5.5. Top 20 biological processes, based on fold enrichment, overrepresented within the transcripts identified by DEXSeq having differential exon usage. Transcripts identified by DEXSeq were uploaded onto the PANTHER Classification system to identify significantly overrepresented biological processes. Within the top 20 processes, based on significance, are many associated with RNA processing.

Table 5.1. RNA processing genes identified by DEXSeq to be alternatively spliced in *n1-src* knockdown embryos.

Gene	Uniprot function
cpsf7	Component of the cleavage factor Im (CFIm) complex that functions as an activator of the pre-mRNA 3'-end cleavage and polyadenylation processing required for the maturation of pre-mRNA into functional mRNAs
dis3	Putative catalytic component of the RNA exosome complex which has 3'→5' exoribonuclease activity and participates in a multitude of cellular RNA processing and degradation events
hnrnpa1	Involved in the packaging of pre-mRNA into hnRNP particles, transport of poly(A) mRNA from the nucleus to the cytoplasm and may modulate splice site selection
ints7	Component of the Integrator (INT) complex, a complex involved in the small nuclear RNAs (snRNA) U1 and U2 transcription
papd5	Terminal nucleotidyltransferase that catalyses preferentially the transfer of ATP and GTP on RNA 3' poly(A) tail creating a heterogeneous 3' poly(A) tail leading to mRNAs stabilization by protecting mRNAs from active deadenylation
papola	Polymerase that creates the 3'-poly(A) tail of mRNA's
qki	RNA-binding protein that plays a central role in myelination. Acts by regulating pre-mRNA splicing, mRNA export and protein translation
rbmx	RNA-binding protein that plays several role in the regulation of pre- and post-transcriptional processes. Implicated in tissue-specific regulation of gene transcription and alternative splicing of several pre-mRNAs. Component of the supraspliceosome complex that regulates pre-mRNA alternative splice site selection.
rprd2	mRNA 3'-end processing
srpk3	Serine/arginine-rich protein-specific kinase which specifically phosphorylates its substrates at serine residues located in regions rich in arginine/serine dipeptides, known as RS domains. Phosphorylates the SR splicing factor SRSF1
srsf1	Plays a role in preventing exon skipping, ensuring the accuracy of splicing and regulating alternative splicing.
ssu72	Protein phosphatase that catalyses the dephosphorylation of the C-terminal domain of RNA polymerase II. Plays a role in RNA processing and termination. Plays a role in pre-mRNA polyadenylation via its interaction with SYMPK.
tarbp2	Required for formation of the RNA induced silencing complex (RISC).
tra2a	Sequence-specific RNA-binding protein which participates in the control of pre-mRNA splicing.
wbscr22	S-adenosyl-L-methionine-dependent methyltransferase that specifically methylates the N7 position of a guanine in 18S rRNA
wdr33	Essential for both cleavage and polyadenylation of pre-mRNA 3' ends.

5.2.2. *Hnrnpa1* and *tra2a* intron retention events identified by DEXSeq

Hnrnpa1 and *tra2a* are present in both the differential expression data and the DEXSeq analysis, suggesting *n1-Src* regulates both their expression and splicing. HnRNPA1 binds to splice silencing elements to inhibit splicing and Tra2a is an SR protein involved in regulating splicing (McLeay and Bailey, 2010).

The first step of the DEXSeq analysis aligns the Illumina sequencing reads to the *X. tropicalis* genome, prior to the generation of the exon counting bins, and this allows for the identification of retained introns within the data set. Within the DEXSeq output are the genome coordinates for all of the exon units within each counting bin. A Track Hub was generated on the UCSC genome browser (<http://genome-euro.ucsc.edu/cgi-bin/hgTracks?db=xenTro9>) to allow the visualisation of the Illumina reads mapped to the *X. tropicalis* genome. This was then used to search for the coordinates of the exon units identified by DEXSeq as being differentially used.

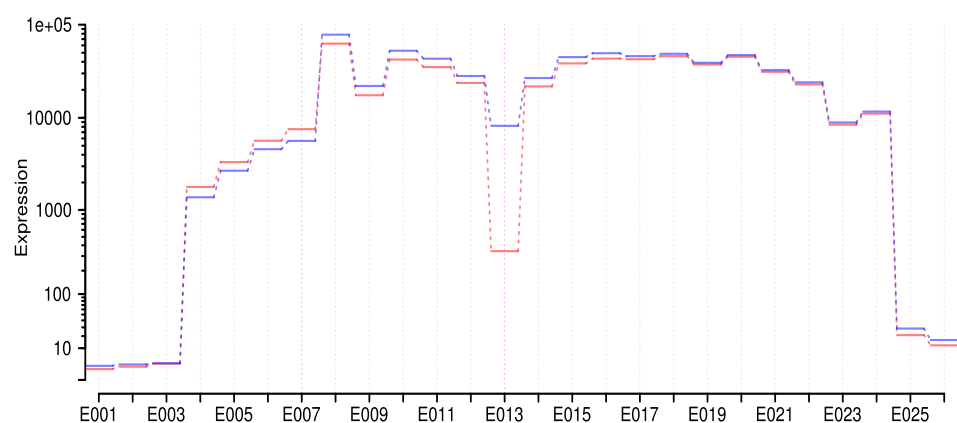
In the case of *hnrnpa1*, DEXSeq identifies exon unit 13 as being differentially used between the two conditions, with increased usage in the *n1-src* knockdown samples (Figure 5.6). This is seen when comparing the DEXSeq plots, with the red plot representing the exon unit usage in control embryos and the blue plot representing the usage in *n1-src* knockdown embryos (Figure 5.6A). DEXSeq identified exon unit 13 as having a Log2 fold change of 4.4 in *n1-src* knockdown embryos compared to controls (Figure 5.6B and 5.6C). Searching the custom track hub in the UCSC genome browser for the coordinates generated by DEXSeq revealed that the exon unit 13 is an intron, between exons 7 and 8, which is retained following *n1-src* knockdown (Figure 5.6D).

Tra2a has two differentially used exon units, 18 and 24, which both show increased usage in the knockdown embryos (Figure 5.7A). The exon units have a Log2 fold change of 3.2 and 3.7 following *n1-src* knockdown (Figure 5.7B and 5.7C). The coordinates of these events map to two retained introns between exons 5-6 and 6-7 (Figure 5.7D). The 5 exon units between units 18 and 24 of *tra2a* combine to form exon 6, which is not differentially used.

The DEXSeq analysis and the mapping of the Illumina short reads to the genome has pointed towards alterations in splicing as a result of *n1-src* knockdown. The Illumina system sequences short reads of about 150 base pairs in length. This system is capable of identifying novel splice junctions and changes in the abundance of these junctions, however, due to the short read length it is unable to characterise how these novel splice

junctions occur in full length transcripts. For example, Illumina sequencing has identified two retained introns in *tra2a*, however, it is unable to detect whether the events occur together in a single transcript or whether *n1-src* knockdown generates two novel *tra2a* isoforms, each with a separate intron retention event. Nanopore long read sequencing was therefore carried out so that full transcripts could be sequenced.

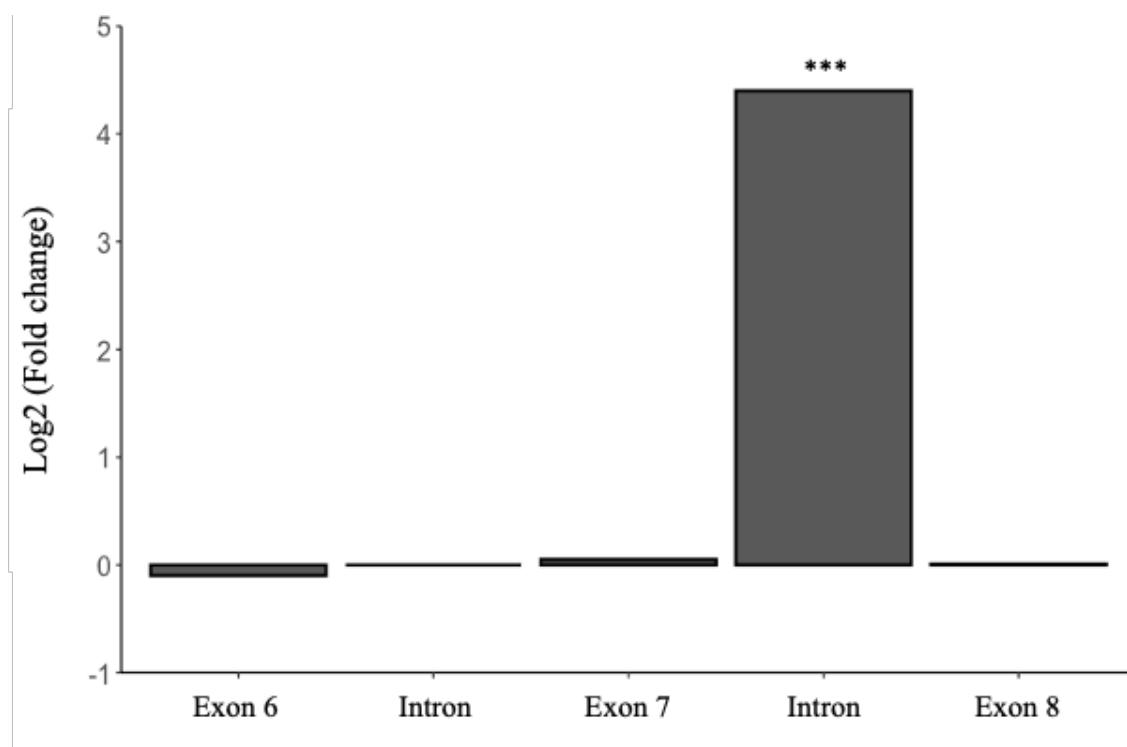
A



B

Exon Unit	Adjusted P-Value	Start	End	CMO	<i>n1-src</i> AMOs	Log2 (Fold Change)
E013	<0.001	142407878	142408035	22.914	43.965	4.401

C



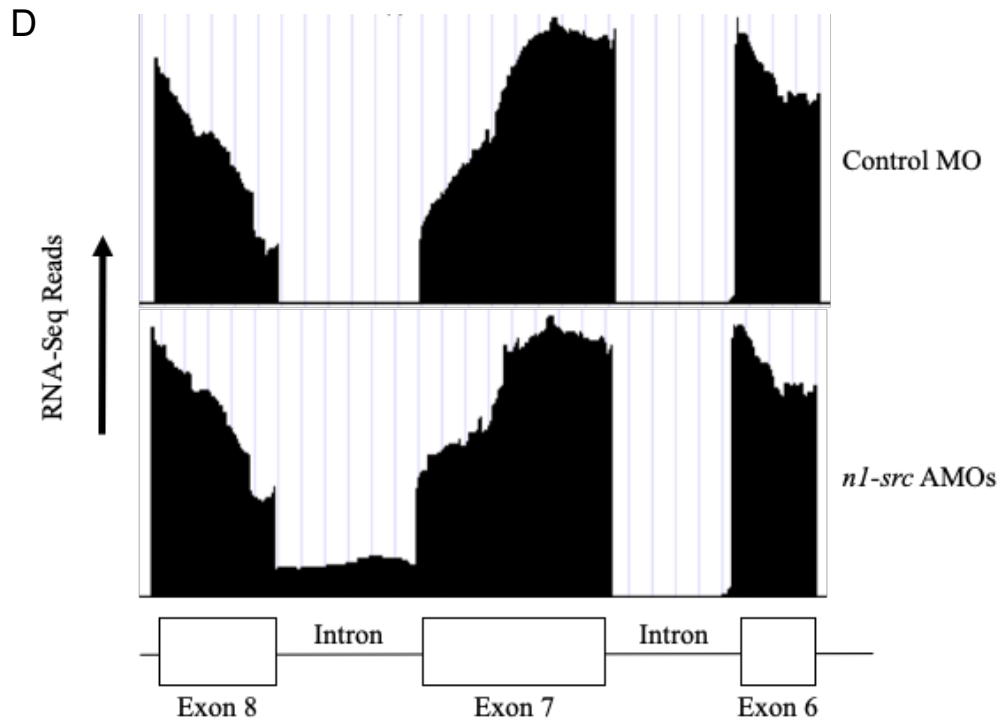
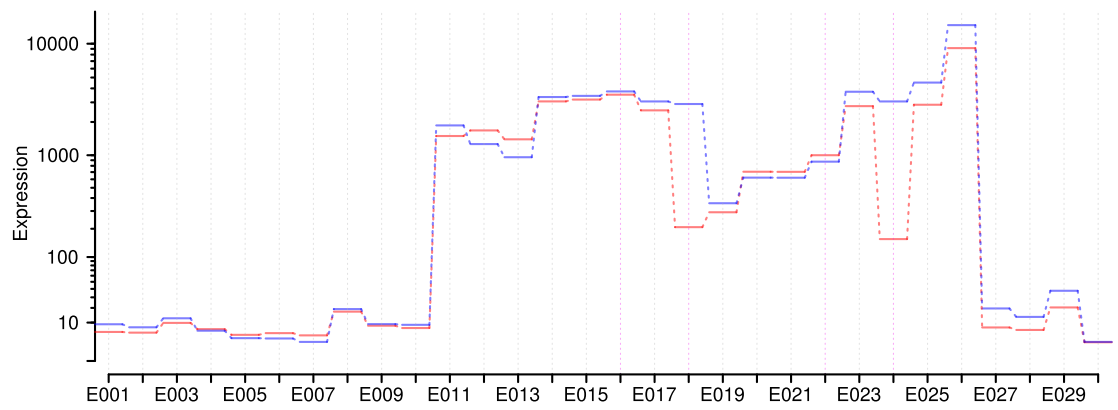


Figure 5.6. *N1-src* knockdown leads to an intron retention event in *hnrnpa1* transcripts. **A)** DEXSeq output showing *hnrnpa1* exon usage events in control (red) and knockdown (blue) samples. There is an increased usage of exon unit 13 in the experimental samples. **B)** DEXSeq output showing the coordinates and usage information for exon unit 13. **C)** Bar chart plotting the Log2 (Fold Change) for exons 6 to 8 and the intervening introns. ***P-adjust<0.001, adjusted for multiple testing using the Benjamini-Hochberg method. **D)** UCSC tracks showing the *hnrnpa1* Illumina short reads for one control sample and one *n1-src* knockdown sample. Knockdown of *n1-src* result in the retention of the intron between exons 7 and 8.

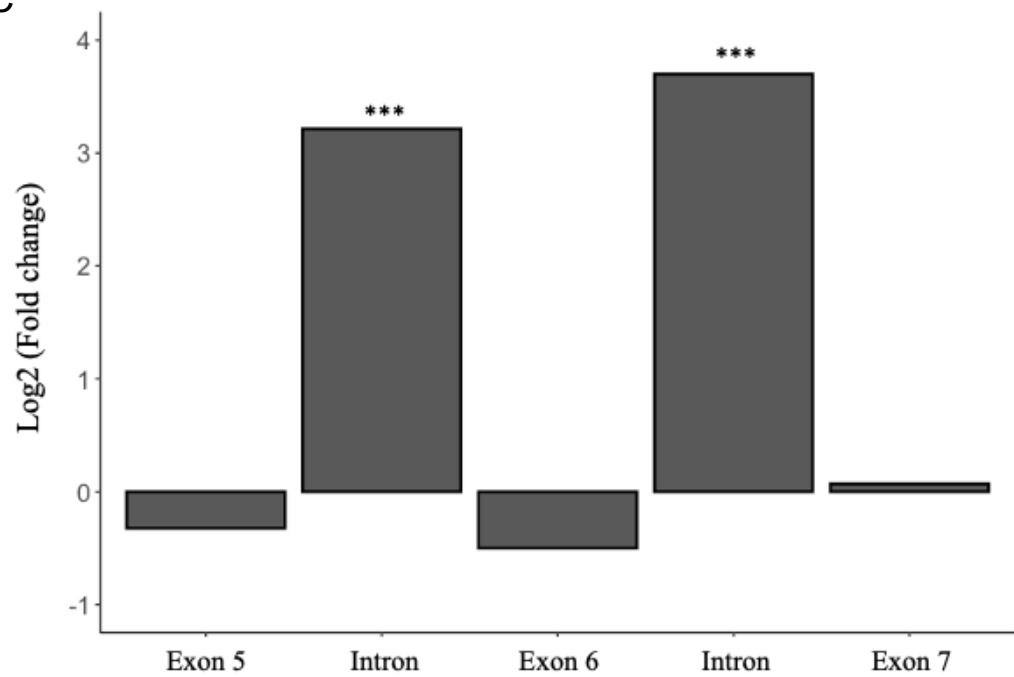
A



B

Exon Unit	Adjusted P-Value	Start	End	CMO	<i>n1-src</i> AMOs	Log2 (Fold Change)
E018	<0.001	34643207	34643207	20.605	35.672	3.214
E024	<0.001	34644670	34645343	18.839	36.042	3.698

C



D

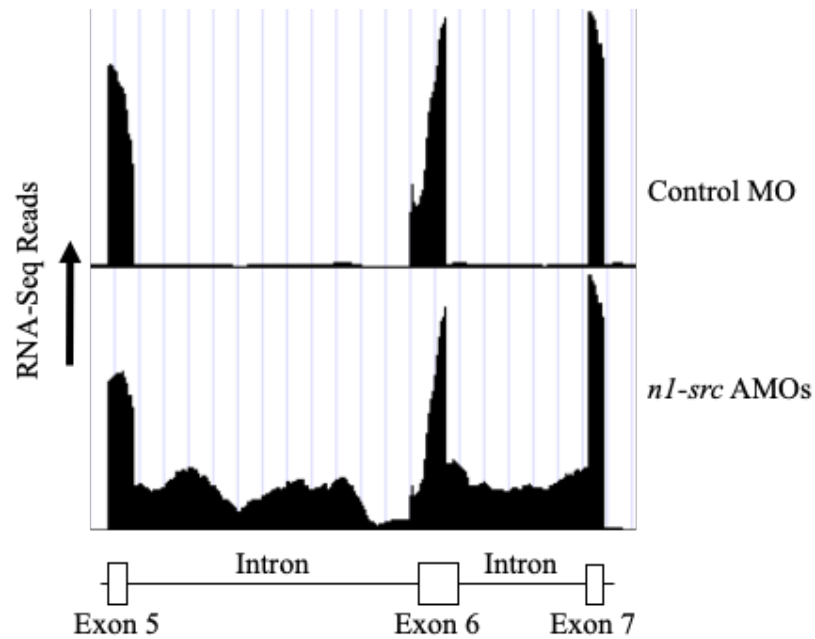


Figure 5.7. *N1-src* knockdown leads to two intron retention events in *tra2a* transcripts. **A)** DEXSeq output showing *tra2a* exon usage events in control (red) and knockdown (blue) samples. There is an increased usage of exon units 18 and 24 in the experimental samples. **B)** DEXSeq output showing the coordinates and usage information for exon units 18 and 24. **C)** Bar chart plotting the Log2 (Fold Change) for exons 5 to 7 and the intervening introns. ***P-adjust<0.001, adjusted for multiple testing using the Benjamini-Hochberg method. **D)** UCSC tracks showing the *tra2a* Illumina short reads for one control sample and one *n1-src* knockdown sample. Knockdown of *n1-src* result in the retention of introns between exons 5 and 6 and exons 7 and 8.

5.2.3. Nanopore long read RNA-Seq sample generation

Further RNA-Seq analysis was carried out at the University of York using the Oxford Nanopore PromethION, which allows the sequencing of full length transcripts. Sibling *X. tropicalis* embryos were bilaterally injected at the one- to two-cell stages with 10ng of *n1-src* AMOs (20ng total), 20ng of control morpholino, or 250pg of FLAG-tagged *n1-src* mRNA. Embryos were cultured to neurula stage 16 and flash frozen on dry ice. After RNA extraction, RT-PCRs were carried out to confirm *n1-src* knockdown in the experimental samples (Figure 5.8). *N1-src* expression was downregulated in the experimental samples compared to the controls in all four repeats. RNA samples were then submitted to the Bioscience Technology Facility at the University of York, where RNA-Seq library preparation and sample sequencing was carried out.

Western blotting was also performed to detect the translated FLAG epitope from *n1-src* mRNA injected samples, to confirm translation of the mRNA. However, the FLAG epitope was only detected in two of the repeats. As this experiment was investigating alterations in splicing following *n1-src* knockdown, rather than differential expression analysis, these samples were still retained in the experiment and used in transcriptome assembly.

Samples were sequenced using the Oxford Nanopore PromethION and raw reads aligned to *X. tropicalis* reference genome v9.1 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000004195.3/). The average sequence length was 860 bases, with the longest being 73,546. StringTie2 was then utilised to assemble a full transcriptome from the long-read RNA-Seq alignments (Kovaka *et al.*, 2019). This was carried out by the Bioscience Technology Facility.

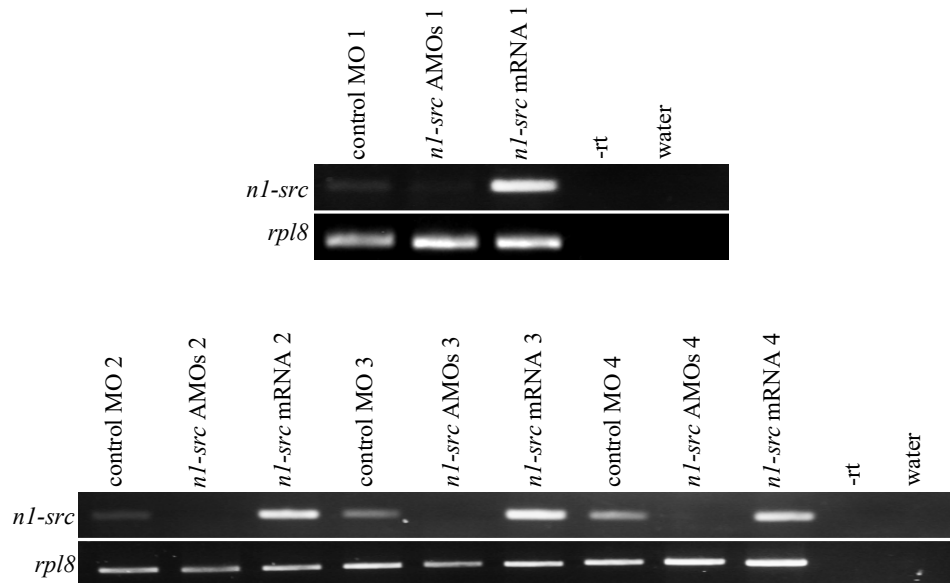


Figure 5.8. RT-PCR analysis for *n1-src* expression. *X. tropicalis* embryos were bilaterally injected, at the 1-2 cell stages, with 10g of *n1-src* AMO A+D (20ng total), 250pg *n1-src* mRNA or 20ng of control morpholino, and cultured to stage 16. RT-PCR was carried out on RNA extracted from these samples to confirm injection of the splice blocking morpholino downregulates *n1-src* expression. However, western blotting showed that only in samples 1 and 3 was the *n1-src* mRNA translated into protein. -rt, No reverse transcriptase control; water, no template control.

Various tools were then used to analyse splicing changes between the control and knockdown samples. Figure 5.9 shows a flow chart of the strategy used.

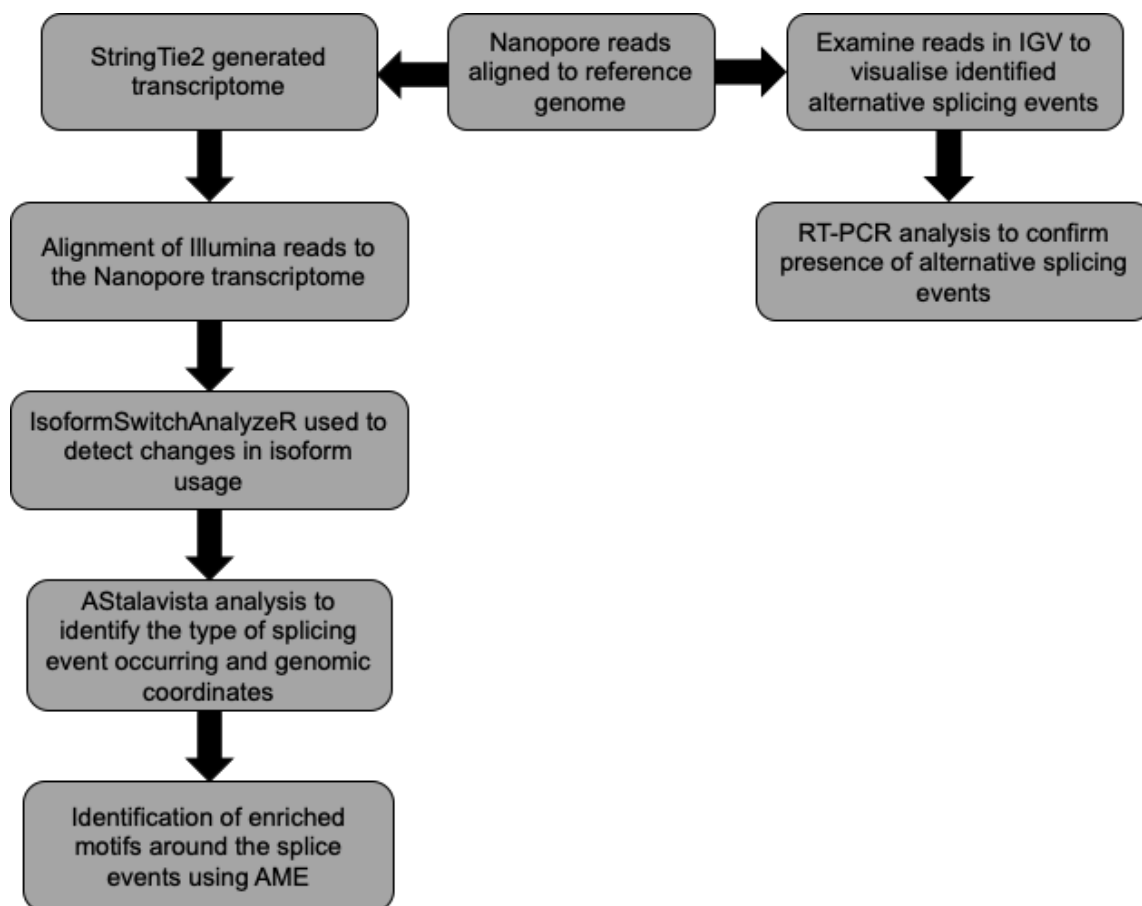


Figure 5.9. Flow chart depicting strategy used to analyse alternative splicing events. Nanopore long reads were aligned to the *X. tropicalis* reference genome. A transcriptome was then constructed with StringTie2 and the Illumina reads aligned to the this Nanopore transcriptome. IsoformSwitchAnalyzeR was then used to detect changes in isoform usage. The presence of enriched RNA binding motifs around the alternative splicing events was then analysed using ASTalavista to determine the genomic coordinates of the events and Analysis of Motif Enrichment (AME) was then used to identify enriched motifs. Meanwhile, the Nanopore long reads were examined in IGV to visualise the splicing events, and events were validated by RT-PCR analysis.

5.2.4. IsoformSwitchAnalyzeR identifies a genome-wide gain in intron retention

IsoformSwitchAnalyzeR was used by the Bioscience Technology Facility at the University of York to examine genome-wide changes in splicing and isoform usage between control and knockdown embryos (McLeay and Bailey, 2010).

IsoformSwitchAnalyzeR identifies pairs of isoforms with opposite changes in usage between two conditions, with one isoforms usage being increased and the others decreased, with at least one of the changes being significant (Vitting-Seerup and Sandelin, 2019). The functional consequence of these isoform switches is then predicted, for example protein domain gain or intron retention gain. Each consequence is part of a pair, for example protein domain gain is paired with protein domain loss and these can then be separated into the number of isoform switches resulting in loss and those resulting in gain. Then number of switches resulting in a gain can then be compared with the number resulting in a loss using a proportion test. This will reveal whether there is a significant enrichment of specific consequence types.

Whilst the Nanopore system is able to sequence full length mRNA transcripts and therefore reveal how novel splice junctions contribute to specific isoforms, it lacks the depth of sequencing required for accurate quantification of splice site usage. This however can be achieved by Illumina short read sequencing. For this reason the two were used together to analyse and quantify the changes in splicing following *n1-src* knockdown. The Illumina short reads, from the three control and three knockdown samples, were aligned to the novel StringTie2 transcriptome assembled from the Nanopore long read data, and IsoformSwitchAnalyzeR was run on these alignments.

IsoformSwitchAnalyzeR identified significant isoform switches in 57 genes at a significance level of $Q < 0.1$ (Table 5.2). Of these 57 genes, 14 were also identified by DEXSeq analysis as having differential exon usage. PANTHER gene ontology analysis was carried out on these 57 genes to identify any enriched biological processes, however none were enriched. There were however, within the 57 genes, 5 that function in RNA processing and splicing, including *hnmpa1* and *tra2a*.

IsoformSwitchAnalyzeR revealed significant increases in intron retention following *n1-src* knockdown, combined with significant protein domain losses (Figure 5.10). IsoformSwitchAnalyzeR uses Pfam to predict the protein domains, such as RNA recognition motifs. 30 different isoforms were identified to have retained introns, whilst 25 exhibit domain loss.

Table 5.2. Genes identified by IsoformSwitchAnalyzeR to have significant changes in isoform usage following *n1-src* knockdown.

Genes with isoform switches	Uniprot function	Present in DEXSeq data
aldoa	Plays a key role in glycolysis and gluconeogenesis	-
ankrd49	May have a role in spermatogenesis	+
arf6	GTP-binding protein involved in protein trafficking that regulates endocytic recycling and cytoskeleton remodeling	-
arhgap8	GTPase activator for the Rho-type GTPases by converting them to an inactive GDP-bound state.	-
c19orf47	Uncharacterised function	-
clip1	Binds to the plus end of microtubules and regulates the dynamics of the microtubule cytoskeleton.	-
dennd5b	Guanine nucleotide exchange factor (GEF) which may activate RAB39A and/or RAB39B	-
diras3	GTP binding	-
eda	Cytokine which is involved in epithelial-mesenchymal signalling during morphogenesis of ectodermal organs	-
eef1d	Stimulates the exchange of GDP bound to EF-1-alpha to GTP	+
epha4	Modulates cell morphology and integrin-dependent cell adhesion through regulation of the Rac, Rap and Rho GTPases activity. Plays an important role in the development of the nervous system controlling different steps of axonal guidance	-
fam124a	Uncharacterised function	-
gaa	Essential for the degradation of glycogen in lysosomes.	-
gmppa	May serve as a regulatory subunit and allow allosteric feedback inhibition of GMPPB by GDP-mannose.	+
hinfp	Transcriptional repressor that binds to the consensus sequence 5'-CGGACGTT-3' and to the RB1 promoter	-

Genes with isoform switches	Uniprot function	Present in DEXSeq data
hnnpa1	Involved in the packaging of pre-mRNA into hnRNP particles, transport of poly(A) mRNA from the nucleus to the cytoplasm and may modulate splice site selection	+
hopx	Required to modulate cardiac growth and development. Acts via its interaction with SRF, thereby modulating the expression of SRF-dependent cardiac-specific genes and cardiac development	-
hs2st1	Catalyses the transfer of sulfate to the C2-position of selected hexuronic acid residues within the maturing heparan sulfate (HS)	-
ivns1abp	Involved in many cell functions, including pre-mRNA splicing	-
kansl3	Involved in acetylation of nucleosomal histone H4	-
kif22	Involved in spindle formation and the movements of chromosomes during mitosis and meiosis.	-
kirrel2	May regulate basal insulin secretion	-
kpna2	Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1	-
kpna4	Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1	+
krt5.1	mRNA processing	+
loc100145764	Uncharacterised function	-
loc100488736	Uncharacterised function	-
loc100489887	Uncharacterised function	-
loc100494719	Uncharacterised function	-
loc100495624	Uncharacterised function	-
loc101730971	Uncharacterised function	-
lrrc16a	Cell membrane-cytoskeleton-associated protein that plays a role in the regulation of actin polymerization	-
lyrm4	R Cell membrane-cytoskeleton-associated protein that plays a role in the regulation of actin polymerization	-
mterf3	Binds promoter DNA and regulates initiation of transcription	-
nol11	Ribosome biogenesis factor	-

Genes with isoform switches	Uniprot function	Present in DEXSeq data
nudcd3	Unfolded protein binding	-
nup210	Nucleoporin essential for nuclear pore assembly and fusion	-
pds5a	Regulator of sister chromatid cohesion in mitosis	-
ppp1r16b	Regulator of protein phosphatase 1 (PP1) that acts as a positive regulator of pulmonary endothelial cell (EC) barrier function.	-
psmc5	Component of the 26S proteasome	-
rpl3l	RNA binding protein	-
rprd2	mRNA 3'-end processing	+
sec22a	Involved in vesicle transport between the ER and the Golgi complex	-
sgsm3	Plays a cooperative role in NF2-mediated growth suppression of cells.	+
spopl	Component of a E3 ubiquitin-protein ligase complex	-
st6gal2	Transfers sialic acid from the donor of substrate CMP-sialic acid to galactose containing acceptor substrates	+
syt8	Involved in the trafficking and exocytosis of secretory vesicles in non-neuronal tissues	+
tctn3	Part of the tectonic-like complex which is required for tissue-specific ciliogenesis	-
tdrd10	RNA binding protein	-
tmem101	May activate NF-kappa-B signalling pathways	+
tra2a	Sequence-specific RNA-binding protein which participates in the control of pre-mRNA splicing.	+
trim33	E3 ubiquitin-protein ligase	+
ttc26	Component of the intraflagellar transport (IFT) complex B required for transport of proteins in the motile cilium	-
usp46	Deubiquitinating enzyme	+
wbp4	Involved in pre-mRNA splicing as a component of the spliceosome	-
wrap73	Act as regulator of spindle anchoring at the mitotic centrosome	-
zbtb3	Involved in transcriptional regulation	-

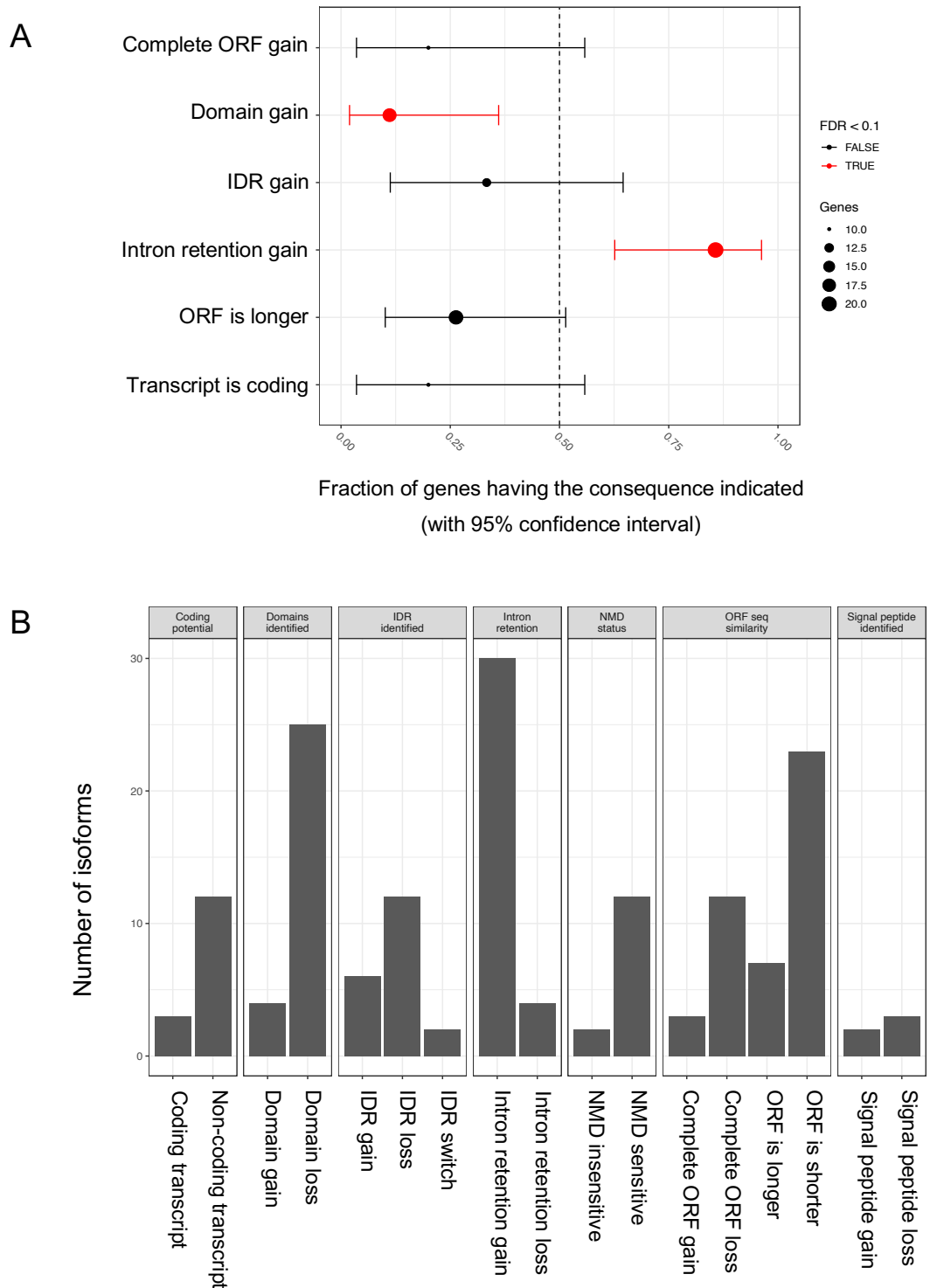


Figure 5.10. Functional consequences of splicing changes identified by IsoformSwitchAnalyzeR. A) Fraction of genes that have the indicated consequence. 6 consequences are shown that could occur from isoform switches. The size of the circle indicates the number of genes effected. If the fraction of genes is above 0.5, then there is an increase in the consequence, for example an increase in retained introns. If below 0.5 then there is an increase in the opposing consequence, for example, the fraction of genes with a domain gain is less than 0.5, meaning there is an increase in domain losses. Red= significant, $Q < 0.1$. ORF=open reading frame. IDR=intrinsically disordered region. **B)** The number of isoforms which contain each consequence, the most abundant consequence is intron retention gain that is seen in 30 isoforms, whilst 25 isoforms show domain loss.

5.2.5. IsoformSwitchAnalyzeR identified splicing changes in *hnrnpa1* and *tra2a*

IsoformSwitchAnalyzeR identified significant changes in isoform usage for both *hnrnpa1* and *tra2a*. I have chosen to examine these in more detail due to them showing some of the most significant changes in expression following *n1-src* knockdown, being 2 of the few genes identified by both DEXSeq and IsoformSwitchAnalyzeR.

There were three isoforms identified for *hnrnpa1*, with one isoform showing no usage change, one with a significant decrease and another with a significant increase in usage (Figure 5.11). The isoform that had a significant increase in usage (MSTRG 6255.9) appeared to contain a retained intron between exons 7 and 8, and IsoformSwitchAnalyzeR identified this isoform as being sensitive to non-sense mediated decay.

Tra2a has six isoforms identified by IsoformSwitchAnalyzeR, with two showing a significant increase in usage (Figure 5.12). The first isoform which is used more following *n1-src* knockdown (MSTRG 15264.2) lacks exon 6 which has been skipped, whilst the other isoform (MSTRG 15264.7) appears to have retained introns between exons 5 and 6, and exons 6 and 7. The latter isoform fails to code for a protein. The intron retention events seen for *tra2a* and *hnrnpa1* in the IsoformSwitchAnalyzeR data matched those identified by the DEXSeq analysis (Figured 5.5 and 5.6). However, the presence of an exon skipping event in an isoform of *tra2a* is a novel observation.

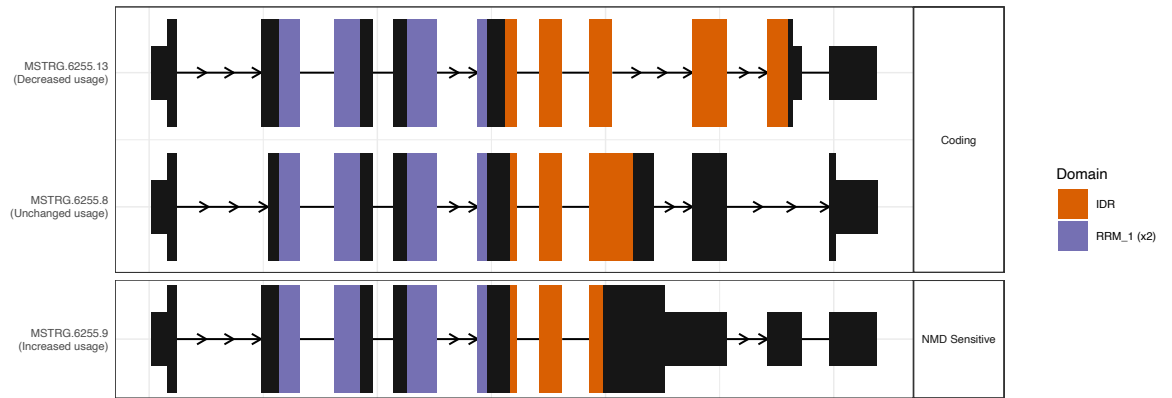


Figure 5.11. IsoformSwitchAnalyzeR identified significant changes in *hnrnpa1* isoform usage. IsoformSwitchAnalyzeR identified 3 *hnrnpa1* isoforms within the dataset. The figure depicts the untranslated regions (UTRs), introns and exons for each isoform. Each cluster of tall vertical rectangles represents an exon, the horizontal line separating these represents introns and the smaller rectangles at each end represent the 5' and 3' UTRs. Isoforms labelled MSTRG.6255.13 and 6255.8 are each made up of 9 exons. IsoformSwitchAnalyzeR attempts to identify the function of each exon. Parts of exons 2-5 have been identified to function as RNA Recognition Motif (RRM) domains (purple). The orange exon regions are thought to contribute to an Intrinsically Disordered Region (IDR). IsoformSwitchAnalyzeR identified isoform MSTRG.6255.13 as having decreased usage and another isoform MSTRG.6255.9 as having increased usage in response to *n1-src* knockdown. MSTRG.6255.9 has a retained intron between exons 7 and 8. This intron probably contains a premature stop codon as its retention results in the subsequent exons being recognised as 3' UTRs rather than coding exons and the transcript is marked as being sensitive to non-sense mediated decay.

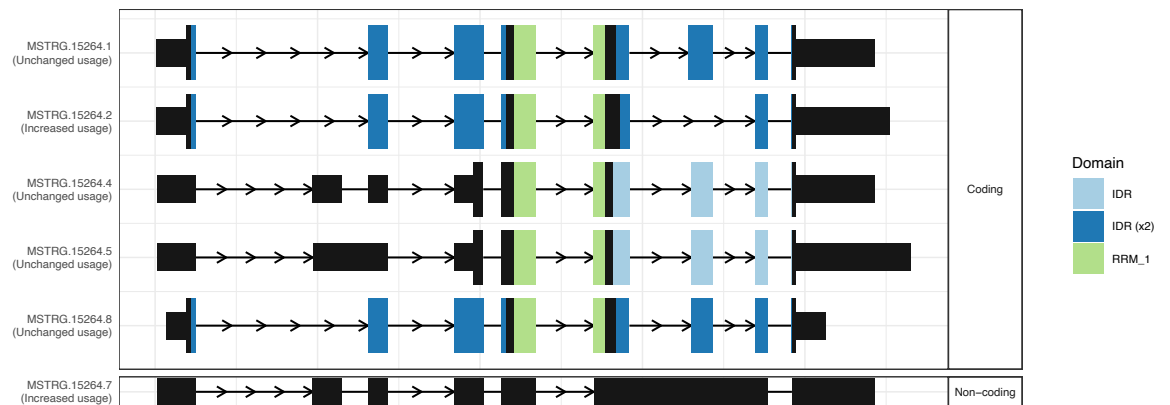


Figure 5.12. IsoformSwitchAnalyzeR identified significant changes in *tra2a* isoform usage. IsoformSwitchAnalyzeR identified 6 *tra2a* isoforms, 2 of which show an increased usage following *n1-src* knockdown. The figure depicts the exons, introns and untranslated regions (UTRs) for each isoform. Each cluster of tall vertical rectangles represents an exon, the horizontal line separating these represents the introns and the smaller rectangles at each end represent the flanking 5' and 3' UTRs. Isoforms MSTRG.15264.1 and 15264.8 contain 8 exons, MSTRG.15264.2 contains 7 exons and MSTRG.15264.4 and 1564.5 contain 6 exons. IsoformSwitchAnalyzeR tries to identify the function of particular exons. In the case of *tra2a*, the light blue and dark blue exon regions contribute to Intrinsically Disordered Regions (IDR), whilst the green exon regions contribute to an RNA Recognition Motif (RRM) domain. Following *n1-src* knockdown, there is an increased usage in MSTRG.15264.2, in which alternative splicing has skipped exon 6. The other isoform identified to have an increase in usage in response to *n1-src* knockdown is MSTRG.15264.7, which contains retained introns between exons 5 and 6 and exons 6 and 7, and this isoform has been identified by IsoformSwitchAnalyzeR as being non-coding.

5.2.6. Alternative splicing of *hnrnpa1* and *tra2a* in the Nanopore long read RNA-Seq data

The DEXSeq analysis carried out on the Illumina short read data, and the mapping of the Illumina short reads to the *X. tropicalis* genome, have shown intron retention events in *hnrnpa1* and *tra2a* in response to *n1-src* knockdown. These events have also been seen in the IsoformSwitchAnalyzeR data when the Illumina short reads were aligned to the novel transcriptome assembled from the Oxford Nanopore full length transcripts.

I next searched for these events within the Nanopore long read data itself to see if full length transcripts containing these alternative splicing events were truly present in the data. The Nanopore long reads were aligned to the *X. tropicalis* genome (v.9.1) and the Integrative Genome Browser 2.5.2 (IGV) from the Broad Institute (<http://software.broadinstitute.org/software/igv/>) was then used to view the alignments.

When examining *hnrnpa1*, full length reads were present in the *n1-src* knockdown samples that contain the retained intron between exons 7 and 8. This intron retention was absent from reads from the control morpholino samples (Figure 5.13). Closer analysis of the sequences revealed that the retained intron contains a premature stop codon, which is in keeping with the IsoformSwitchAnalyzeR results. IsoformSwitchAnalyzeR identified the isoform with the retained intron as being sensitive to non-sense mediated decay (Figure 5.9).

In the *n1-src* knockdown samples there were also full length *tra2a* transcripts that have retained introns between exons 5 and 6 and exons 6 and 7 (Figure 5.13). There were also many *tra2a* transcripts within the *n1-src* knockdown samples that have skipped exon 6, which fits with the results seen in the IsoformSwitchAnalyzeR data.

Analysis of the long-read RNA-Seq alignments confirmed that knockdown of *n1-src* leads to alterations in splicing of *hnrnpa1* and *tra2a*, with full length transcripts from both genes displaying the same alterations detected by the DEXSeq and IsoformSwitchAnalyzeR analysis.

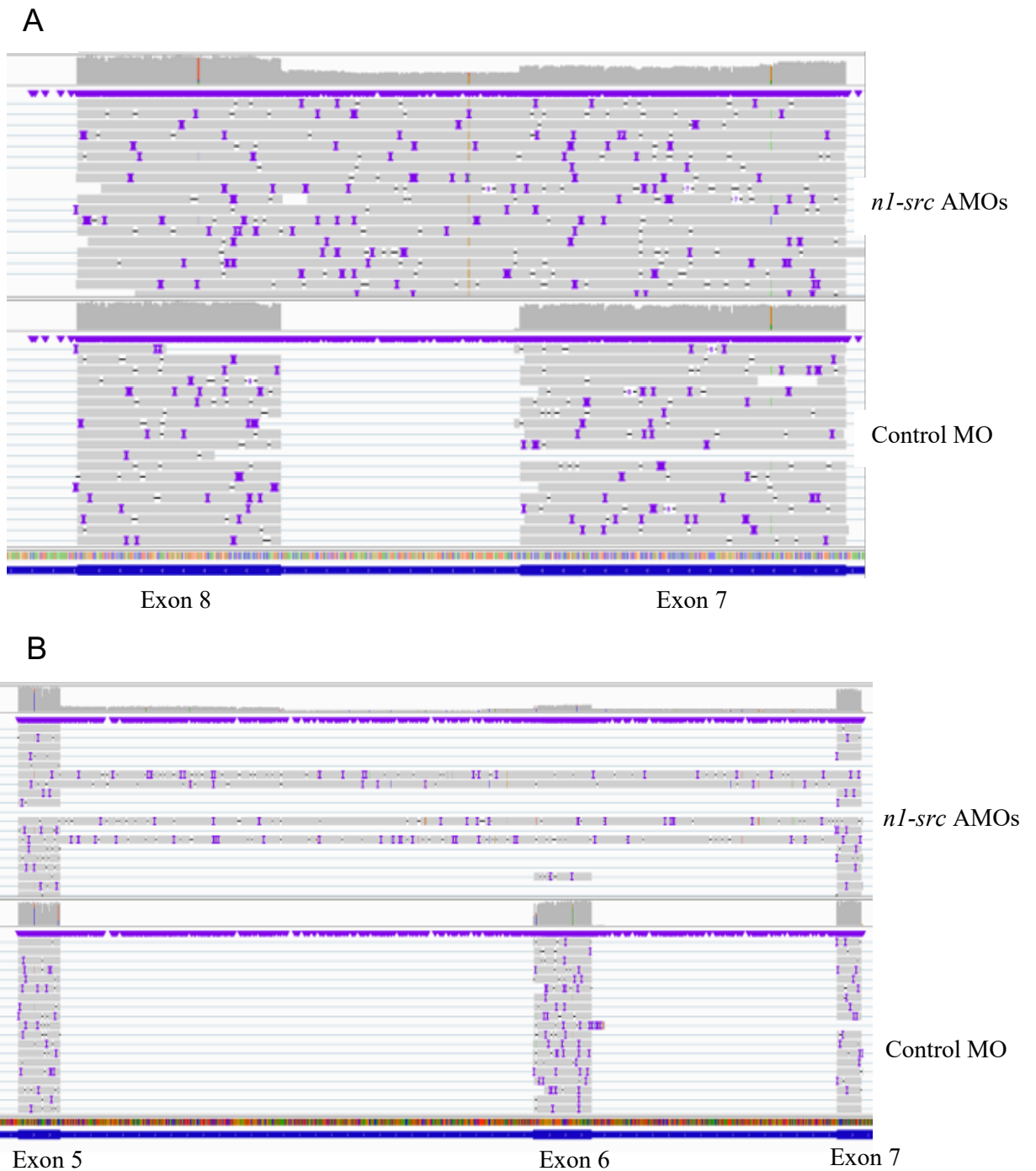


Figure 5.13. Nanopore long reads confirm the splicing events identified by DEXSeq and IsoformSwitchAnalyzerR. Nanopore full length transcripts from a single *n1-src* knockdown and control sample were aligned to the *X. tropicalis* genome in IGV. These transcripts confirm the results from the DEXSeq and Isoform Switch Analysis. **A)** Individual full length transcripts can be seen in for *hnrnpa1* that retain the intron between exons 7 and 8 following *n1-src* knockdown. Such reads are absent from the control sample. **B)** *Tra2a* transcripts can be seen in the *n1-src* knockdown sample that either skip exon 6, or contain exon 6 and retain the introns flanking either side.

5.2.7. RT-PCR analysis verifies the *hnrnpa1* and *tra2a* intron retention events

To verify the intron retention events observed in the analysis of the RNA-Seq data, RT-PCR analysis was carried out on *X. tropicalis* embryos injected with 10ng AMO A+D (20ng total), 20 ng CMO or 250pg FLAG-tagged *n1-src* mRNA. RNA was extracted from embryos collected at early neurula stages 14-16. PCR primers were designed to anneal within exons 7 and 8 of *hnrnpa1* and exons 6 and 7 of *tra2a*, and consequently large or small products would be produced depending on whether or not the introns are retained.

RT-PCR analysis detected the retention of introns in both *hnrnpa1* and *tra2a* following *n1-src* knockdown (Figure 5.14). Overexpression of FLAG-tagged *n1-src* mRNA had no observable effect. Knockdown of *n1-src* also resulted in a smaller *hnrnpa1* product being generated, suggesting that in addition to transcripts with retained introns there were also mRNA transcripts that experienced other splicing alterations.

Examination of the *hnrnpa1* long read transcripts found that there were full length transcripts in which the knockdown of *n1-src* had led to the use of an alternative 5' splice donor site and this resulted in the majority of exon 7 being spliced out along with the intron (Figure 5.14).

All the PCR products for *tra2a* and *hnrnpa1* were then isolated and sent for sequencing by GATC/Eurofins. The sequence alignments to the *X. tropicalis* genome are in Appendix A-E. The sequencing of these products confirmed the splicing events shown here are the same as those seen in the long read nanopore data. These intron retention events have been repeatedly seen by RT-PCR analysis, thereby confirming the alternative splicing events shown previously in this chapter for *hnrnpa1* and *tra2a*.

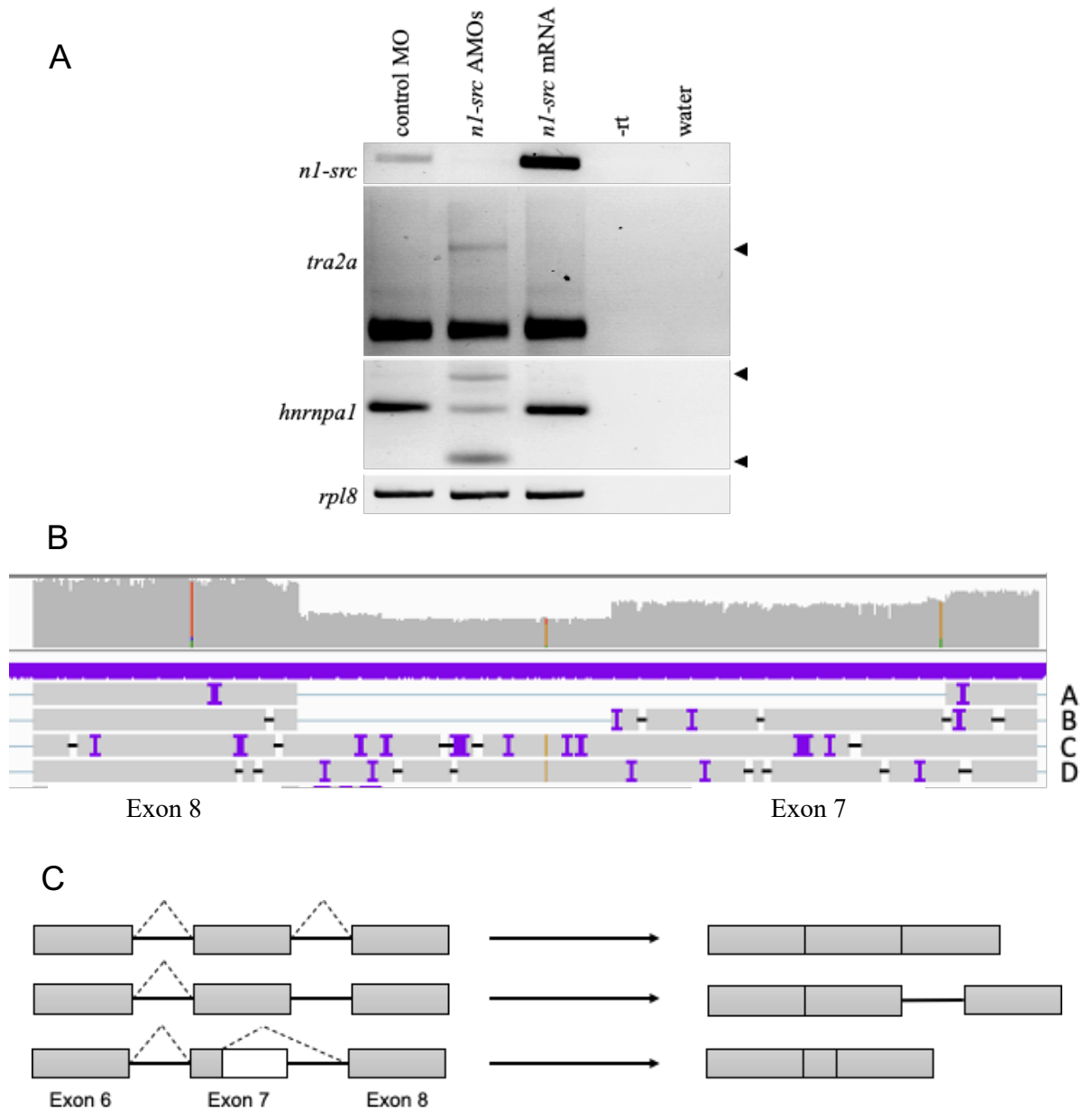


Figure 5.14. RT-PCR analysis verifies the changes in *hnrnpa1* and *tra2a* splicing following *n1-src* knockdown. **A)** *X. tropicalis* embryos were bilaterally injected, at the 1-2 cell stages, with 10g of *n1-src* AMO A+D (20ng total), 250pg *n1-src* mRNA or 20ng of control morpholino, and cultured to stages 14-16. RT-PCR were then carried out for *n1-src*, *tra2a* and *hnrnpa1*. Knockdown of *n1-src* lead to intron retention in both *tra2a* and *hnrnpa1*. A smaller *hnrnpa1* product was also detected. *N1-src* overexpression had no effect on splicing of either gene. *Rpl8* is a loading control. -rt, No reverse transcriptase control; water, no template control. **B)** *Hnrnpa1* transcripts from the Nanopore long read RNA-Seq experiment. 4 individual transcripts from 1 of the *n1-src* knockdown samples are shown. Alongside the normal transcript (B) and transcripts with the retained intron (C and D), is a *hnrnpa1* in which the majority of exon 7 has been spliced out (A). This corresponds to the smaller product seen in the RT-PCR experiment. **C)** Schematic visualising the 3 different *hnrnpa1* products formed. The top is the normal spliced product, the middle contains the retained intron and the bottom depicts the alternative 5' splice site in exon 7 which results in the generation of the smallest PCR product.

5.2.8. Overexpression of *n1-src* mRNA rescues the *hnmpa1* splicing changes following *n1-src* knockdown

To confirm that the changes in alternative splicing observed are a specific result of *n1-src* knockdown, rather than an off-target effect of the splice blocking morpholinos, work was carried out to rescue the splicing changes. *Hnmpa1*, rather than *tra2a*, was chosen as the primary target for a rescue attempt as it's alternative splicing events were more sensitive to *n1-src* knockdown, meaning that a lower concentration of morpholinos could be utilised.

Firstly, the minimum concentration of *n1-src* morpholinos that would still knockdown *n1-src* and cause changes in the splicing of *hnmpa1* was optimised through microinjections and RT-PCR analysis. Once, this had been ascertained, various concentrations of *n1-src* mRNA were then titrated in to try to rescue the events. All of the tested mRNA concentrations at least partially rescued the splicing events, and ultimately 2ng of mRNA was determined to be optimal.

X. tropicalis embryos were bilaterally injected at the one- to two-cell with either 4ng control morpholino, 2ng of each *n1-src* splice blocking morpholino, 2ng of FLAG-tagged *n1-src* mRNA or a combination of 2ng of each *n1-src* morpholino and 2ng of *n1-src* mRNA. Embryos were collected at stage 14 for RT-PCR and western blot analysis.

As previously observed, knockdown of *n1-src* results in the retention of the intron between exons 7 and 8, and also the generation of a smaller product through the use of an alternative 5' splice site (Figure 5.15). Injection of *n1-src* mRNA alongside the *n1-src* morpholinos is able to rescue these events, proving that they are specific to *n1-src* knockdown. Western blotting confirmed the translation of the injected mRNA (Figure 15). The *hnmpa1* splicing events have been successfully rescued 2 times using the concentrations of morpholinos and mRNA listed above, along with another 2 times using different concentration combinations.

Therefore, the alterations in *hnmpa1* splicing are a specific result of *n1-src* knockdown rather than an off-target event.

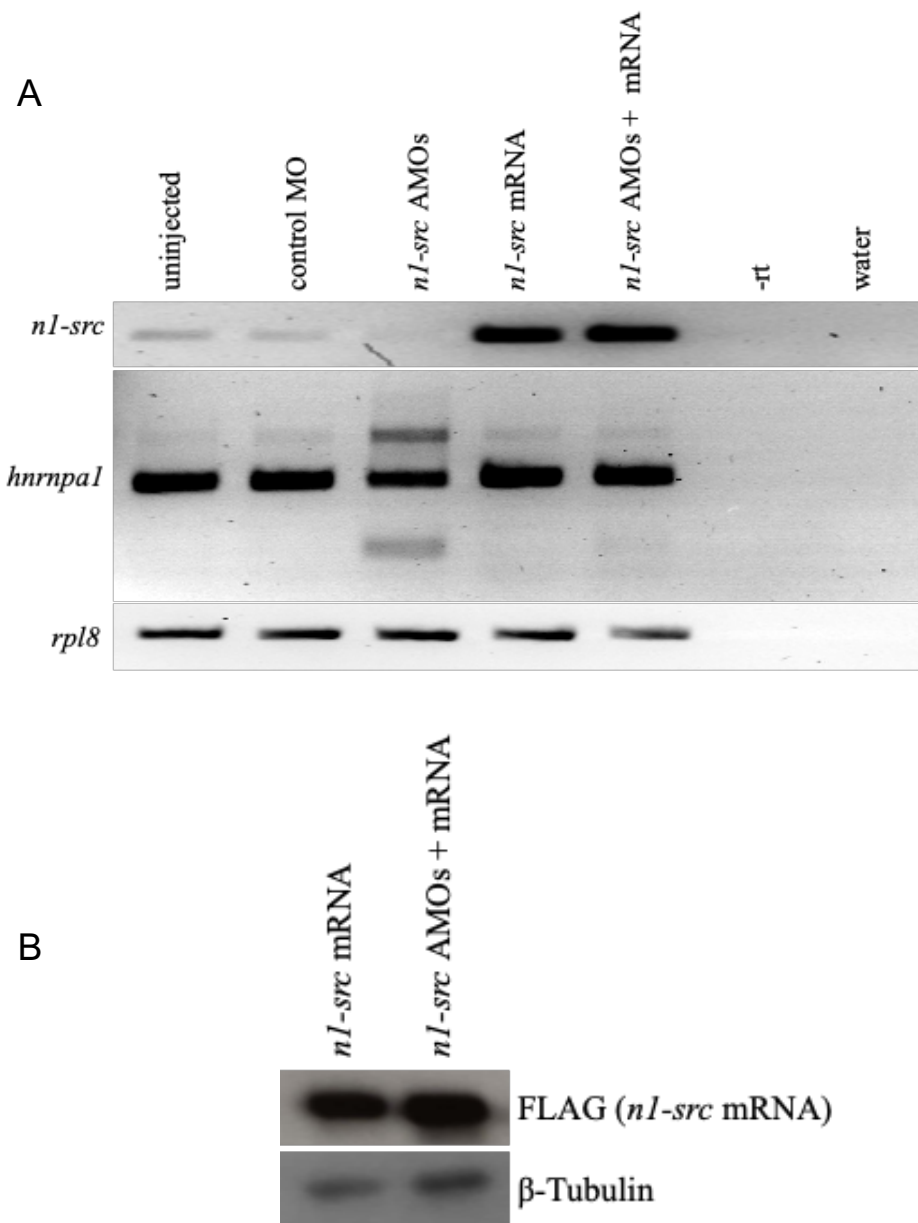


Figure 5.15. *N1-src* mRNA rescues the *hnnpa1* mis-splicing caused by *n1-src* knockdown **A)** *X. tropicalis* embryos were bilaterally injected, at the 1-2 cell stages, with 2g of *n1-src* AMO A+D (4ng total), 2ng *n1-src* mRNA, 4ng of control morpholino or a combination of the *n1-src* morpholinos and mRNA. Embryos were cultured to stages 14-16. RT-PCR were then carried out for *n1-src* and *hnmpa1*. Knockdown of *n1-src* leads to the mis-splicing of *hnmpa1* and the generation of 3 different products. *N1-src* mRNA injected alongside the *n1-src* morpholinos is able to rescue the mis-splicing events. *Rpl8* is a loading control. -rt, No reverse transcriptase control; water, no template control. **B)** Western blotting for the FLAG epitope, confirming translation of the *n1-src* mRNA.

5.2.9. 14 motifs for RNA binding proteins were enriched in the sequences surrounding the alternative splicing events

The results in this chapter so far show that n1-Src is involved in the regulation of alternative splicing, with factors such as *hnmpa1* and *tra2a* being alternatively spliced following *n1-src* knockdown. The mechanistic details behind this regulation however remain unknown. To try to obtain a view on how n1-Src regulates these splicing events, analysis was carried out to identify any enriched motifs for RNA binding proteins proximal to the splice junctions.

Whilst IsoformSwitchAnalyzeR can identify global changes in isoform usage such as domain loss or gain, because it uses DEXSeq during its analysis it is unable to properly identify the underlying splicing events occurring in each isoform change. Its output also does not contain genomic coordinates for the events. Due to these limitations, AStalavista (<http://astalavista.sammeth.net/>) was used by the bioinformatics team to further analyse the splicing changes. AStalavista characterises the alternative splicing events in a given transcript annotation (McLeay and Bailey, 2010). Full length transcripts from the 57 genes identified by IsoformSwitchAnalyzeR were extracted from the StringTie2 transcriptome file and inputted into AStalavista. By comparing the transcripts, AStalavista detected the variations in splicing structure and identified the type of alternative splicing event and provided its coordinates.

For 11 of the 57 IsoformSwitchAnalyzeR genes, AStalavista failed to identify the type of alternative splicing event occurring between their different transcripts. For the other 46 genes AStalavista identified one or more alternative splicing events in the analysed transcripts (Table 5.3). The dominant form of events were intron retentions and exon skipping, although there were also events where alternative splice acceptor and splice donor sites were used.

AStalavista identified the same intron retention event in *hnmpa1* that was seen in all the previous analyses. The intron retention events that DEXSeq identified in *tra2a* transcripts were also detected by AStalavista, along with the exon skipping event seen in the IsoformSwitchAnalyzeR data. The coordinates confirmed that exon 6 of *tra2a* is being skipped. Interestingly, AStalavista identified another exon skipping event in *tra2a*, however the coordinates of this event did not match to an exon but instead the intron and splice site upstream of exon 2.

Table 5.3. The type of alternative splicing event occurring and the genome coordinates of each event as identified by AStalavista

Gene	Alternative splicing event	Genome coordinates
ankrd49	Exon skipping	NC_030678.1; 162075260; 162075367
arf6	Intron retention	NC_030684.1; 59443251; 59443394
arhgap8	Alternative splice donor	NW_016685428.1; 4807; 4867
c19orf47	Alternative splice donor Alternative splice acceptor	NC_030684.1; 56789356; 56789371 NC_030684.1; 56790568; 56790690
clip1	Exon skipping	NC_030677.1; 153170952; 153171056
dennd5b	Intron retention	NC_030679.1; 354331; 354413
eda	Alternative splice donor	NC_030684.1; 22516656; 22516662
eef1d	Exon skipping	NC_030682.1; 134417541; 134417615
epha4	Alternative splice acceptor	NC_030681.1; 121191082; 121191085
fam124a	Exon skipping	NC_030678.1; 146970093; 146970124
gaa	Intron retention	NC_030686.1; 12678048; 12678143
gmppa	Intron retention	NC_030685.1; 78252005; 78252561
hinfp	Exon skipping	NC_030683.1; 85717157; 85717309
hnrrpa1	Intron retention	NC_030678.1; 142407877; 142408036
kansl3	Exon skipping Alternative splice donor	NC_030679.1; 109044903; 109045041 NC_030679.1; 109026116; 109026215
kif22	Intron retention	NW_016683721.1; 7078; 7174
kirrel2	Intron retention	NW_016683384.1; 841940; 842239
kpna2	Intron retention	NW_016683548.1; 183144; 183222
kpna4	Exon skipping Intron retention	NC_030681.1; 103300772; 103300801 NC_030681.1; 103309760; 103310207
loc100488736	Alternative splice donor	NC_030684.1; 4642044; 4642569
loc100489887	Intron retention	NC_030684.1; 24713897; 24714538
loc100494719	Exon skipping	NC_030678.1; 34220019; 34220042
loc100495624	Alternative splice donor	NC_030679.1; 10042738; 10044261
loc101730971	Intron retention	NC_030685.1; 1757139; 1757277
lrrc16a	Alternative splice acceptor	NC_030682.1; 29385310; 29385313
lyrm4	Alternative splice donor	NC_030682.1; 50162840; 50163118
mterf3	Exon skipping	NC_030682.1; 115002853; 115003014
nol11	Intron retention	NW_016683548.1; 271445; 271540
nup210	Intron retention	NW_016683948.1; 10051; 10456

Gene	Alternative splicing event	Genome coordinates
pds5a	Exon skipping Alternative splice donor	NC_030677.1; 27105834; 27105972 NC_030677.1; 27166387; 27166456
ppp1r16b	Alternative splice acceptor	NC_030686.1; 31929801; 31929857
psmc5	Exon skipping	NC_030686.1; 8620596; 8620694
rpl3l	Intron retention	NC_030685.1; 11154843; 11154991
rprd2	Alternative splice donor	NC_030684.1; 107028545; 107028551
sec22a	Alternative splice donor	NW_016683995.1; 7178; 7213
sgsm3	Other event	NC_030680.1; 111375858; 111376897
syt8	Exon skipping	NC_030680.1; 12527165; 1252720
tctn3	Intron retention	NW_016683904.1; 15505; 15581
tldr10	Alternative splice acceptor	NW_016683840.1; 60535; 60568
tmem101	Exon skipping	NC_030686.1; 13716996; 13717176
tra2a	Exon skipping	NC_030682.1; 34638900; 34639207
		NC_030682.1; 34644511; 34644669
	Intron retention	NC_030682.1; 34643206; 34644508
		NC_030682.1; 34643206; 34645344
trim33	Exon skipping Alternative splice acceptor	NC_030678.1; 67270734; 67270784 NC_030678.1; 67258085; 67258157
ttc26	Exon skipping	NC_030680.1; 104753321; 104753413
usp46	Alternative splice donor	NC_030684.1; 25994057; 25994065
wbp4	Alternative splice acceptor	NC_030678.1; 129054685; 129054688
wrap73	Exon skipping	NC_030683.1; 93279371; 93279457

The next step was to examine the sequences surrounding these coordinates for any enriched RNA protein binding conserved motifs. For each event, there would be 4 regions of sequence to analyse (Figure 5.16). The 5 bp immediately upstream and downstream of the splicing events were ignored as these would be the canonical sites where general splicing factors, required for every splicing event, would bind. The regions analysed were instead the next 100 bp up and downstream.

These sequences were extracted from the *X. tropicalis* genome and uploaded into the Analysis of Motif Enrichment (AME) tool (McLeay and Bailey, 2010), which is part of the MEME suite (<https://meme-suite.org/meme/tools/ame>). AME searches for enriched motifs within the submitted sequences, from a motif database available within the MEME suite (Ray *et al.*, 2013). Random 100 bp sequences from the *X. tropicalis* genome were used as control sequences.

Motifs for 14 different RNA binding proteins were found to be enriched across the splice events ($P_{adj} < 0.05$) (Figure 5.16). Whilst these 14 different RNA binding proteins had motifs enriched in the data, these motifs were only present in 21 of the 46 genes in which IsoformSwitchAnalyzeR and AStalavista identified splicing changes. The most abundant motif was that for the RNA binding protein Srsf1 which was found in 16 sites across 10 different genes. Table 5.4 lists the RNA binding proteins, the enriched motifs to which they bind, and the targets in which these motifs were located. Interestingly, none of the motifs from the Ray 2013 database that were enriched in the data were located in *hnrnpa1*, however the *tra2a* splicing sites had motifs recognised by Rmb45 and Srsf7.

N1-Src therefore regulates splicing in neuronal development, and there are RNA binding protein motifs that are enriched among some of the targets that it regulates, in particular motifs for Srsf1. This suggests that n1-Src might be capable of regulating the binding of these RNA binding proteins to these motifs, however the exact mechanism by which it does this is unknown and requires further research. Furthermore, the enriched motifs were only present in a subset of the genes identified by IsoformSwitchAnalyzeR and AStalavista as being alternatively spliced in *n1-src* knockdown embryos. The RNA binding proteins that could regulate the splicing events in the other 25 genes remain unknown and requires further research.

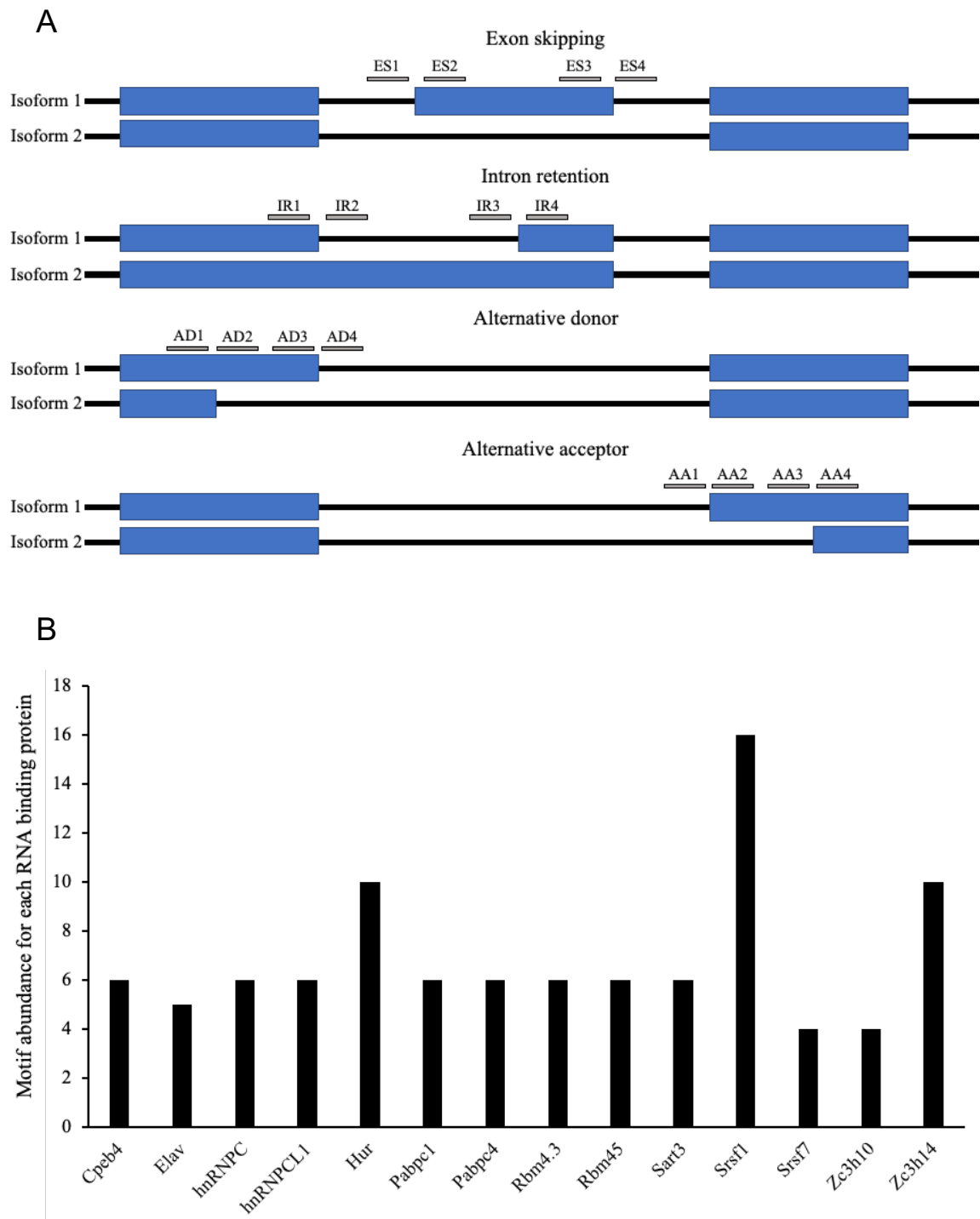


Figure 5.16. Analysing the sequences around the alternative splicing events for enriched motifs. A) Schematic showing the four regions of sequence, for each type of alternative splicing event, that would be analysed for enriched motifs. Each sequence is 100 base pairs long, and 50 base pairs up or downstream of the splicing events. **B)** Motifs for 14 RNA binding proteins were enriched within the analysed sequences. The most abundant motif was for Srsf1.

Table 5.4. The RNA binding proteins, their relevant enriched binding motif and the transcripts in which the motifs are located. There are 14 different RNA binding proteins with enriched motifs that are present in 21 of the transcripts ($P_{adj} < 0.05$). The RNA binding protein with the most targets was Srsf1 with 10.

RNA binding protein	Motif sequence	Associated splicing event	Transcripts affected
CPEB4	CTTTTTT	Exon skipping	kpna4, LOC100494719, trim33, mterf3, wrap73, syt8
ELAV	TTTKKTT	Exon skipping	kpna4, LOC100494719, trim33, mterf3, kansl3
HNRNPC	ATTTTTK	Exon skipping	kpna4, LOC10049471, trim33, mterf3, wrap73, syt8
HNRNPL1	ATTTTTT	Exon skipping	kpna4, LOC10049471, trim33, mterf3, wrap73, syt8
HUR	TTTTTTT	Exon skipping	kpna4, LOC10049471, trim33, mterf3, wrap73, syt8
HUR	TTATTTT	Exon skipping	kpna4, LOC10049471, trim33, mterf3
PABPC1	ARAAAAA	Exon skipping	kpna4, LOC100494719, trim33, mterf3, wrap73, syt8
PABPC4	AAAAAAA	Exon skipping	kpna4, LOC100494719, trim33, mterf3, wrap73, syt8
RBM4.3	NACGACGM	Intron retention	rpl3l, tra2a, LOC101730971, nup210
RBM45	GACGACM	Intron retention	LOC100489887, LOC101730971, rpl3l, tra2a, nup210, arf6
SART3	ARAAAAA	Exon skipping	LOC100494719, syt8, kpna4, trim33, wrap73, mterf3

RNA binding protein	Motif sequence	Associated splicing event	Transcripts affected
SRSF1	MAGGACAV	Exon skipping	hinf, kpna4, ttc26, ankrd49, fam124a, tmem101, pds5a, trim33, eef1d, syt8
SRSF7	GACGACGR	Intron retention	LOC101730971, tra2a, rpl3l, LOC100489887
ZC3H10	SCAGCGC	Alternative donor	kansl3, usp46, pds5a, arhgap8
ZC3H14	TTTGTTT	Exon skipping	LOC100494719, kpna4, kansl3, trim33, mterf3, syt8, wrap73, hinf, eef1d, ttc26

5.2.10. N1-Src regulates *n1-src* splicing

Data presented in this thesis indicates that n1-Src regulates the splicing of a variety of splicing factors, alongside interacting with and/or phosphorylating various splicing and mRNA processing proteins. Since *n1-src* is a neuronal-specific alternative splice variant of *src*, I hypothesised that n1-Src might regulate its own splicing in *X. tropicalis*.

To investigate further, embryos were bilaterally injected at the one- to two-cell stages with either 500pg or 1000pg of mammalian *n1-src* mRNA. Embryos were cultured to stage 14 and RNA was extracted. There are significant enough differences between the mammalian and *X. tropicalis* *n1-src* sequences to allow for the PCR amplification of one, without amplifying the other. RT-PCRs were carried out on the RNA samples to detect *X. tropicalis* *n1-src* and *c-src* expression (Figure 5.17).

Overexpression of mammalian *n1-src* led to the significant downregulation of *X. tropicalis* *n1-src* expression without affecting *c-src* expression (Figure 5.17). The lack of effect on *c-src* expression suggests that the regulation is happening at the level of the pre-mRNA, rather than transcription of the gene. N1-Src therefore appears to act in a negative feedback loop, regulating its own splicing levels and inhibiting the inclusion of the *n1* microexon when expression levels get too high. However, the exact mechanism through which it acts requires further research. Interestingly, factors that are involved in inhibiting inclusion of the *n1* microexon include hnRNPA1 and Srsf1, which I have shown to also be regulated by n1-Src.

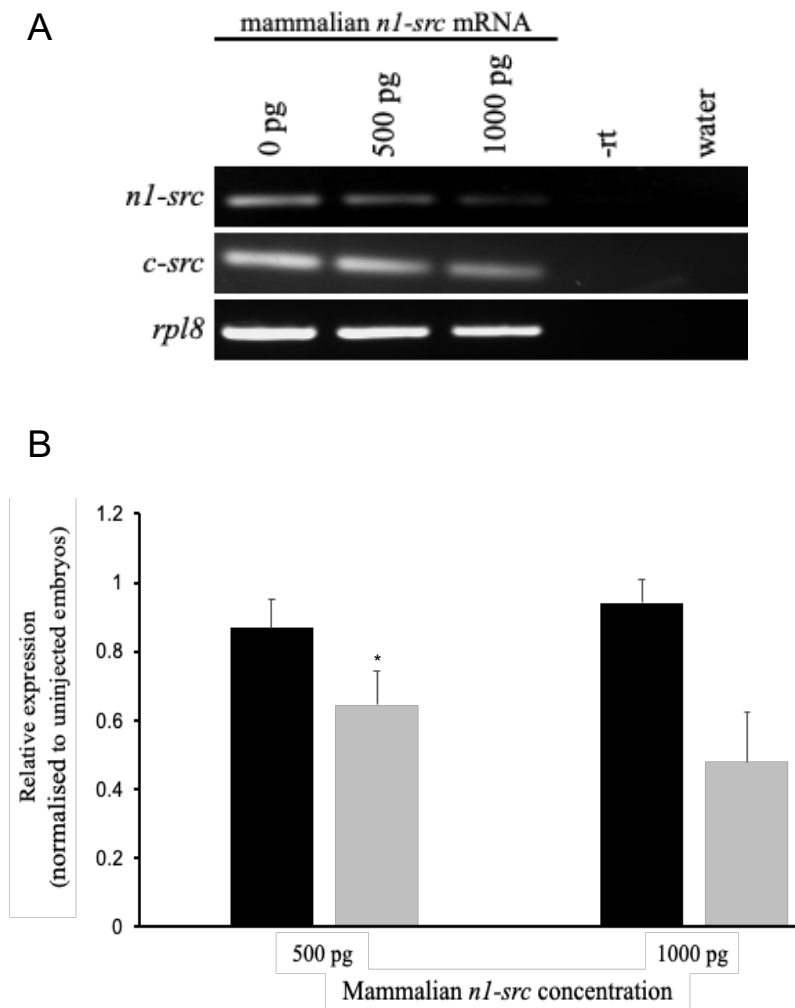


Figure 5.17. N1-Src regulates its own splicing. A) RT-PCR analysis was carried out for *n1-src* and *c-src* expression in *X. tropicalis* embryos injected with various concentrations of mammalian *n1-src* mRNA. The more mammalian *n1-src* mRNA that was injected, the less *X.tropicalis* *n1-src* expression. However, *c-src* expression was not affected. Therefore, transcription of the *src* gene was not affected by overexpressing *n1-src*, but splicing of the *n1* microexon was reduced. **B)** Quantification of *n1-src* and *c-src* RT-PCR expression levels following *n1-src* overexpression, normalised to uninjected embryos. Data is plotted as mean $\pm$ SEM ($n = 3$ independent experiments, analysed by t-test, * $p < 0.05$).

5.3. Discussion

The data in this chapter have shown that n1-Src has a role in splicing regulation, including regulating the splicing of splicing factors. This is seen in the Illumina short read and Nanopore long read RNA-Seq data which both show alterations in splicing, particularly intron retention and exon skipping events, in response to *n1-src* knockdown.

The role for n1-Src in regulating alternative splicing is supported by the differential expression of splicing factors in *n1-src* knockdown *X. tropicalis* embryos (Chapter 4), and the ability of n1-Src to interact with, and phosphorylate various splicing factors (West, 2019).

5.3.1. N1-Srcs regulation of alternative splicing

Alternative splicing is a method of increasing the transcriptome diversity, and can occur in a tissue or developmental-specific manner, or in response to cellular signals (Nilsen and Graveley, 2010). The mammalian brain and nervous system has exceptionally high levels of alternative splicing, with many genes having neural specific splice variants, such as *src* (Yeo *et al.*, 2004). Alternative splicing is involved in all steps of nervous system development such as during axon guidance, synaptogenesis and neuron differentiation (Li, Lee and Black, 2007).

This thesis has shown that n1-Src is required for neuronal differentiation in *X. tropicalis* embryos (Chapter 3) and we have previously shown that neuronal Srcs drive neurite outgrowth in COS7 fibroblasts, and *n1-src* knockdown inhibits neurite outgrowth (Keenan *et al.*, 2017; Lewis *et al.*, 2017). This chapter has shown that knockdown of *n1-src* leads to splicing alterations in *X. tropicalis* embryos, with one of the most common forms being the skipping of exons.

Neural-specific SR-related protein of 100kDa (nsR100; SRRM4) is a neural specific splicing factor required for neuronal differentiation, and its inhibition in N2a cells prevents differentiation and neurite extension (Calarco *et al.*, 2009). Inhibiting nsR100 in N2a cells prevents differentiation and neurite extension. Furthermore, depletion of nsR100 in mouse neuroblastoma cells and tissues results in the skipping of numerous alternatively spliced exons that are normally included in transcripts in the brain (Calarco *et al.*, 2009). Many of the affected genes function in regulating neuronal differentiation. The fact that nsR100 and n1-Src both function in neuronal differentiation, and the downregulated expression of both leads to an increase in exon skipping events suggests that n1-Src

might regulate a specific alternative splicing profile in order to drive neuronal differentiation in *X. tropicalis* embryos.

The other most common form of alternative splicing identified in *n1-src* knockdown embryos is the retention of introns. This observation is of particular interest because a common feature shared across different cancers is abnormal RNA splicing, with these most often being intron retention events (Dvinge and Bradley, 2015). Neuronal srcs have been associated with a positive prognosis in neuroblastoma patients and patients who have higher expression levels have more favourable outcomes (Mellström *et al.*, 1987; Matsunaga *et al.*, 1993). IsoformSwitchAnalyzeR identified genome-wide intron retention events following *n1-src* knockdown and as intron retention events commonly correlate with tumorigenesis, potentially in neuroblastoma patients with a poor prognosis, the absence of *n1-src* expression leads to genome-wide intron retention events.

In this chapter, the approaches taken broadly identified the changes in splicing resulting from *n1-src* knockdown. Analysis of motif enrichment (AME) was then used to identify whether any motifs for RNA binding proteins are enriched around these alternative splicing events. This would hopefully reveal more information about how n1-Src regulates these splicing events. The sequences analysed had 14 different RNA binding protein motifs significantly enriched, with the most abundant motifs being for Serine/arginine-rich splicing factor 1 (Srsf1). Srsf1, or SF2/ASF, is a splicing factor that interacts with the splicing components, U1-snRNP and U2AF, to prevent exon skipping (Kohtz *et al.*, 1994). As previously mentioned, alternative splicing is important in neuronal differentiation, and it also functions in differentiation of neuroblastoma cells, as retinoic acid driven differentiation of neuroblastoma cells leads to the downregulation of Srsf1 levels (Meseguer *et al.*, 2011). As alternative splicing appears to function in neuroblastoma differentiation, and n1-Src has been shown to drive neurite like outgrowths in neuroblastoma cells (Lewis, 2014), it could be that n1-Src drives these neurites by regulating alternative splicing.

Another factor that was identified by AME to have binding motifs enriched in the sequences surrounding the alternative splicing events is RNA-binding motif protein 45 (RBM45). *Rbm45* expression is detected in fetal rat brain neurons and expression gradually reduces through development (Tamada *et al.*, 2002). In *X. tropicalis*, elevated expression levels of *rbm45* is observed during the primary neurogenesis (Owens *et al.*, 2016). Rbm45 motifs are enriched around intron retention events, suggesting that n1-Src regulates the removal of these introns from mature mRNA transcripts through Rbm45

activity. Alongside roles in regulating splicing, Rbm45 has been associated with the neurodegenerative diseases, amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), where it has been found to accumulate in protein aggregations (Collins *et al.*, 2012).

A study in HEK293 cells found there are 132 proteins that specifically interact with Rbm45 (Li *et al.*, 2016). Included amongst these 132 proteins are a variety of other RNA processing and splicing factors, with many members of the hnRNP family interacting, including hnRNPA1. Two other factors found to interact with Rbm45 are Splicing factor proline- and glutamine-rich (SFPQ) and Non-POU domain-containing octamer-binding protein (NONO), which often function together as a heterodimer (Knott, Bond and Fox, 2016). In the Evans lab, a GST-n1-Src SH3 domain pulldown using P1 rat brain lysates identified 33 ligands that bind to the n1-Src SH3 domain (West, 2019). Included in these 33 ligands were SFPQ and NONO. Along with binding to the SH3 domain of n1-Src, SFPQ is also a target for n1-Src phosphorylation (West, 2019). It is possible therefore that these intron retention events occurring following *n1-src* knockdown, which have motifs for Rbm45 in the surrounding sequence, occur due to the lack of interaction and phosphorylation of SFPQ and NONO by n1-Src and this affects their interactions with Rbm45.

Analysis of the transcriptome in *n1-src* knockdown embryos compared to control embryos has revealed that amongst the transcripts alternatively spliced following *n1-src* knockdown are a variety of splicing factors. DEXSeq and IsoformSwitchAnalyzeR were used to examine the alterations in splicing in the data. Whilst they both map the same Illumina short reads, DEXSeq utilised the *X. tropicalis* genome to identify alterations in splicing, whilst IsoformSwitchAnalyzeR used a novel transcriptome generated from Nanopore long read RNA sequencing to align the Illumina reads. This resulted in both methods returning different factors as being alternatively spliced, however, there were 14 genes in common between the two methods, including *tra2a* and *hnrnpa1*.

Off-target mis-splicing has previously been reported as a common side effect of using antisense morpholinos in *X. tropicalis* (Gentsch *et al.*, 2018). The presence of mis-splicing events in the transcriptomic data in this thesis could therefore have been an off-target side effect that came from using the *n1-src* splice blocking morpholinos. One of the recommendations for a control morpholino is a 5 base mismatch morpholino (Eisen and Smith, 2008). Analysis of the sequences around the observed mis-splicing events in my data revealed more than 5 bases mismatched, therefore the morpholino should not

anneal to those sites. Furthermore, *hnrnpa1* was selected as a target to rescue with co-injection of synthetic *n1-src* mRNA which is not recognised by the morpholino. The co-injection of mRNA was able to rescue the mis-splicing events in *hnrnpa1*, showing that the effects of the morpholino are specific (Figure 5.13).

The data presented in this chapter has shown that n1-Src is involved in regulating alternative splicing during *X. tropicalis* neuronal development, and splicing factors such as *hnrnpa1* and *tra2a* are amongst the targets whose splicing is regulated.

5.3.2. hnRNPA1 in the nervous system

Heterogeneous nuclear ribonucleoprotein A1 is an RNA binding protein that has repeatedly been identified throughout this thesis as being regulated by n1-Src. It appears to be both transcriptionally regulated, and also be a splicing factor whose splicing is regulated by n1-Src. hnRNPA1 functions as an inhibitor of splicing by binding to exon splicing silencer (ESS) elements and it is highly expressed in differentiated neurons (Mayeda and Krainer, 1992; ; Kamma, Portman and Dreyfuss, 1995; Del Gatto-Konczak *et al.*, 1999).

The Illumina differential expression data in chapter 4 showed the significant upregulated expression of a *hnrnpa1* transcript following *n1-src* knockdown. However, IsoformSwitchAnalyzeR identified the upregulated *hnrnpa1* isoform retains intron seven, and this transcript is susceptible to non-sense mediated decay due to a premature stop codon at the beginning of the intron. Further examination of the Illumina transcriptomic data identified a *hnrnpa1* transcript that is significantly downregulated following *n1-src* knockdown, and this is also seen in the IsoformSwitchAnalyzeR data. Therefore, it could be that expression of the *hnrnpa1* gene is not affected by *n1-src* knockdown, but the splicing of the pre-mRNA is affected. As the transcript that is upregulated is sensitive to non-sense mediated decay, the overall result of *n1-src* knockdown could be a decrease in the total amount of hnRNPA1 protein.

During *X. laevis* development, *hnrnpa1* expression is enriched in the neural tube, and overexpression of *hnrnpa1* mRNA leads to a variety of embryo phenotypes such as kinking of tail, neural mispatterning and pigmentation defects (Dichmann, Fletcher and Harland, 2008). These are phenotypes shared by *n1-src* knockdown embryos, suggesting both hnRNPA1 and n1-Src function in the same developmental processes.

The importance of hnRNPA1 and alternative splicing and in neuronal development is illustrated by the linking of several neurodegenerative diseases to the dysfunction of hnRNPA1. Neurodegeneration is believed to be one of the primary contributors to the pathogenesis of multiple sclerosis (MS), and it has been observed that patients often develop antibodies to hnRNPA1 (Douglas *et al.*, 2016). Furthermore, injection of such antibodies into MS mice models leads to a worsening of the disease and degeneration of neurons. Meanwhile, studies in patients with the fatal neurodegenerative disease ALS, a disease characterised by a loss of motor neurons, found depleted levels of hnRNPA1 in patients motor neurons (Honda *et al.*, 2015). The progressive loss of motor neurons in ALS is therefore thought to be a result of, at least in part, a disruption in normal mRNA processing. N1-Src could therefore be important in maintaining neuronal health, by regulating the splicing of *hnrnpa1* transcripts, ensuring the translation of a functional protein.

This chapter has shown that *n1-src* knockdown in *X. tropicalis* results in the retention of an intron between exons 7 and 8 of *hnrnpa1*. The corresponding intron in humans contains a cassette exon, exon 7b (Buvoli *et al.*, 1990; Berenstein and Soreq, 2013). The protein generated when alternative splicing includes this cassette exon in humans is hnRNPA1B, which appears in a relatively low abundance but has also been linked to ALS (Deschaies *et al.*, 2018). Cytoplasmic inclusions combined with nuclear depletions of the RNA binding protein, TDP-43, are observed in motor neurons in most ALS cases and there is concomitant marked loss of hnRNPA1 protein (Honda *et al.* 2015). However, it has more recently been shown that TDP-43 can bind to hnRNPA1 to modulate its alternative splicing (Deschaies *et al.*, 2018). The nuclear depletion of TDP-43 results in increased inclusion of exon 7b in mature *hnrnpa1* transcripts and therefore increased levels of the hnRNPA1B protein and reduced hnRNPA1.

hnRNPA1B is more prone to form protein aggregations, and in ALS patients with TDP-43 cytoplasmic inclusions there are pronounced cytoplasmic aggregates (Deschaies *et al.*, 2018). In *X. tropicalis*, the intron between exons 7 and 8 of *hnrnpa1* is retained following *n1-src* knockdown. In humans this intron contains the cassette exon 7b. If n1-Src is involved in inhibiting the inclusion of the intron in *X. tropicalis*, then potentially in humans n1-Src could be involved in regulating the exclusion of the cassette exon contained within the intron, and thereby maintain neuronal health.

Interestingly hnRNPA1 has also been linked to neuroblastomas (Zhang *et al.*, 2016). In this study researchers found that in stage 4 MYCN-amplified neuroblastoma tumours, 6

splicing factors displayed differential expression. High-throughput RNA sequencing found elevated expression levels of *hnrnpa1*, and ChIP-Seq analysis found MYCN can bind directly to the promoter of *hnrnpa1*. There is an inverse correlation between MYCN gene amplification in human neuroblastomas and the expression of *n-srcs* (Matsunaga *et al.*, 1994), meaning that neuroblastoma tumours with high levels of MYCN and *hnrnpa1* expression have low levels of *n1-src* expression. Zhang *et al.* also utilised siRNA to knockdown *hnrnpa1* expression, and this significantly suppressed proliferation rates of MYCN-amplified IMR5 neuroblastoma cells.

The results from Zhang *et al.* suggest that low levels of hnRNPA1 is a positive sign for neuroblastoma patients. Results in this chapter however might suggest the opposite. Expression of neuronal *srcs* is associated with a positive prognosis for neuroblastoma patients (Mellström *et al.*, 1987; Matsunaga *et al.*, 1993), and it appears knockdown of *n1-src* in *X. tropicalis* downregulates hnRNPA1 protein levels. This would suggest that patients with a positive neuroblastoma prognosis would have high levels of hnRNPA1.

The results in this chapter show that n1-Src is involved in regulating the splicing of the splicing factor, *hnrnpa1*. The correct splicing of *hnrnpa1* is important for maintaining neuron health as many neurodegenerative diseases have associated *hnrnpa1* defects. The exact relationship between hnRNPA1 and n1-Src is an area that requires more research, particularly in the context of neuroblastomas.

5.3.3 Tra2a in the nervous system

Transformer-2 protein homolog alpha (Tra2a) is another factor that was repeatedly identified in this chapter as being regulated by n1-Src. Tra2 proteins are SR related proteins that contain Arg/Ser-rich domains, and they function in alternative splicing by binding to exon splicing enhancers (Tacke *et al.*, 1998; Lam *et al.*, 2003). The spatial expression of *tra2a* suggests it has role in nervous system development. In *X. tropicalis* embryos, at tailbud stage 30, *tra2a* expression is detected in the developing brain (Chen *et al.*, 2005), and work in our lab has shown its expression is enriched in the neural plate at stage 14 (Patel, 2019). The overlapping spatial expression patterns of *n1-src* and *tra2a* support the results in this thesis that show n1-Src regulates *tra2a* expression and splicing.

Much like hnRNPA1, Tra2a has been linked with neurodegenerative diseases like Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) and ALS (Markmiller *et al.*, 2018; Gohel *et al.*, 2020). As the results in this chapter show that n1-Src has an important role in regulating the splicing of *tra2a*, it suggests that n1-Src activity is important for maintaining neuronal health.

Tra2a activity has also been heavily linked with cancers, with studies finding that it facilitates the proliferation and migration of various different cancer cells, including triple negative breast cancer cells and glioma cells (Liu *et al.*, 2017; Tan *et al.*, 2018). Glioma is the most common form of malignant cancer in the central nervous system and therefore Tra2a is an important factor in healthy nervous system development.

Studies have also implicated Tra2a in neuroblastomas (Duan *et al.*, 2018). Therapy-resistant relapse is the main cause of mortality in neuroblastoma patients being treated with chemotherapy. In patients who developed resistance to chemotherapy, recurrent new mutations were detected in several genes, including *tra2a*. As *n1-src* knockdown in *X. tropicalis* embryos leads to alterations in *tra2a* splicing, alterations in *tra2a* splicing may also occur in neuroblastoma patients, as those with a poor prognosis have reduced levels of neuronal *src* expression. Alterations in *tra2a* isoforms through mis-splicing may result in increased mortality in patients, in a similar way to the *tra2a* mutations appear to do in chemotherapy patients who go through a therapy resistance relapse.

Along with being associated with neuroblastomas, n1-Src is required for neuronal differentiation, and work in this thesis has suggested it interacts with the Fgf/Erk signalling pathway in this process. Retinoic acid (RA) signalling is also involved in neuronal differentiation, and RA treatment drives the differentiation of neuroblastoma cells (Jones-Villeneuve *et al.*, 1982; Pählman *et al.*, 1984; Lippman, Kessler and Meyskens Jr, 1987), and I have proposed that n1-Src may also interact with this pathway and that is an area for future research. Tra2a has also been implicated in RA induced neural differentiation (Chen *et al.*, 2005). RA treatment of P19 carcinoma cells leads the transient upregulation of *tra2a* expression, and overexpression of *tra2a* is able to increase the amount of RA-induced differentiated P19 cells *in vitro*. The results in this chapter show that n1-Src is involved in regulating *tra2a* splicing and as both n1-Src and Tra2a are involved in neural differentiation it suggests that n1-Src regulates a specific alternative splicing profile in order to drive neuronal differentiation in *X. tropicalis* embryos.

5.3.4. Conclusions

The results in this chapter have shown that knockdown of *n1-src* in *X. tropicalis* leads to the mis-splicing of a variety of genes, including splicing genes. Alternative splicing is crucial to all major steps of nervous system development including neuronal differentiation. N1-Src is also required for the differentiation of primary neurons in *X. tropicalis* and therefore during this process it could be driving a specific alternative splicing profile. This is strengthened by the fact that one of the most prominent factors to be mis-spliced following *n1-src* knockdown is *tra2a*, which has also been shown to function in neural differentiation. However, the exact mechanisms through which n1-Src regulates alternative splicing remains unknown, and how this affects differentiation of neurons, remains unknown.

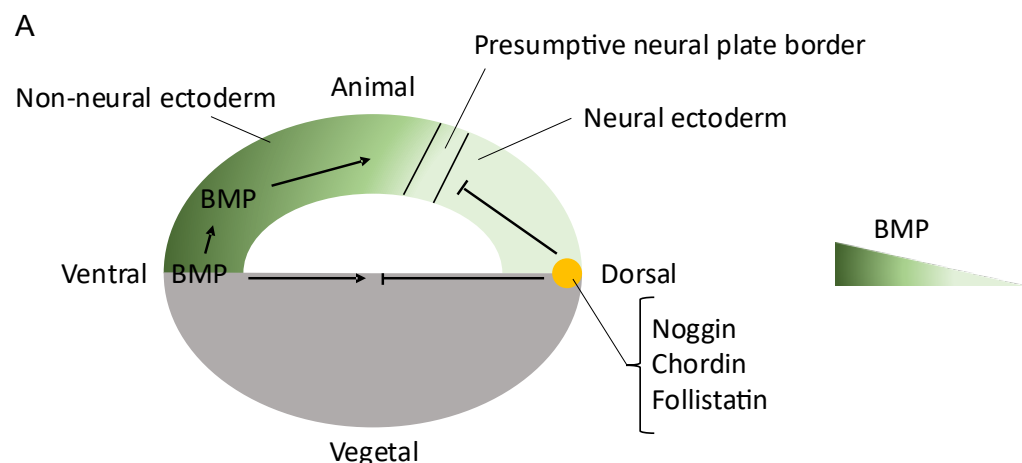
6. The role of n1-Src in neural crest development in *X. tropicalis*

6.1. Introduction

Transcriptomic analysis on *n1-src* knockdown *X. tropicalis* embryos identified processes involved in neural crest development enriched within the downregulated transcript list (Table 4.4). The neural crest is a transient, multipotent population of cells that arises from the neural plate border and after specification, will migrate to various regions of the embryo and ultimately give rise to, amongst other things, much of the peripheral nervous system, craniofacial cartilage and bone, smooth muscle and melanocytes (Mayor and Theveneau, 2013).

6.1.1. Neural plate border specification

The *Xenopus* ectoderm is patterned by a gradient of bone morphogenic proteins (BMP) released from cells of the underlying ventral mesoderm and the ventral ectoderm itself. The ventral non-neural ectoderm is exposed to high activity levels, whilst the dorsal neural ectoderm is protected from BMP activity by inhibitors like noggin, chordin and follistatin released from the dorsal mesoderm (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou & Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999). It is proposed that at the neural plate border, between the neural and non-neural ectoderms, a threshold level of BMP activity is met, which whilst not high enough to induce non-neural ectoderm, is sufficient to induce the neural crest (Wilson *et al.*, 1997; Marchant *et al.*, 1998; Nieto, 2001) (Figure 6.1).



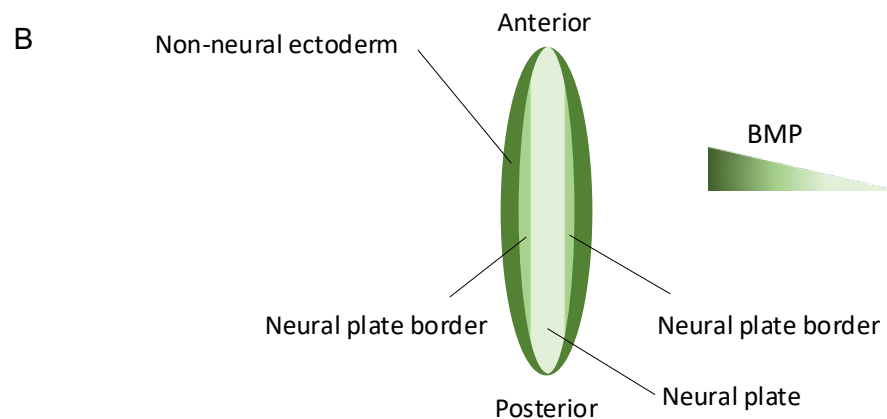


Figure 6.1. Schematic of BMP induction of the neural plate border in *Xenopus* embryos. **A)** Schematic of a blastula *Xenopus* embryo. The endoderm is established in the vegetal hemisphere (grey), the ectoderm in the animal hemisphere (green) and the mesoderm in the interface between the two. A gradient of BMP signalling, illustrated by the green shading, is observed in the ectoderm. BMP signals are produced by the ventral mesoderm and ventral ectoderm. BMP signalling is inhibited in the dorsal ectoderm (light green) by molecules such as chordin released from the Spemann-Mangold Organiser in the dorsal mesoderm (yellow). BMP inhibition in the dorsal ectoderm gives rise to the neural ectoderm. High levels of BMP signalling in the ventral ectoderm (dark green) induces the non-neural ectoderm. The neural plate border is induced at the interface between the two, where a threshold of level of BMP activity is met which whilst not high enough to induce non-neural ectoderm, is sufficient to induce the neural crest. **B)** Dorsal view schematic of an early neurula *Xenopus* embryo showing the tissues formed following exposure to various BMP activity levels during the blastula stage. The neural plate develops from the neural ectoderm that receives minimal BMP signalling (light green). The non-neural ectoderm forms after receiving the highest levels of BMP signalling (dark green), whilst at the interface between the two, the neural plate border develops after receiving medium levels of BMP signalling.

The requirement for BMP antagonists, such as noggin and chordin, in neural plate border induction has been known since the 1990s (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou & Melton, 1997; Weinstein and Hemmati-Brivanlou, 1999), and further BMP antagonists have been identified in recent years. For example, Gremlin 1 is a BMP antagonist required for neural plate border induction, and manipulation of its expression levels is sufficient to expand or shrink the neural plate border domain (Pegge *et al.*, 2020).

However, experiments using the *Xenopus* animal cap assay have shown that whilst the attenuation of BMP signalling alone is able to induce the expression of neural crest

markers, their expression levels fail to reach those seen *in vivo* (LaBonne and Bronner-Fraser, 1998).

A two-step model for neural crest induction was therefore proposed, which involved BMP signalling being initially inhibited to levels that specify the neural plate border, and this region is then competent to respond to further signals that enhance and maintain crest induction (LaBonne and Bronner-Fraser, 1998). Two factors thought to be involved in second step specification of the neural plate border are fibroblast growth factor (Fgf) and Wnt signalling (Figure 6.2)

Recombinant experiments using presumptive paraxial mesoderm and *Xenopus* animal cap explants induces expression of neural crest markers, however this induction is blocked if Fgf signalling is inhibited in animal cap explants (Monsoro-Burq, Fletcher and Harland, 2003). Furthermore, inhibition of Fgf signalling in whole *Xenopus* embryos by overexpressing *dominant-negative Fgf-receptor-1* mRNA inhibits expression of neural crest markers (Mayor, Guerrero and Martinez, 1997).

Induction of neural crest markers is also observed following the conjugation of *Xenopus* animal caps expressing *chordin* with those expressing *wnt8*, and the observed expression levels are much greater than those seen with chordin treatment alone (LaBonne and Bronner-Fraser, 1998). In avians, *wnt6* expression is detected in the ectoderm adjacent to the neural plate and inhibition of wnt signalling perturbs neural crest formation (Garcia-Castro *et al.*, 2002). The need for Wnt signalling was further tested through injection of a *dominant negative wnt1* construct into the embryos adjacent to the neural plate. A marked inhibition of expression of the neural crest marker *snai2*, a zinc finger transcription factor, was seen in the region surrounding the injection site when assayed 12-18 hours later.

Specification of the neural plate border therefore is thought to require Bmp signalling being initially inhibited to levels that specify the neural plate border, followed by Fgf and Wnt signals in the region to enhance crest induction.

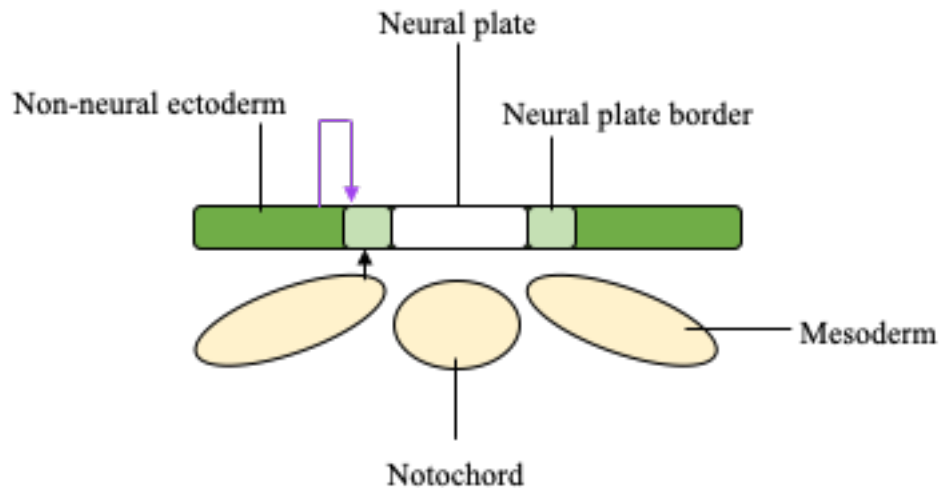


Figure 6.2. Schematic of proposed signals to induce the neural plate border in *Xenopus* embryos. The neural plate border arises at the junction of the neural plate and the non-neural ectoderm. Following Bmp signalling, Fgf and Wnt signals, released from either the non-neural ectoderm (purple arrow) or the underlying paraxial mesoderm (black arrow), function as a second step in neural plate border induction.

The neural plate border expresses a group of genes which define its territory and drive specification of the neural crest through the activation of a complex gene regulatory network (Simões-Costa and Bronner, 2015). Two genes expressed in this region are the paired box transcription factor, *pax3* and the zinc finger transcription factor, *zic1* (Figure 6.3), and the combined activity of both these factors is required for specification of the neural crest (Meulemans and Bronner-Fraser, 2004; Hong and Saint-Jeannet, 2007), with *pax3* enhancers being identified in zebrafish as a direct target of canonical wnt signalling (Plouhinec *et al.*, 2017).

6.1.2. Neural crest specification

Following specification of the neural plate border and the expression of neural plate border specifying genes, a second set of genes is activated, termed the neural crest specifiers, which are specific to the neural plate border and absent from the neural plate and non-neural ectoderm (Pla and Monsoro-Burq, 2018). Examples of neural crest specifier genes include the basic helix-loop-helix transcription factor *twist1*, the zinc finger transcription factors *snai1* and *snai2*, and the SRY-related HMG-box transcription factors, *sox9* and *sox10* (Simões-Costa and Bronner, 2015) (Figure 6.3). Some of these genes, including *twist1* and *snai2* are directly activated by *pax3* and *zic1* (Plouhinec *et al.*, 2014).

Neural crest specifier genes regulate each other's expression, within the neural plate border *Snai1* and *2* are some of the earliest activated neural crest specifiers and are able to induce the expression of other crest markers like *twist1* (LaBonne and Bronner-Fraser, 2000; Aybar, Nieto and Mayor, 2003). *Snai2*, despite being directly activated by Pax3, is also activated by another crest marker, Sox10 and it was observed that antisense morpholino-mediated inhibition of *sox10* blocks *snai2* expression in a dose dependent manner (Honoré, Aybar and Mayor, 2003). *Sox10* expression in the neural crest however appears to be dependent on expression of *snai2* as injection of *dominant negative snai2* mRNA results in a severe loss of *sox10* expression (Aoki *et al.*, 2003). Meanwhile, *sox9* expression is essential for Wnt mediated crest induction and its expression is regulated by *snai2*, with reduced expression seen in embryos injected with *dominant negative snai2* mRNA (Lee *et al.*, 2004). Interestingly, transient knockdown of *sox9* using antisense morpholinos prevents *snai2* expression in a dose dependent manner, indicating *sox9* expression regulates expression of *snai2* (Spokony *et al.*, 2002).

The specification of the neural crest at the neural plate border is therefore a complicated event, with neural plate border specifiers like Pax3 and Zic1 inducing expression of neural crest specifying genes, which appear to then regulate each other's expression via feedback loops (Figure 6.3). The full, direct hierarchy of gene expression here is still unknown.

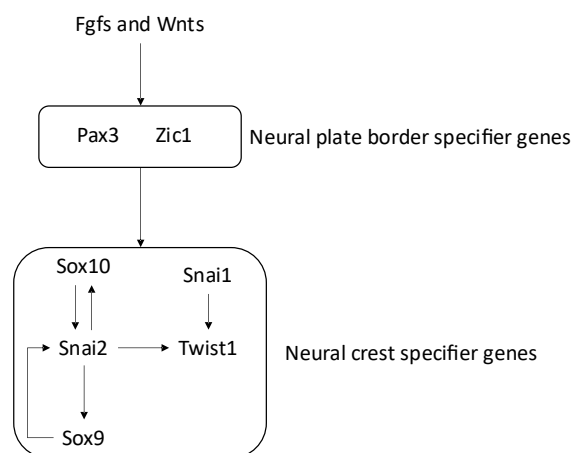


Figure 6.3. Schematic showing the complexity of gene expression involved in specification of the neural crest. Fgf and Wnt signalling leads to the expression of the neural plate border specifier genes, *pax3* and *zic1*. These then induce the expression of the neural crest specifier genes, including *snai1*, *snai2*, *sox9*, *sox10* and *twist1*. Various feedback loops exist amongst these neural crest specifier genes to maintain their expression and drive crest formation, for example Sox9 regulates *snai2* expression, which in turn regulates *sox9* expression, and *sox10* and *snai2* have been identified to drive each other's expression.

6.1.3. Neural crest migration

Following the specification of the neural crest cells, the cells delaminate from the neural tube and begin to migrate to diverse locations throughout the embryo (Bronner, 2012) (Figure 6.4). In order to migrate, the neural crest cells go through an epithelial to mesenchymal transition (EMT).

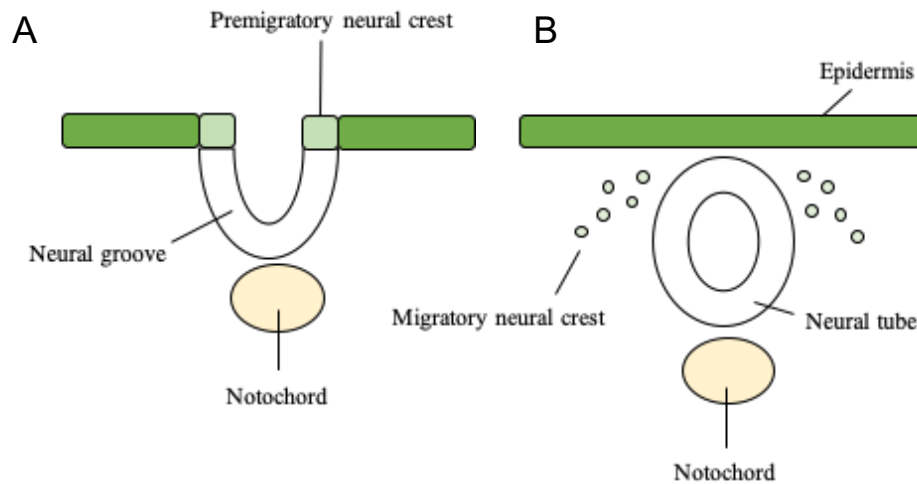


Figure 6.4. Schematic showing migration of the neural crest cells in *Xenopus*.

A) After induction of the neural crest at the neural plate border and differentiation of the primary neurons in the neural plate, the plate begins to infold. **B)** After closure of the neural groove into the neural tube, the neural crest cells undergo an epithelial to mesenchymal transition and migrate away from the neural tube. The environment in which they come to reside in determines their final differentiation.

One critical change in gene expression during the EMT is the switch from cells expressing *N-cadherin* to *E-cadherin* (Kalluri and Weinberg, 2009). EMT is triggered by a combination of signals from the extracellular matrix as well as secreted Tgf- β and Fgf ligands (Sauka-Spengler and Bronner-Fraser, 2008). Many of the neural crest specifier genes are required for the EMT and migration of neural crest cells. For example, *Snai2* has been shown to directly represses N-cadherins in the EMT observed in chicken embryos (Taneyhill, Coles and Bronner-Fraser, 2007; Ferronha *et al.*, 2013). Expression of *sox9* and *sox10* is also maintained within the migrating neural crest cells, and loss of *sox9* expression results in the apoptosis of neural crest cells undergoing EMT (Cheung *et al.*, 2005).

Following delamination, the migrating neural crest cells receive a variety of cues to help direct them to their final destination. The cells interact with the surrounding tissues as

they migrate, with ephrins and semaphorins signalling to prevent cells from migrating off course (Szabo and Mayor, 2018). Crest migration is also controlled by chemotaxis, with various molecules attracting and repelling the migrating cells, including vascular endothelial growth factors (Vegfs) and Fgfs (McLennan *et al.*, 2010; Sato *et al.*, 2011). The final differentiation of neural crest cells is largely determined by the environment they finally reside in.

6.1.4. Neural crest and neuroblastoma

The failure of precursor cells in the sympathetic nervous system – itself derived from the neural crest – to differentiate, leads to the development of neuroblastoma, a developmental cancer (Ciccarone *et al.*, 1989; Brodeur, 2003). Src kinase activity has been associated with neuroblastoma malignancy, with less malignant and more differentiated cell lines exhibiting a 30-fold higher level compared to less differentiated lines (Mellström *et al.*, 1987). Furthermore, *n1-src* expression was only detected in neuroblastoma cell lines with the ability to differentiate, and in neuroblastoma cell lines that were estimated to have a good prognosis, n1-Src was identified as the dominant form of src (Bjelfman *et al.*, 1990; Matsunaga *et al.*, 1993).

With neuroblastoma tumours arising from cells in the neural crest derived sympathetic nervous system, and a link between n1-src and neuroblastoma differentiation, it was hypothesised that n1-src could potentially be involved in neural crest development. Transcriptomic analysis on *n1-src* knockdown embryos revealed significant downregulation of transcripts involved in neural crest development, however these data requires validation.

6.1.5. Aims

The aims of this chapter are:

1. Characterise the effect that *n1-src* knockdown has on the expression of neural crest markers by semi-quantitative PCR and *in situ* hybridisation analysis.
2. Characterise the effects of *n1-src* overexpression on neural crest markers expression.
3. Identify whether *n1-src* is expressed in neural crest tissue.

6.2. Results

6.2.1. Knockdown of *n1-src* downregulates the expression of neural plate border specifiers

To investigate whether *n1-src* functions in development of the neural crest, the *n1-src* splice blocking antisense morpholinos were used to knockdown *n1-src* expression.

X. tropicalis embryos were bilaterally injected at the one- to two-cell stages with 10ng AMO A+D (20ng total) or 20ng CMO. RT-PCRs were carried out on RNA extracted from these embryos at early neurula stage 14, and band intensities were quantified using ImageJ, to analyse the expression of the neural plate border specifier genes, *pax3* and *zic1*. Gel pictures of a representative experiment are shown (Figure 6.5). Knockdown of *n1-src* expression leads to the significant downregulation of both of the markers.

Expression of *pax3* was further analysed by *in situ* hybridisation analysis. *X. tropicalis* embryos were unilaterally injected in one-cell at the two-cell stage with 5ng AMO A+D (10ng total). Embryos were fixed at stage 14 and *in situ* hybridisations were carried out to analyse *pax3* expression (Figure 6.5). Knockdown of *n1-src* results in a reduction or absence of expression of *pax3* 7/9 samples (n=9). *N1-src* expression is therefore required for the expression of neural plate border specifier genes.

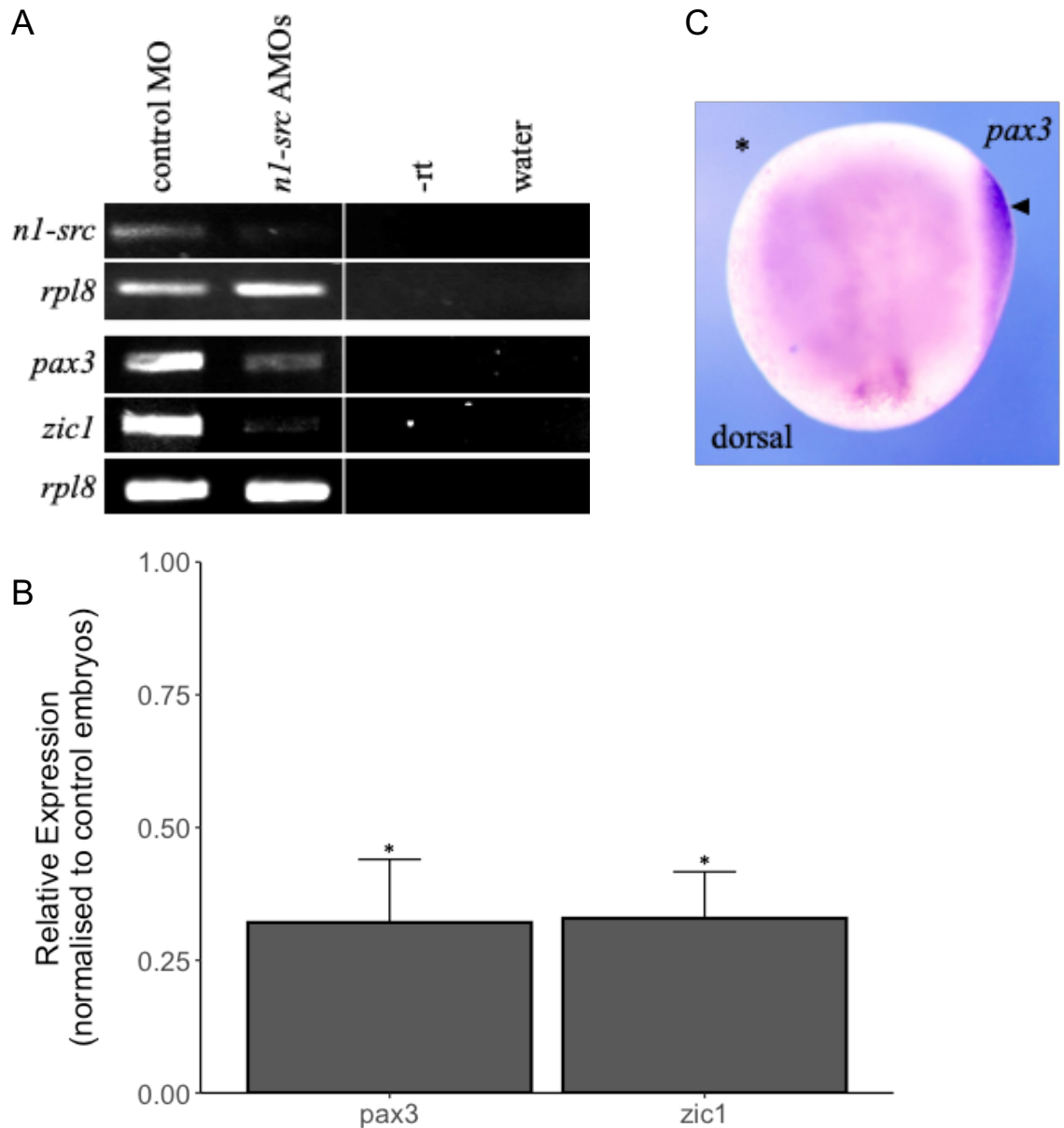


Figure 6.5. *n1-src* knockdown downregulates expression of neural plate border specifier genes. A) RT-PCR analysis of *n1-src*, *pax3* and *zic1* mRNA expression in neurula stage 14 embryos injected with 20ng CMO or 10ng AMO A+D. Knockdown of *n1-src* leads to a reduced expression of both genes. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. **B)** Quantification of *pax3* and *zic1* RT-PCR expression levels in *n1-src* knockdown embryos, normalised to control embryos (Control = 1.0). Data is plotted as mean $\pm$ SEM (n = 3 independent experiments, analysed by t-test, *p < 0.05). **C)** *in situ* hybridisation for *pax3* expression. Dorsal view of stage 14 *X. tropicalis* embryo, anterior to the top. Embryos were unilaterally injected at the two-cell stage with 5ng AMO A+D. Asterisk indicates the injected side. Arrowhead labels expression in neural plate border. Knockdown of *n1-src* leads to a loss or reduction of *pax3* expression compared to the uninjected control hemisphere.

6.2.2. Knockdown of *n1-src* downregulates the expression of neural crest specifier genes

RT-PCR analysis for neural crest specifier genes was carried out on stage 14 *X. tropicalis* embryos that had been injected at the one- to two-cell stage with 10ng AMO A+D or 20ng CMO. Band intensities were quantified using ImageJ and gel pictures of representative experiments are shown (Figure 6.6). The neural crest specifier genes, *snai2*, *twist1* and *sox10* are significantly downregulated in response to *n1-src* knockdown. The downregulation of *twist1* expression supports the transcriptomic data in Chapter 4 of this thesis, which also identified *twist1* as being significantly downregulated in response to *n1-src* knockdown. The other analysed genes – *sox9* and *phox2a* – are not significantly downregulated when assayed by RT-PCR analysis, however, there is a downward trend in their expression levels.

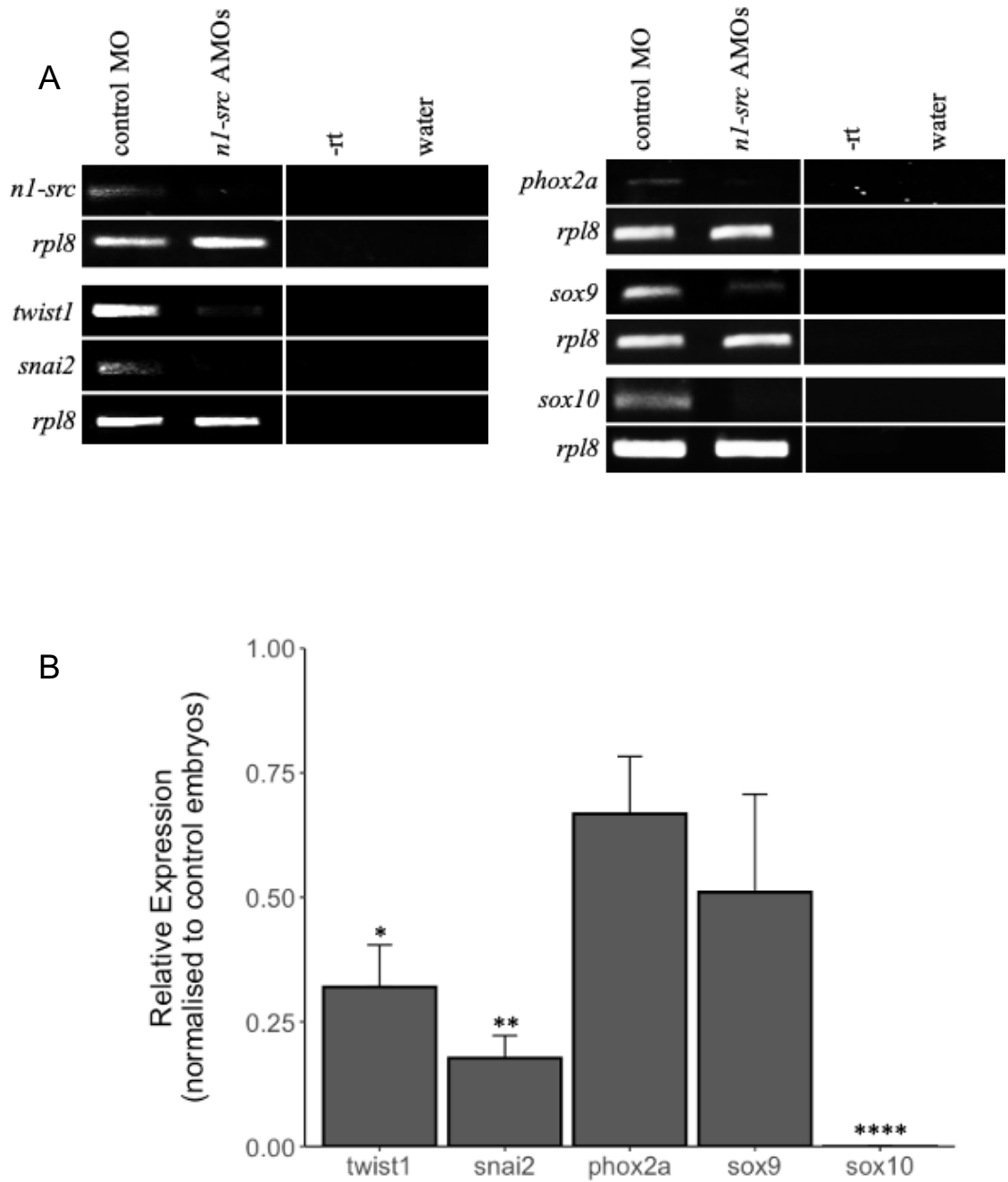


Figure 6.6. *n1-src* knockdown downregulates expression of neural crest specifier genes. RT-PCR analysis of **A)** *n1-src*, *twist1*, *snai2*, *phox2a*, *sox9* and *sox10* mRNA expression in neurula stage 14 embryos injected with 20ng CMO or 10ng AMO A+D. *rpl8* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. **B)** Quantification of RT-PCR expression levels in *n1-src* knockdown embryos, normalised to control embryos (Control = 1.0). Data is plotted as mean $\pm$ SEM (n = 3 independent experiments, analysed by t-test, *p < 0.05, **p<0.01, ****p<0.0001).

Expression of *snai2*, *twist1* and *phox2a* was further analysed by *in situ* hybridisation analysis. *X. tropicalis* embryos were unilaterally injected in one-cell at the two-cell stage with 5ng AMO A+D and fixed at stage 14. Knockdown of *n1-src* results in a reduction or absence of expression of: *twist1* in 79% of samples (n=14), *snai2* in 75% of samples (n=8) and *phox2a* in 80% of samples (n=5) (Figure 6.7). *N1-src* expression is therefore required for the expression of a variety of neural crest genes, and the correct development of the neural crest.



Figure 6.7. *n1-src* knockdown inhibits neural crest specifier gene expression. *In situ* hybridisations for *twist1*, *snai2* and *phox2a* expression. Dorsal view of stage 14 *X. tropicalis* embryos, anterior to the top. Embryos were unilaterally injected at the two-cell stage with 5ng AMO A+D. Asterisk indicates the injected side. Arrowhead labels expression in the neural plate border. Knockdown of *n1-src* leads to a loss or reduction of expression of all three genes compared to the uninjected control hemisphere.

6.2.3. *N1-src* overexpression results in an upwards trend in *phox2a* expression

I next investigated the effect that overexpressing synthetic FLAG-tagged *Xenopus n1-src* mRNA would have on development of the neural crest in *X. tropicalis* embryos. Embryos were injected at the one- to two-cell stage with 250pg of *n1-src* mRNA or the equivalent volume of water. To confirm translation of the FLAG-tagged mRNA, stage 11 embryos were analysed by western blotting, with no FLAG signal observed in the water injected samples, but a band is present in the *n1-src* overexpressing embryos (Figure 6.8).

RT-PCRs were carried out on RNA extracted from embryos at stage 14 to examine the relative expression levels of a variety of neural crest markers. There is no significant change in expression of any of the crest markers when assayed by this method (Figure 6.7). This experiment was repeated four times and there was much variation in the data, with markers which appeared to be upregulated in one experiment being downregulated in the next. The only consistent marker was *phox2a* which always showed an upwards trend in expression, however, it was still not significant.

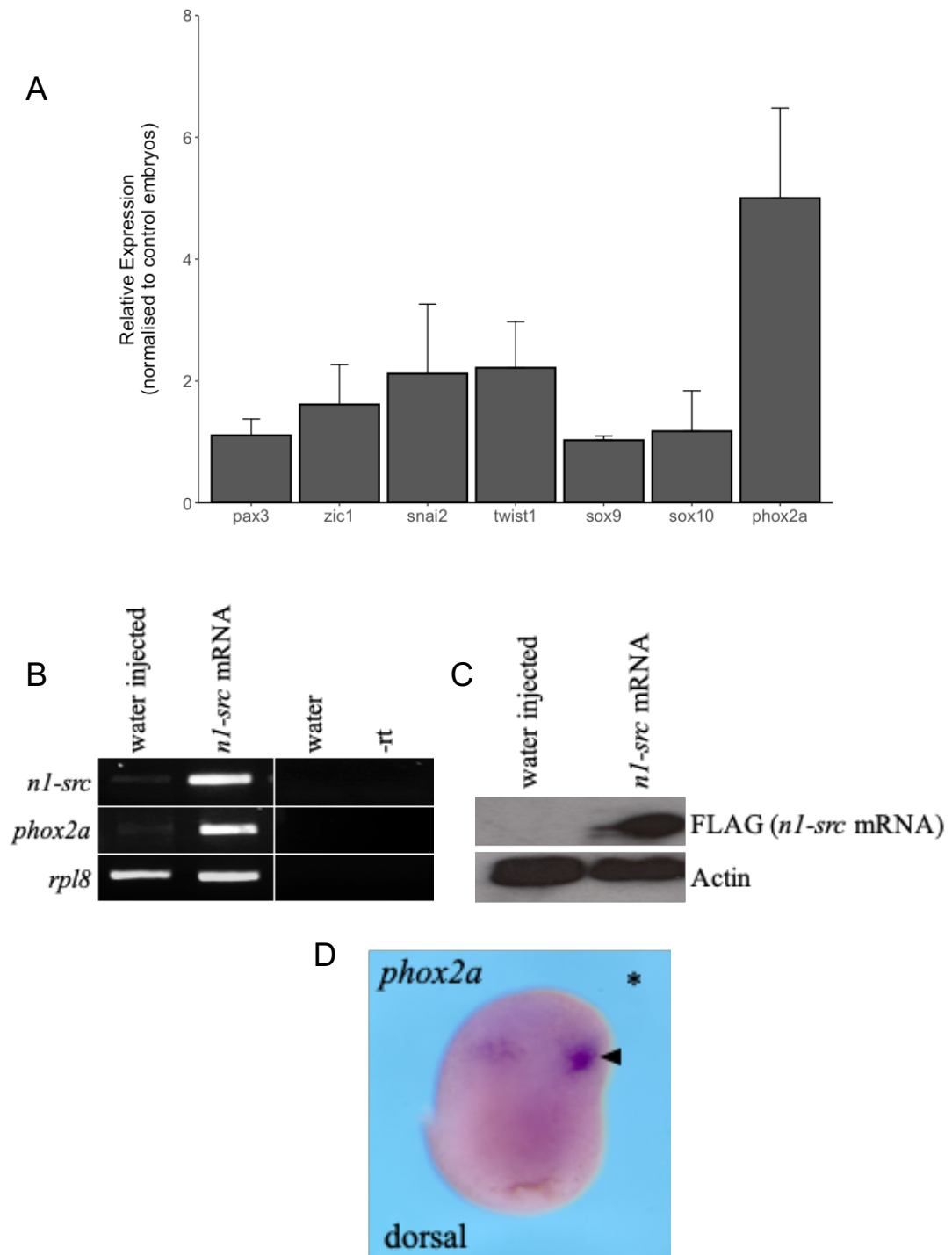


Figure 6.8. Effects of *n1-src* overexpression on neural crest genes **A)** Quantification of RT-PCR expression levels at stage 16, in *X. tropicalis* embryos injected with 250pg *n1-src* mRNA, normalised to control embryos (Control = 1.0). Data is plotted as mean $\pm$ SEM ($n = 4$ independent experiments, analysed by t-test) **B)** RT-PCR analysis for *n1-src* and *phox2a* mRNA expression in neurula stage 16 embryos injected with 250pg *n1-src* mRNA or the equivalent volume of water. *Rpl8* is used as a loading control. -rt, No reverse transcriptase control; water, no template control. **C)** Western blot probing for FLAG epitope in stage 11 *X. tropicalis* embryos injected with 250pg FLAG-tagged *n1-src* mRNA or the equivalent volume of water. **D)** *in situ* hybridisation for *phox2a* expression. Dorsal view of stage 14 *X. tropicalis* embryo, anterior to the top. Embryos were unilaterally injected at the two-cell stage with pg *n1-src* mRNA. Asterisk indicates the injected side. Arrowhead labels expanded expression in neural plate border.

Further analysis of *phox2a* expression was carried out by *in situ* hybridisations. Embryos were injected in one-cell at the two-cell stage with 125pg of FLAG-tagged *n1-src* mRNA and embryos were collected at around stage 14 for *in situ* hybridisation analysis. Increased *phox2a* expression was observed in 66% of injected embryos (n=9) (Figure 6.8).

Whilst the data presented here does not provide any clear and significant results, it does point towards the overexpression of *n1-src* in the developing *X. tropicalis* embryo upregulating *phox2a* expression. qPCR analysis, along with further *in situ* hybridisations, is required to investigate this further.

6.2.4. *N1-src* mRNA overexpression rescues *phox2a* expression in *n1-src* knockdown embryos

Knockdown of *n1-src* expression in *X. tropicalis* leads to the downregulated expression of neural plate border and neural crest genes. I have attempted the rescue of one of these markers, *phox2a*, to confirm that this downregulated expression is a specific result of *n1-src* knockdown. This marker was chosen as it was the only gene assayed that showed a consistent trend in response to *n1-src* overexpression.

Embryos were bilaterally injected at the one-to two-cell stage with the control morpholino, *n1-src* splice blocking morpholinos, *n1-src* mRNA or the *n1-src* morpholinos and mRNA in combination. Embryos were collected at stage 14 for RT-PCR and western blot analysis.

Four different concentration combinations of morpholino and mRNA were tested, and here I show RT-PCRs and western blot analysis carried out on embryos injected with 4ng of control MO, 2ng *n1-src* AMOs, 2ng *n1-src* mRNA or 2ng *n1-src* AMOs A+D + 2ng mRNA (Figure 6.9). When tested with these concentrations, overexpression of *n1-src* mRNA rescues the downregulated expression of *phox2a* caused by knockdown of *n1-src*. The other three combinations of morpholino and mRNA also all rescued *phox2a* expression. Therefore, downregulation of *phox2a* seen in *n1-src* knockdown embryos is confirmed as a specific response to *n1-src* knockdown.

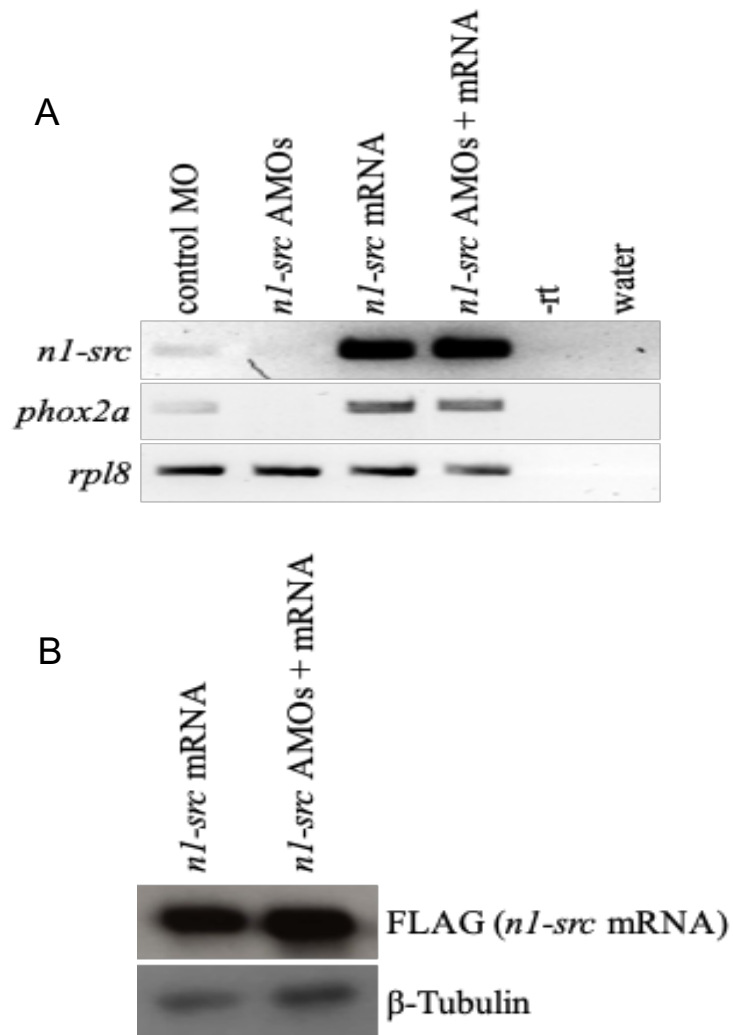


Figure 6.9. Rescue of *phox2a* expression in *n1-src* knockdown embryos A) RT-PCR analysis for *n1-src* and *phox2a* mRNA expression in neurula stage 14 *X. tropicalis* embryos injected with 4ng control morpholino, 2ng *n1-src* AMOs, 2ng *n1-src* mRNA or 2ng *n1-src* AMOs + 2ng *n1-src* mRNA. *Rpl8* is used as a loading control. Overexpression of *n1-src* mRNA is able to rescue the downregulation of *phox2a* expression following *n1-src* knockdown. -rt, No reverse transcriptase control; water, no template control. **B)** Western blot probing for the FLAG epitope in stage 14 *X. tropicalis* embryos. Beta-tubulin is used as a loading control.

6.2.5. *N1-src* is expressed in neural crest induced animal caps

Pax3 and *zic1*, each fused in frame to the human glucocorticoid receptor (GR) ligand binding domain, can be used to induce neural crest gene expression in animal cap explants (Hong and Saint-Jeannet, 2007). Synthetic mRNAs transcribed from these constructs are injected together into the animal pole of embryos, and animal cap explants are then cultured in normal amphibian medium (NAM) containing 10 μ M dexamethasone, leading to the strong induction of neural crest genes, including *snai2* and *twist1*.

Animal caps explants were dissected from *X. laevis* embryos that were either untreated, injected with 50pg of *noggin* mRNA or injected with 250pg of both *pax3GR* and *zic1GR* mRNA. Explants from *noggin* injected embryos were cultured to neurula stage 17 in 1/2X NAM, whilst explants from embryos injected with *pax3GR* and *zic1GR* mRNA were cultured to stage 17 in 1/2X NAM supplemented with 10 μ M dexamethasone.

RT-PCR analysis on RNA from these samples shows expression of the neural crest genes *twist1* and *snai2* in the *pax3GR/zic1GR* treated animal caps (Figure 6.10). Expression of *n1-src* is also detected in these neural crest induced samples. This preliminary result, from a single experiment, suggests that in addition to neural plate, *n1-src* is expressed in the *Xenopus* neural plate border which gives rise to the neural crest.

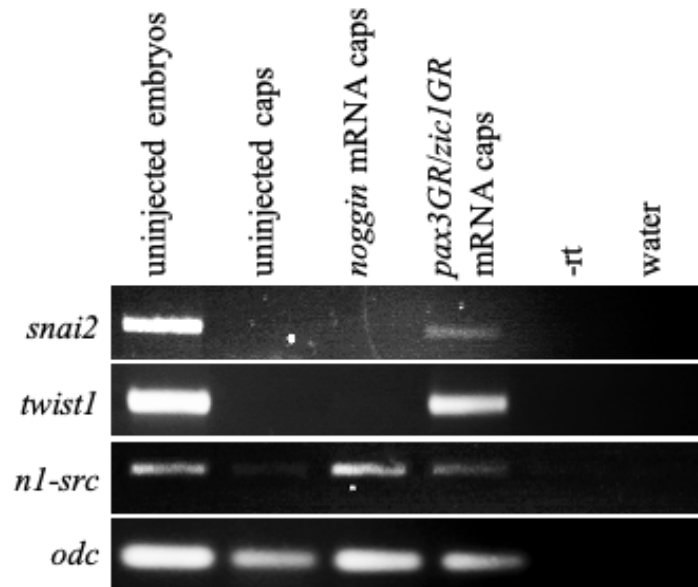


Figure 6.10. RT-PCR analysis showing *n1-src* expression in neural crest induced animal cap explants. RT-PCR analysis of *n1-src*, *twist1* and *snai2* mRNA expression at stage 16 in control uninjected embryos and animal caps, and animal caps explants from embryos injected with either 50pg *noggin* mRNA or 250pg *pax3GR* and *zic1GR* mRNA. Caps were then cultured to stage 17 in NAM/2 (supplemented with 10μM dexamethasone for *pax3GR/Zic1GR* caps). Injection of *noggin* mRNA induces neural tissue in animal caps, as shown by the expression of *n1-src*. Injection of *pax3GR/zic1GR* and culturing in dexamethasone induces the expression of the neural crest markers *snai2* and *twist1*. *N1-src* expression is also detected in these crest induced animal cap explants. *Odc* is used as a ubiquitously expressed loading control. -rt, No reverse transcriptase control; water, no template control. n=1.

6.3. Discussion

Transcriptomic analysis in this thesis revealed that knockdown of *n1-src* in *X. tropicalis* embryos resulted in the downregulated expression of transcripts involved in development of the neural crest (Figure 4.7, Tables 4.5, 4.6, 4.7, 4.8,). A potential role for n1-Src in regulating neural crest development is an interesting hypothesis due to the relationship between n1-src and the crest derived cancer, neuroblastoma. Neuroblastoma tumours arise from sympathetic neuron progenitors, which are derived from the neural crest (Ciccarone *et al.*, 1989; Brodeur, 2003;), and *n1-src* has been linked with neuroblastoma patient prognosis, with higher expression levels of *n1-src* relative to *c-src* expression being associated with a positive prognosis (Bjelfman *et al.* 1990). Experiments by Phil Lewis in the Evans lab showed that neuronal srcs are able to induce the growth of neurite-like processes in a neuroblastoma cell line, suggesting a potential role for n-Srcs in neuroblastoma differentiation (Lewis, 2014). Work in this chapter therefore examines whether *n1-src* expression is required for correct development of the neural crest.

6.3.1. *N1-src* knockdown downregulates the expression of neural plate border specifier genes and neural crest specifiers

Expressed in the neural plate border are the neural plate border specifier genes which drive specification of the neural crest. Two of these genes are *pax3* and *zic1*, and the combined activity of both these factors is required for specification of the neural crest (Garnett, Square and Medeiros, 2012). Antisense morpholino mediated knockdown of *n1-src* expression leads to a significant downregulation in the expression of *pax3* and *zic1* in *X. tropicalis* embryos, assayed by RT-PCRs and *in situ* hybridisations (Figure 6.5). N1-Src activity therefore functions in specification of the neural plate border.

Knockdown of *n1-src* expression also leads to a significant down regulation of a variety of neural crest specifier genes including *snai2*, *twist1* and *sox10* (Figure 6.6). Two other markers analysed – *phox2a* and *sox9* – show a downwards trend in expression but are not significant. The analysis of *phox2a* expression by *in situ* hybridisation shows reduced expression following *n1-src* knockdown compared to controls. More quantitative qPCR analysis of these markers is required to analyse their expression. As the neural crest specifiers function downstream of the neural plate border specifiers, downregulation of their expression was anticipated considering the knockdown of *n1-src* downregulated both *pax3* and *zic1* expression.

Multiple signalling pathways function to induce the neural plate border and in turn, the neural crest, including Bmp, Wnt and Fgf signalling (LaBonne and Bronner-Fraser 1998). The requirement for Fgf signalling in this process, combined with results in this thesis revealing the ability of n1-Src to activate the Fgf/Erk pathway (Chapter 3) and its potential role in regulating neural plate border specification leads to the hypothesis that n1-Src is involved in inducing the neural plate border through regulating Fgf signalling.

However, the exact manner in which Fgf signalling functions in plate border development is not fully known.

The underlying mesoderm is involved in development of the neural plate border and it has been proposed as the site of Fgf signalling. Recombination assays involving *Xenopus* paraxial mesoderm and ectodermal animal cap explants revealed that the mesoderm is able to induce expression of a variety of neural crest markers (Monsoro-Burq, Fletcher and Harland, 2003). This induction can be blocked by inhibiting Fgf signalling and *fgf8* is expressed in the paraxial mesoderm. Fgf8 in the underlying mesoderm is proposed to function indirectly in the induction of the neural plate border, by activating Wnt8, which then induces neural crest in the overlying ectoderm (Hong, Park and Saint-Jeannet, 2008). This is supported by *wnt8* expression being able to restore neural crest progenitors in Fgf8-deficient embryos, but Fgf8 is unable to rescue crest formation in Wnt8-deficient embryos. However, other work suggests that Fgf8 and Wnt8 act in parallel at the neural plate border, as expression of a neural plate border specifier, *msx1*, can be induced by Fgf8 when Wnt signalling is inhibited (Monsoro-Burq, Wang and Harland, 2005).

Earlier experiments using *Xenopus* in the 1990s implicated the neural plate as the site of Fgf/Erk signalling in neural plate border induction. Grafting experiments showed that the interaction between the neural plate and the non-neural ectoderm could induce neural crest marker expression (Mancilla and Mayor, 1996). Further work found that this induction could be repressed by inhibiting Fgf signalling in the neural plate but not in the epidermis, suggesting that Fgf signals from the epidermis into the neural plate are required for neural plate border specification (Mayor, Guerrero and Martinez, 1997). Fgfs are expressed in both the presumptive ectoderm and presumptive neural plate during early *Xenopus* development (Friesel and Sharron, 1992; Tannahill *et al.*, 1992; Song and Slack, 1996). Similar observations have been seen following grafting experiments using chick and quail embryos, with the juxtaposition of neural plate and non-neural ectoderm inducing expression of neural crest markers (Selleck and Bronner-Fraser, 1995).

Further experiments using the chick model found that the targeted blocking of Fgf receptors or targeted inhibition of Erk phosphorylation at the neural plate border, inhibits the expression of neural plate border genes like *snai2* supporting a direct role for Fgf signalling in plate border induction (Stuhlmiller and García-Castro, 2012). A direct role for Fgf signalling in neural plate border specification is also proposed in zebrafish, with both *pax3* and *zic1* having enhancers that are responsive to Fgf signalling (Garnett, Square and Medeiros, 2012).

Induction of the neural plate border at the lateral edges of the neural plate therefore requires Fgf signals from the underlying mesoderm, the non-neural ectoderm or both. If Fgf signals are released from surrounding tissues to the lateral neural plate, as suggested by the experiments carried out by Mayor *et al.* (1997) and Monsoro-Burq *et al.* (2003, 2005), and n1-Src is capable of interacting with and regulating the Fgf/Erk pathway as data in this thesis shows, then one hypothesis is that n1-Src's regulation of the Fgf/Erk pathway is required for plate border induction.

6.3.2. *N1-src* overexpression effects on the expression of neural crest genes

The overexpression of FLAG-tagged *n1-src* mRNA did not result in significant upregulation of any of the neural plate border or neural crest specifier genes when assayed via RT-PCRs (Figure 6.8). There was a noticeable, but not-significant upregulation in *phox2a* expression. This observation led to the examination of *phox2a* expression via *in situ* hybridisation analysis, and overexpression of 125pg of mRNA resulted in an expansion of the domain of *phox2a* expression (Figure 6.8). Further *in situ* hybridisations, and q-PCR analysis on *n1-src* overexpressing embryos is needed to investigate *phox2a* expression more intensively, perhaps with a higher concentration of mRNA overexpressed.

The upregulation of *phox2a* expression is a noteworthy observation, and requires further analysis due to its potential links with neuroblastomas. Firstly, *phox2a* expression is detected in anteroventral noradrenergic (AVNA) cells, which are derived from the neural crest and share many characteristics with neuroblastoma cells (Wylie *et al.*, 2015). AVNA cells have been proposed as a developmental model in which to study the differentiation of neuroblastomas presumptive cell of origin. Secondly, *phox2a* expression has been linked with neuroblastoma differentiation. RA treatment drives the differentiation of neuroblastoma cell lines and is used as a treatment for neuroblastoma patients, and the RA driven differentiation of SK-N-BE(2)C neuroblastoma cells leads to the upregulation

of *phox2a* expression (Sidell, 1982; Reynolds *et al.*, 2003; Maris *et al.*, 2007; di Lascio *et al.*, 2016) Manipulation of *n1-src* expression in *Xenopus* embryos leads to alterations in *phox2a* expression and overexpression of *n1-src* in neuroblastoma cells drives differentiation of neurite like outgrowths (Lewis, 2014). Retinoic acid signalling also functions in the differentiation of primary *Xenopus* neurons, and alongside Fgf and Wnt signalling in induction of the *Xenopus* neural crest (Blumberg *et al.*, 1997; Villanueva *et al.*, 2002). Results from this thesis and Lewis *et al.* (2017) show that n1-Src functions in both of these processes. These observations suggest a potential interaction between n1-Src and retinoic acid regulated pathways.

6.3.3. N1-src expression in the neural crest

Expression of *n1-src* is required for induction of the neural plate border and the neural crest (Figures 6.5, 6.6 and 6.7). RA signalling also functions in the induction of the neural crest (Villanueva *et al.*, 2002) and results so far lead to the hypothesis that there may be interactions between n1-Src and members of pathways regulated by RA. As RA signalling can induce differentiation of neuroblastoma tumours, that arise from tissues derived from the neural crest, could n1-Src expressed and active in the later stages of neural crest development and differentiation alongside its expression in the developing nervous system?

Locked nucleic acid *in situ* hybridisations for *n1-src* expression in *X. tropicalis* show a broad expression domain throughout the neural plate (Lewis *et al.*, 2017). Closer examination suggests that *n1-src* expression might not be confined to the neural plate but could also be expressed in the plate border. To investigate this *pax3* and *zic1* constructs were used in the induction of neural crest in the *X. laevis* animal cap assay (Hong and Saint-Jeannet, 2007).

Injection of mRNA encoding for glucocorticoid receptor linked *pax3* and *zic1*, followed by culturing of caps in dexamethasone, induced the expression of the neural crest specifier genes, *snai2* and *twist1*. Interestingly, *n1-src* expression was also detected in these caps. This experiment was only carried out once and therefore needs repeating but preliminary results suggest that *n1-src* expression occurs in the neural plate border in response to the Pax3 and Zic1 activity. Analysis of a previously published RNA-Seq data set has supported the results from this assay. Anterior and posterior neural plate and neural border regions were dissected from *X. laevis* embryos at early neurula stage 14, along with dissections of non-neural ectoderm (Plouhinec *et al.*, 2017). RNA extracted from

these dissections was then sequenced on an Illumina HiSeq 2000. The Evans lab used HISAT2 to align the published quality checked reads to the *X. laevis* genome v9.2 (<https://www.ncbi.nlm.nih.gov/genome/?term=Xenopus+laevis>) and then visualised the alignments in IGV. Reads were observed aligning to the *n1-src* microexon in the neural plate and neural plate border samples, but not in the non-neural ectoderm samples (Figure 6.11). These observations together indicate *n1-src* is expressed in the neural plate border, however the animal cap experiment needs repeating and further experiments are also required to analyse migratory and differentiated neural crest cells to determine if *n1-src* is expressed in them.

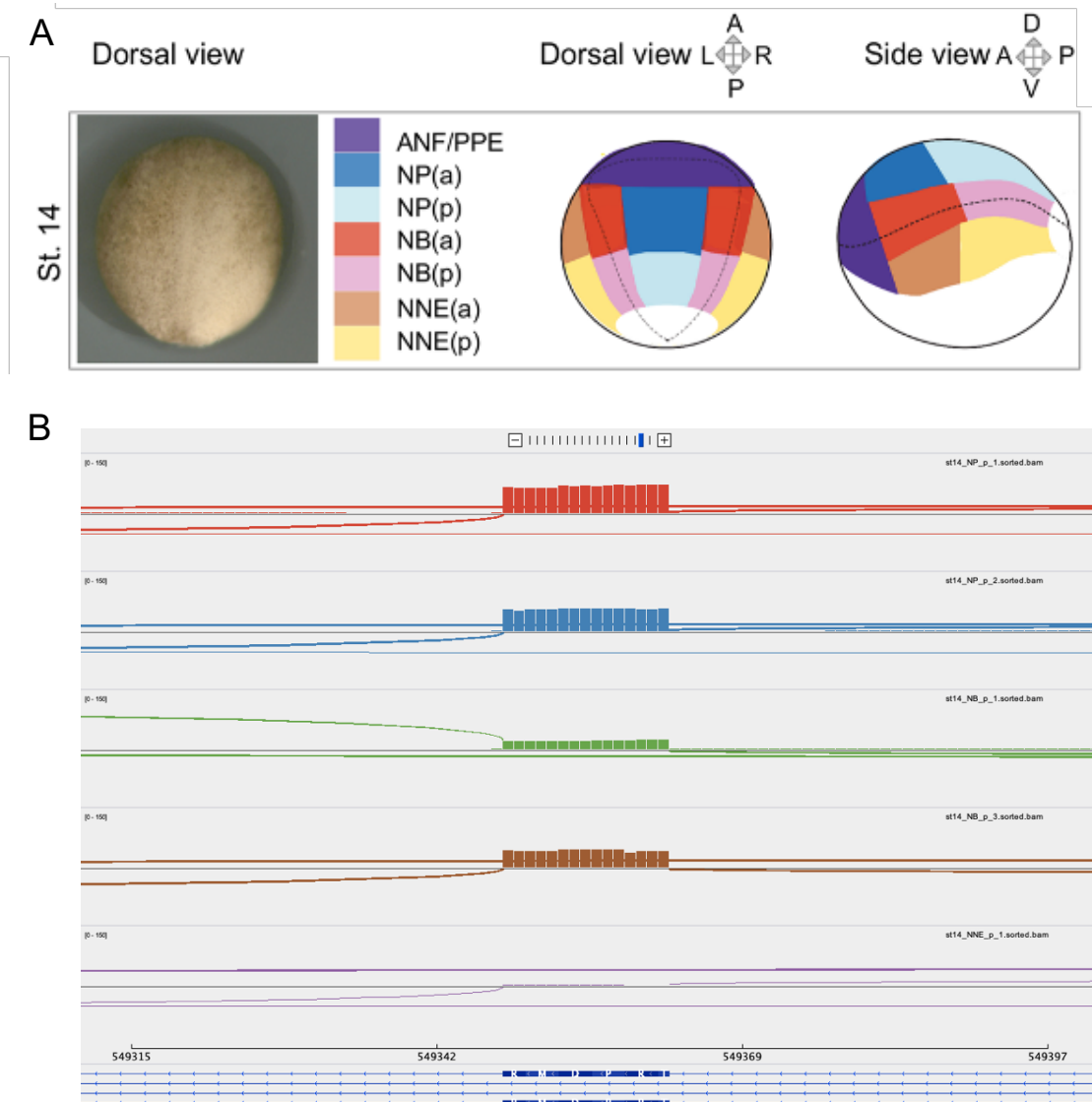


Figure 6.11. Detecting *n1-src* expression in the neural plate border using previously published RNA-Seq data. **A)** Figure taken from Plouhinec *et al.* (2017). Schematic displaying regions they dissected for RNA-Seq analysis from *X. laevis* embryos at stage 14. **B)** Reads were aligned to the *X. laevis* genome by the Evans lab and visualised in IGV. Analysis of reads present in the neural plate dissections shows reads aligning to the *n1-src* microexon (red and blue). Reads present in the non-neural ectoderm (purple) do not align to the microexon, indicating a lack of *n1-src* expression in this region. Analysis of the neural plate border dissections (green and brown/orange) shows reads aligning to the *n1-src* microexon, indicating expression of *n1-src* within the neural plate border as well as the neural plate.

6.3.4. Conclusion

Transcriptomic analysis on *n1-src* knockdown embryos revealed a downregulation of transcripts involved in development of the neural crest. In this chapter, I have further analysed this and shown that knockdown of *n1-src* expression in *X. tropicalis* embryos results in a significant downregulation of expression of a variety of markers for the neural plate border and the neural crest. This shows that expression of *n1-src* is required for the earliest steps of neural crest induction.

Furthermore, I have shown that induction of the neural crest in *Xenopus* animal cap explants leads to an upregulation of *n1-src* expression, suggesting that n1-Src potentially functions in later crest development, and its role is not restricted to just the earliest induction steps.

Fgf and RA signals are both involved in development of the amphibian neural crest. Work in this thesis has suggested shown that n1-Src is capable of interacting with the Fgf/Erk signalling pathway and potentially with members of pathways regulated by RA and therefore interactions between n1-Src and these signalling pathways could be integral for neural crest development, though more work is needed to investigate this.

7. Discussion

7.1. Summary of findings

The aims of this PhD thesis were to investigate the role that n1-Src plays in regulating neuronal specification and differentiation in the developing nervous system, and also investigate links between n1-Src and the amphibian neural crest. Chapter 3 of this thesis presents data that shows n1-Src is key in development of the *X. tropicalis* nervous system. The transcriptomic effects of *n1-src* knockdown were then analysed in chapter 4 of this thesis and revealed links between n1-Src and both alternative splicing and neural crest development. Chapters 5 and 6 investigated each of these links respectively.

The knockdown of *n1-src* leads to an aberrant touch responses in *X. tropicalis*, that is due to the inhibition of the primary neuron differentiation, and these results in chapter 3 this have been published in the Journal of Neuroscience (Lewis *et al.*, 2017). Further work identified that n1-Src acts upstream of the proneural genes in the hierarchical cascade of neural gene expression, and n1-Src interacts with the Fgf/Erk intracellular signalling cascade. The Fgf/Erk signalling pathway is an important factor in nervous system development (Ford-Perriss, Abud and Murphy, 2001; Delaune, Lemaire and Kodjabachian, 2005).

The role for n1-Src in neuronal differentiation was supported by the RNA-Seq data in this thesis. These transcriptomic data also show significant differential expression of transcripts involved in the splicing of pre-mRNA, and in development and migration of the neural crest. Analysis of the *n1-src* transcriptomic data was therefore expanded, and changes in splicing in response to *n1-src* knockdown was examined in both Illumina short read and Nanopore long read data. This identified the mis-splicing of a variety of splicing factors in knockdown embryos.

Validation of the n1-Src/neural crest link identified in the transcriptomic data was carried out in chapter 6, with results showing that expression of *n1-src* is vital for induction of the neural plate border and subsequent neural crest.

7.2. N1-Src in vertebrate development

N1-src expression has been detected in central nervous system (CNS), with activity particularly enriched in the forebrain and hippocampus of mature rat brains (le Beau, Wiestler and Walter, 1987). Its expression is however absent from the peripheral nervous system (PNS). Meanwhile, *c-src* expression is seen at equal levels in the mature CNS and PNS. This lead to the suggestion that c-Src functions in events that are shared

between the CNS and PNS whilst n1-Src activity is required for events that are unique to the CNS. With that in mind the authors proposed that the difference in *n1-src* expression in the CNS and PNS could suggest an important role of n1-Src in neuronal plasticity.

The central nervous system is able to modify its neural networks in response to injury and experience (Stiles, 2000). These modifications can include changes to preexisting synapses and individual neurons making new synaptic connections. A role for n1-Src in generating new synaptic connections is supported by its enriched expression in nerve growth cone membranes (Maness *et al.*, 1988) and its ability to modulate neurite outgrowth in *Xenopus* cells (Worley, Cornel and Holt, 1997).

In addition to work examining n1-Src in mature postmitotic neurons, it has been proposed to function in neuron differentiation (Schartl and Barnekow, 1984; Fults *et al.*, 1985). However, the exact role and mechanisms through which n1-Src function in the early stages of nervous system development remained unknown and work in this thesis aimed to answer this.

7.2.1. N1-Src is required for neuronal differentiation

Development of the amphibian nervous system involves two waves of neurogenesis, primary neurogenesis that gives rise to the neurons required for early larval behaviour and survival, and secondary neurogenesis which gives rise to the adult nervous system (Hartenstein, 1989).

The neurons that differentiate during primary neurogenesis form a simple reflex arc that allow for the embryos to unconsciously responds to touch and dart away from the stimuli (Boothby and Roberts, 1995; Roberts, Li and Soffe, 2010). In this thesis we observed embryos displaying an absent or aberrant response to touch following *n1-src* knockdown (Figure 3.7). Based on the expression patterns of *tubb2b*, knockdown of *n1-src* is causing a reduction or total loss of differentiated primary neurons in the neural plate of *X. tropicalis* embryos. (Figure 3.8). The differentiation of primary neurons is the result of a hierarchy of transcriptional activation and repression. Work in this thesis has identified that n1-Src functions between the neural prepattern and proneural genes to drive neuronal differentiation (Figure 3.11).

The failure of neurons to differentiate following *n1-src* knockdown explains the lack of touch response in the early tailbud stages. Later in development, the ability to sense and

respond to touch returns but it is an aberrant and uncoordinated response. This is believed to be due to the transient nature of the morpholinos, which wear off, allowing for neurogenesis to proceed but as the process has been delayed the resultant nervous system is incomplete.

Neurons have been observed at these late tailbud stages, but they are sparse compared to control embryos, and their neurites do not appear to have projected as far (Mole, 2020). With the neuronal projections not being as prominent in *n1-src* knockdown embryos, the touch response defects observed could be due to their failure to innervate muscles correctly.

Alongside neuronal defects, muscle abnormalities could also potentially be contributing to the aberrant touch response. Whilst *n1-src* is thought to be a neural specific kinase, mesodermal transcripts were seen in the RNA-Seq data to be downregulated following *n1-src* knockdown. These mesodermal transcripts include, amongst others, a variety of troponin and myosin transcripts. They have been attributed to function in a variety of processes including skeletal muscle contraction, musculoskeletal movement, muscle cell differentiation and sarcomere organisation. *N1-src* is expressed broadly throughout the neural plate of stage 14 *X. tropicalis* embryos. At this stage, mesoderm is underlying the neural plate (Keller, 1976) and in the *in situ* hybridisations figures presented there is no means of determining if *n1-src* expression is restricted to the overlying neural tissue or if it is also present in the mesoderm. Dissections or histology of such *in situ* would reveal the depth of *n1-src* expression within the embryo, and if expression is detected in the mesoderm then much more work is required here to investigate the role of n1-Src in the development of tissues derived from the mesoderm. I have carried out RT-PCR analysis on *X. laevis* embryos to examine *n1-src* expression in mesoderm dissections but at this point the results remain inconclusive.

7.2.2. N1-Src is required for neural crest development

The ability of neuronal Srcs to drive the differentiation of neuroblastoma cells (Lewis, 2014), a cancer that arises in cells derived from the neural crest, leads to the suggestion that n1-Src might regulate neural crest development.

Knockdown of *n1-src* inhibits the expression of numerous neural crest specific genes in *X. tropicalis* embryos, as shown by RT-PCR, *in situ* hybridisation and RNA-Seq analysis. In addition to being required for the induction of the neural plate border, from where the

neural crest arises, preliminary work in this thesis suggests *n1-Src* might be expressed in the developing neural crest.

Expression of *n1-src* within the developing neural crest fits with its ability to drive the differentiation of neuroblastoma cells. I have shown that *n1-Src* is required for the differentiation of primary neurons in *X. tropicalis* embryos and it may well be that neuroblastomas arise from the failure of *n1-Src* to drive the differentiation of the crest derived sympathetic neurons. The potential expression of *n1-src* in the neural crest and roles it might have in crest development is an area that needs further research.

N1-src expression is absent from the mature neurons of the neural crest derived PNS (le Beau, Wiestler and Walter, 1987). The absence of expression from the mature neurons of the PNS does not eliminate the potential for *n1-Src* to drive the differentiation of these neurons. *N1-Src* carries out multiple roles in an organism, I have shown its required early in development to drive differentiation of neural precursor cells into primary neurons in *X. tropicalis* and *n1-Src* could be expressed in the neural crest derived tissues, driving differentiation of PNS neurons. Following their differentiation, *n1-src* expression could be downregulated.

Whilst I have shown that *n1-Src* is involved in neuronal differentiation and development of the neural crest, the only phenotype detected in *src* ^{-/-} mice is osteopetrosis (Soriano *et al.*, 1991). The absence of any neuronal or neural crest phenotype could be due to the redundant nature of *c-Src* and *Fyn*, as *fyn* ^{-/-} mice are also viable, but the *Src/Fyn* double mutants die perinatally (Stein *et al.*, 1994; Morse *et al.*, 1998). Examination of the double mutants however revealed axonal defects. Phenotypic differences between by gene knockouts and knockdowns have been observed in multiple different model organisms, and these differences have been attributed to various factors, including the off-target effects of antisense reagents and their toxicity to embryos (El-Brolosy and Stainier, 2017). However, experiments in zebrafish have supported the hypothesis that knockout embryos exhibit genetic compensation, which is absent from knockdowns (Rossi *et al.*, 2015). In this study, knockdown of the extracellular-matrix gene, *egfl7*, led to vascular defects in zebrafish embryos, however *egfl7* mutants displayed no obvious defects. *Egfl7* mutants displayed an increased expression of other extracellular-matrix genes, thought to compensate for *egfl7*. Furthermore, injection of *egfl7* morpholinos into *egfl7* mutants led to no vascular defects, indicating the phenotype was not due to the morpholino toxicity or off-targeting events. Therefore, the absence of a neuronal or neural crest phenotype in

src ^{-/-} mice could be due to genetic compensation, that is not exhibited following the morpholino mediated knockdown of *n1-src* in *X. tropicalis* embryos.

7.3. Mechanisms by which n1-Src regulates development

This thesis has shown that n1-Src is an important regulator of early development in *X. tropicalis*, with both neuronal differentiation (Figure 3.8) and neural crest induction (Figure 6.4) dependent on *n1-src* expression.

I have also investigated the mechanisms through which n1-Src might be acting to regulate such processes, with the regulation of intracellular signalling and the regulation of splicing both implicated.

7.3.1. N1-Src regulates intracellular signalling

Both the differentiation of primary neurons in *X. tropicalis* and the induction of the neural crest involve the expression of complex gene regulatory networks and require the activation of intracellular signalling pathways. Such pathways often induce or repress transcription factor activity and thus regulate gene expression (Hill and Treisman, 1999).

One intracellular signalling pathway that is utilised in both neuronal and neural crest development is the Fgf/Erk pathway. Fgf signalling has been implicated in neural induction, but its role in later neural development is not as well established, with results implicating Fgfs in neural induction, but not in differentiation and other results showing a role for Fgfs in the proliferation of neural progenitors (Storey *et al.*, 1998; Diez del Corral and Morales, 2017). However, other research suggests Fgf signalling does function in the differentiation of neurons (Kosaka *et al.*, 2006; Goldshmit *et al.*, 2018). Fgf signalling has been linked to the neurogenic pathway with the expression of the neural prepatter genes, *sox2* and *sox3* and the differentiated neuron marker, *tubb2b*, all sensitive to Fgf signalling (Hardcastle, Chalmers and Papalopulu, 2000; Rogers *et al.*, 2008). Likewise in neural crest development, induction of the neural plate border, from which the neural crest will arise, requires Fgf signalling and Fgf inhibition inhibits the expression of neural crest specifier genes (Mayor, Guerrero and Martinez, 1997).

Other intracellular signalling cascades function in nervous system development, and whilst I have shown in this thesis that *n1-src* expression is required to induce the expression of neuronal differentiation genes and also regulate Erk activity, I postulate that

during neuronal differentiation n1-Src regulates Akt activity and it is this regulation that is more important for neuronal differentiation. The Phosphoinositide 3-kinase (PI3K)/Akt signalling pathway is an intracellular signal transduction pathway whose activation by extracellular ligands results in changes in target gene expression (Cooper, 2000). Along with activating the Erk signalling pathway, Fgf ligands can activate the PI3K/Akt pathway, and this activation has critical roles in early development (Carballada, Yasuo and Lemaire, 2001; Dailey *et al.*, 2005; Geary and LaBonne, 2018). Another extracellular signal that activates the PI3K/Akt pathway is nerve growth factor (Ngf). Ngf has a well-documented role in controlling the differentiation and survival of neurons (Levi-Montalcini, 1987; Matsushima and Bogenmann, 1990; Aloe *et al.*, 2016). Furthermore, the differentiation of neuroblastoma cells with retinoic acid is dependent on PI3K/Akt signalling, and the ability of neuronal Srcs to drive the differentiation of RA-resistant neuroblastoma cells suggests they can also activate the same pathway (López-Carballo *et al.*, 2002; Qiao *et al.*, 2012; Lewis, 2014). Experiments had been planned to investigate the potential regulation of the PI3K/Akt pathway via n1-Src by examining the levels of phosphorylated Akt.

The differentiation of primary neurons in *X. tropicalis* embryos is the result of a hierarchy of transcriptional activation and repression. I hypothesise that in cells fated to become neurons, n1-Src controls the expression levels of proliferative and differentiation genes through the regulation of PI3K/Akt signalling. *N1-src* is expressed broadly throughout the neural plate, and acts within the hierarchical neurogenic pathway. This model suggests that those cells in the neural plate that are destined to differentiate into neurons express elevated levels of *n1-src* compared to cells that remain proliferative. Differences in n1-Src levels within the neural plate could be investigated via whole-mount immunostaining using an antibody against the *X. tropicalis* n1-Src insert. The use of antibodies specific to the n1-Src insert have been previously utilised to detect mammalian n1-Src (Sugrue *et al.*, 1990; Keenan, 2012). Increased levels and activity of n1-Src in particular cells in the neural plate would lead to increased phosphorylation of members of the Akt signalling pathway, and the phosphorylation of target transcription factors, and the elevated expression of differentiation genes. This would be accompanied by the downregulated expression of the proliferative neural prepattern genes such as *sox3*. This is in keeping with results showing overexpression of neuronal Srcs in SK-N-AS cells decreases the number of proliferating cells (Lewis, 2014).

Whilst it is possible that n1-Src is regulating Akt activity to drive neuronal differentiation, that does not exclude n1-Src from regulating Erk activity in the same process. Alongside

potential regulation in neuronal differentiation, I predict that n1-Src is regulating the Fgf/Erk signalling pathway at much later stages in nervous system development, with Fgf signalling important in axonal growth and synaptic plasticity, two processes that Src activity is implicated in (Zechel *et al.*, 2010; dos Santos *et al.*, 2019).

Alongside roles in nervous system development, Fgf signalling is required in neural crest development, with cells in the neural plate border being subject to Fgf signals emanating from either the neural plate or the underlying mesoderm (Mayor, Guerrero and Martinez, 1997; Monsoro-Burq, Fletcher and Harland, 2003). These signals are required for the transcription of neural crest specifier genes. The results showing *n1-src* knockdown downregulates expression of the crest specifier genes, combined with the preliminary data showing expression of *n1-src* in the neural plate border. again leads to the hypothesis that n1-Src is a key regulator of intracellular signalling pathways downstream of Fgf signalling.

Therefore, I propose that n1-Src regulates the Akt and Erk activity during early vertebrate development. The potential for n1-Src to regulate Akt activity during neuronal differentiation is a key area of future research. Furthermore, the exact mechanisms through which n1-Src could be regulating these intracellular signalling pathway remains to be determined. The requirement of n1-Src in induction of the neural crest is also an area where much more work is required, with investigations needed into whether it regulates the activity of Erk during this process.

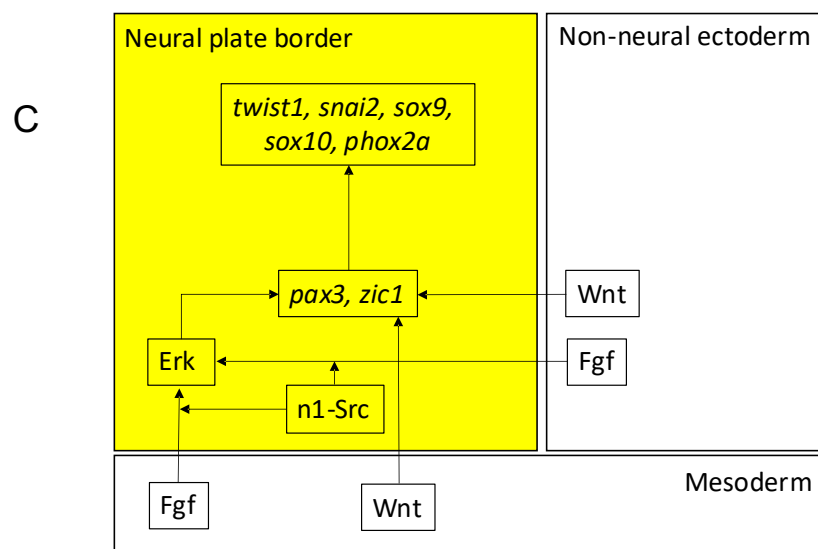
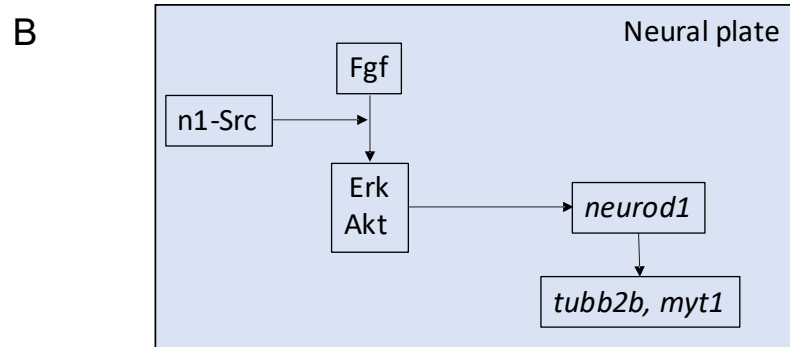
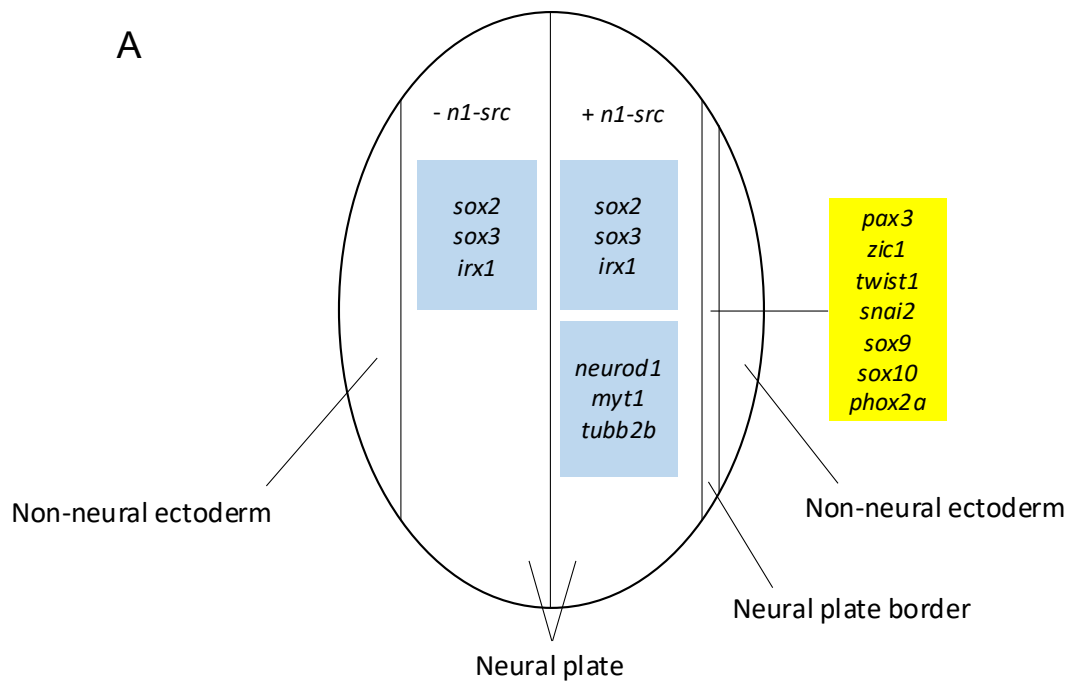


Figure 7.1. A summary of the proposed role for n1-Src in regulating the expression of genes required for neurogenesis and neural crest development. **A)** N1-Src is required for differentiation of *Xenopus* primary neurons within the neural plate, and induction of the neural plate border and subsequent neural crest. Knockdown of *n1-src* does not affect expression of the neural prepatter genes *sox2*, *sox3*, and *irx1*. However, expression of the proneural gene, *neurod1*, and the differentiated neuron markers, *myt1* and *tubb2b*, is significantly downregulated following *n1-src* knockdown. Neural plate border induction is also impeded following *n1-src* knockdown. The neural plate border is marked by the expression a variety of neural plate border and neural crest specifier genes (yellow), which are downregulated in response to *n1-src* knockdown. **B)** Fgf signalling has been implicated in neuronal differentiation. This thesis proposes that n1-Src modulates the activity of the Fgf/Akt and/or the Fgf/Erk signalling pathways within cells of the neural plate, to upregulate expression of the proneural genes such as *neurod1* and drive neuronal differentiation. **C)** N1-Src is required for induction of the neural plate border and development of the neural crest. Neural plate border induction requires the expression of neural plate border specifier genes, such as *pax3* and *zic1*, in response to Fgf and Wnt signalling from the adjacent non-neural ectoderm and/or the underlying mesoderm. It is proposed that n1-Src activity is required in the presumptive neural plate border, to modulate Fgf/Erk signalling and drive expression of the neural plate border specifier genes. This is required for the subsequent downstream expression of the neural crest specifier genes such as *twist1*, *snai2*, *sox9*, *sox10* and *phox2a*.

7.3.2. N1-Src regulates alternative splicing

Results in this thesis show that knockdown of *n1-src* leads to a variety of splicing defects in *X. tropicalis* embryos. Alternative splicing plays a critical role in development of the nervous system (Raj and Blencowe, 2015; Su, Dhananjaya and Tarn, 2018). Alongside transcriptional regulation through the modulation of intracellular signalling pathways, n1-Src could also be directing alternative splicing throughout neural development.

Pre-mRNA splicing functions as a level of control in gene expression in both neural progenitors and non-neural cells. In non-neural cells, Ptb1 has been found to drive intron retention events in at least 4 neuron-specific genes, which leads to the degradation of these incompletely spliced mRNAs via nonsense mediated decay (Yap *et al.*, 2012). A similar mechanism is used in differentiated neurons, where intron retention is utilised to downregulate the expression of genes that are required in neural progenitors but not in mature neurons (Braunschweig *et al.*, 2014).

Alongside the alternative splicing data presented in chapters 2 and 3 of this thesis, work in the Evans lab has shown that n1-Src interacts with and phosphorylates a cluster of RNA processing and splicing factors (West, 2019). One interactant is Splicing Factor,

Proline And Glutamine Rich (SFPQ). SFPQ activity has been linked with neuronal development.

Sfpq displays high expression levels in the developing *D. rerio* brain, particularly in regions enriched for neuronal progenitors (Lowery, Rubin and Sive, 2007). In motor neurons, in addition to nuclear pools, SFPQ protein was also detected in the axons (Thomas-Jinu *et al.*, 2017). Furthermore, *sfpq* *-/-* *D. rerio* embryos exhibited motor neurons with axonal defects, a phenotype similar to that seen in *n1-src* knockdown *x. tropicalis* embryos. The similar phenotypes between embryos deficient in SFPQ or n1-Src, and their observed interaction *in vitro*, adds support for a role for n1-Src in regulating alternative splicing in nervous system development. The shared axonal defects also suggest that the aberrant touch response seen in *n1-src* knockdown embryos is due to the lack of muscle innervation by motor neuron axons, brought about by defects in splicing.

Alternative splicing also appears to be an important factor in development of the neural crest, another process that this thesis has shown n1-Src to function in. Mutations that disrupt development of the neural crest are collectively known as neurocristopathies (Beauchamp *et al.*, 2020). Many neurocristopathies lead to craniofacial malformations, a phenotype that is shared by spliceosomopathies. One such neurocristopathy is CHARGE syndrome, a complex genetic syndrome that mainly impacts cranial and cardiac neural crest derivatives (Bérubé-Simard and Pilon, 2019). Whilst CHARGE syndrome displays a wide variety of symptoms, the underlying genetics are not well known, with only 1 or 2 genes being associated with the disease (Bélanger *et al.*, 2018). One such gene is *fam172a*, which plays an important role in cotranscriptional alternative splicing and the analysis of cell lines from CHARGE syndrome patients and their unaffected parents found dysregulated splicing of a cluster of genes in patients. This suggests that alternative splicing plays an important role in correct neural crest development. As I have shown that n1-Src is required for correct neural crest development, and also regulates alternative splicing, I postulate that n1-Src regulates a neural crest specific splicing profile, and that the potential dysregulation of n1-Src function could be a component in the development of some neurocristopathies.

Investigations into the expression and activity of n1-Src in neurocristopathies could be an avenue of future research. This would first require confirmation that *n1-src* is expressed in the developing neural crest as my research suggests it is and then confirmation that n1-Src regulates splicing in the developing neural crest. The analysis of neural crest

splicing could be carried out in *Xenopus* with through RNA-Seq analyses of neural plate border dissections. Sequencing of such explants from control and *n1-src* knockdown embryos would give insight into whether n1-Src regulates a crest specific splicing profile. However, due to size limitations such an approach may be a struggle with *X. tropicalis* embryos and *X. laevis* would be an easier model to work with. The tetraploid nature of *X. laevis* however would might present a challenge, with the potential need for four different morpholinos required to effectively knockdown *n1-src*, rather than the two needed in *X. tropicalis*.

7.4. N1-Src and neuroblastoma therapies

Neuroblastoma is another example of a neurocristopathy that arises when neural crest derived cells of the sympathetic nervous system fail to differentiate (Boland, 1974; Ratner *et al.*, 2016). Neuronal Srcs has been shown to drive differentiation of neuroblastoma cells *in vitro*, and their elevated expression compared to c-Src has been associated with a positive prognosis in neuroblastoma patients (Bjelfman *et al.*, 1990; Lewis, 2014).

I have shown that n1-Src is required for the differentiation of primary neurons in *X. tropicalis* embryos, and for the induction of the neural crest. Furthermore, preliminary data suggests *n1-src* is expressed in the crest itself. The hypothesis that *n1-src* is expressed in the neural crest is supported by its ability to differentiate neuroblastoma cells and its association with a positive prognosis. These observations suggest that neuroblastomas may arise by the failure of n1-Src to drive the differentiation of the crest derived sympathetic neurons.

Treatments for neuroblastomas are constantly being searched for. One treatment is with Retinoic acid (RA), the active form of vitamin A, which binds to nuclear retinoic acid receptors, however it is by no means 100% effective (Reynolds *et al.*, 2003). RA activity induces the expression of genes required for neuronal differentiation (Tonge and Andrews, 2010). When used to differentiate embryonal carcinoma cells, RA treatment leads to an 8-20 fold increase neuronal *src* expression (Lynch, Brugge and Levine, 1986). A similar increase in *n1-src* expression could also occur in RA treated neuroblastoma cells. The ability of N-Srcs to drive the differentiation of RA-resistant neuroblastoma cell lines supports n1-Src acting downstream of RA in neuroblastomas.

The similarities between the cellular effects of RA treatment, and the results in this thesis examining n1-Src support that n1-Src functions in the same pathway as RA. Alongside upregulating the activity of intracellular signalling pathways, RA treatment has been shown to affect splicing in neuroblastoma cells, with a variety of splicing factors being phosphorylated in response, including hnRNPA1 (Laserna *et al.*, 2009). Furthermore, miRNAs are also activated in response to RA treatment and they regulate the activity of factors such as SRSF1 (Meseguer *et al.*, 2011).

If n1-Src is acting downstream of RA during the neuronal differentiation and in the differentiation of neuroblastoma cells, then it could potentially be a therapeutic target, especially in patients where RA treatment is ineffective.

Any potential therapy could either target n1-Src itself, or target factors downstream of n1-Src. The more feasible option would probably be designing drugs to work downstream of n1-Src. Work in this thesis, and other work in the lab has identified phosphorylation targets for n1-Src (West, 2019), and also targets whose transcription is regulated by n1-Src. Drugs that increase the phosphorylation of key n1-Src targets, or inhibit their dephosphorylation, could therefore be one potential therapy. Another therapy that could be considered could be attempting to increase the expression of genes downstream of n1-Src that drive differentiation, such as *neurod1*. Such therapies could potentially increase the activity of intracellular signalling pathways that regulate expression of differentiation genes. An opposite approach could be attempting therapies that inhibit the activity of proteins that inhibit neuronal differentiation. Much more work however is required to gain a deeper understanding of how n1-Src functions in the developing embryo before such therapies can be developed. For example, the targets of n1-Src phosphorylation have been identified by an *in vitro* screen (West, 2019), but such phosphorylation events remain to be confirmed *in vivo*.

Therapies that target *n1-src* expression or activity could also be an avenue of research. Any treatment would have to work post-transcriptionally as merely attempting to increase expression of the *src* gene would lead to increased c-Src activity. The regulation of alternative splicing as a therapeutic target is becoming an area of increased research interest (Bates *et al.*, 2017). Factors that are involved in the splicing of the microexon could therefore be a potential target for neuroblastoma therapy, however a much deeper understanding of the n1-Src splicing mechanism would be required for such a treatment. Furthermore, administration of the treatment would also be a major obstacle to overcome, due the likelihood of off-targeting splicing effects (Bates *et al.*, 2017).

The method of administration of any potential therapy developed would have to be considered, to ensure that the treatment effected the malignant cancer cells whilst minimizing off-targeted side effects. Nanoparticles are one of the most rapidly developing areas of research, as a method of delivering therapeutic drugs to specific targets (Patra *et al.*, 2018). Ganglioside GD2 is expressed on the plasma membrane of stem cells, neurons and various types of malignant cells, including neuroblastoma tumours (Doronin *et al.*, 2014; Suzuki and Cheung, 2015). Nanoparticles designed to recognise GD2 could therefore be a potential method of delivering a therapeutic that would limit off-targeting effects.

Whilst I have proposed broad mechanisms by which neuroblastoma therapies could be utilised to target n1-Src or its downstream effectors, a much deeper understanding of n1-Src is required before the development of any therapies, in particular the signals required for the induction of the n1-Src microexon splicing machinery and regulation of the activity of the machinery.

7.5. Conclusions

Neuronal Src activity has long been correlated with a positive prognosis for neuroblastoma patients, but the role that n1-Src plays in early development is mostly unknown. This thesis has looked to increase our understanding of the role of n1-Src during development. Utilising *X. tropicalis* as a model organism, a variety of techniques have been used to demonstrate that n1-Src has essential roles in neuronal development, where its activity is required for the expression of proneural genes and the differentiation of primary neurons. This study has also revealed n1-Src activity is essential for development of the neural crest. Furthermore, I have shown that n1-Src is a regulator of alternative splicing and therefore driving these processes through regulation of the splicing machinery. This work has greatly added to our knowledge of the role of n1-Src in early development and identified several avenues which could be considered for future research.

Appendices

Appendix A – Alignment of sequenced *hnrnpa1* medium sized product to *X. tropicalis hnrnpa1* genomic sequence. Sequence in bold is the sequenced PCR product. Highlighted in red is the intron between exons 7 and 8 that is not retained.



170 180 190 200 210 220 230

GCCGCCATATCCACCTCCTTGGCCACCTCCATAGCCTTGGCCACCACCACCACCATAGCCTCTGT
CGGCGGTATAGGTGGAGGAACCGGTGGAGGTATCGGAACCGGTGGTGGTGGTATCGGAGACA

Exon 7

GCCGCCATATCCACCTCCTTGGCCACCTCCATAGCCTTGGCCACCACCACCACCATAGCCTCTGT
GCCGCCATATCCACCTCCTTGGCCACCTCCATAGCCTTGGCCACCACCACCACCATAGCCTCTGT

240 250 260 270 280 290

TACCCCCAGAGTATGGAGGGCTGCCACCGTAACCACCATCATTGCCAAAGCCATTGTAGCCATCT
ATGGGGGTCTCATACCTCCCGACGGTGGCATTGGTGGTAGTAACGGTTTCGGTAACATCGGTAGA

Exon 7

TACCCCCAGAGTATGGAGGGCTGCCACCGTAACCACCATCATTGCCAAAGCCATTGTAGCCATCT
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300

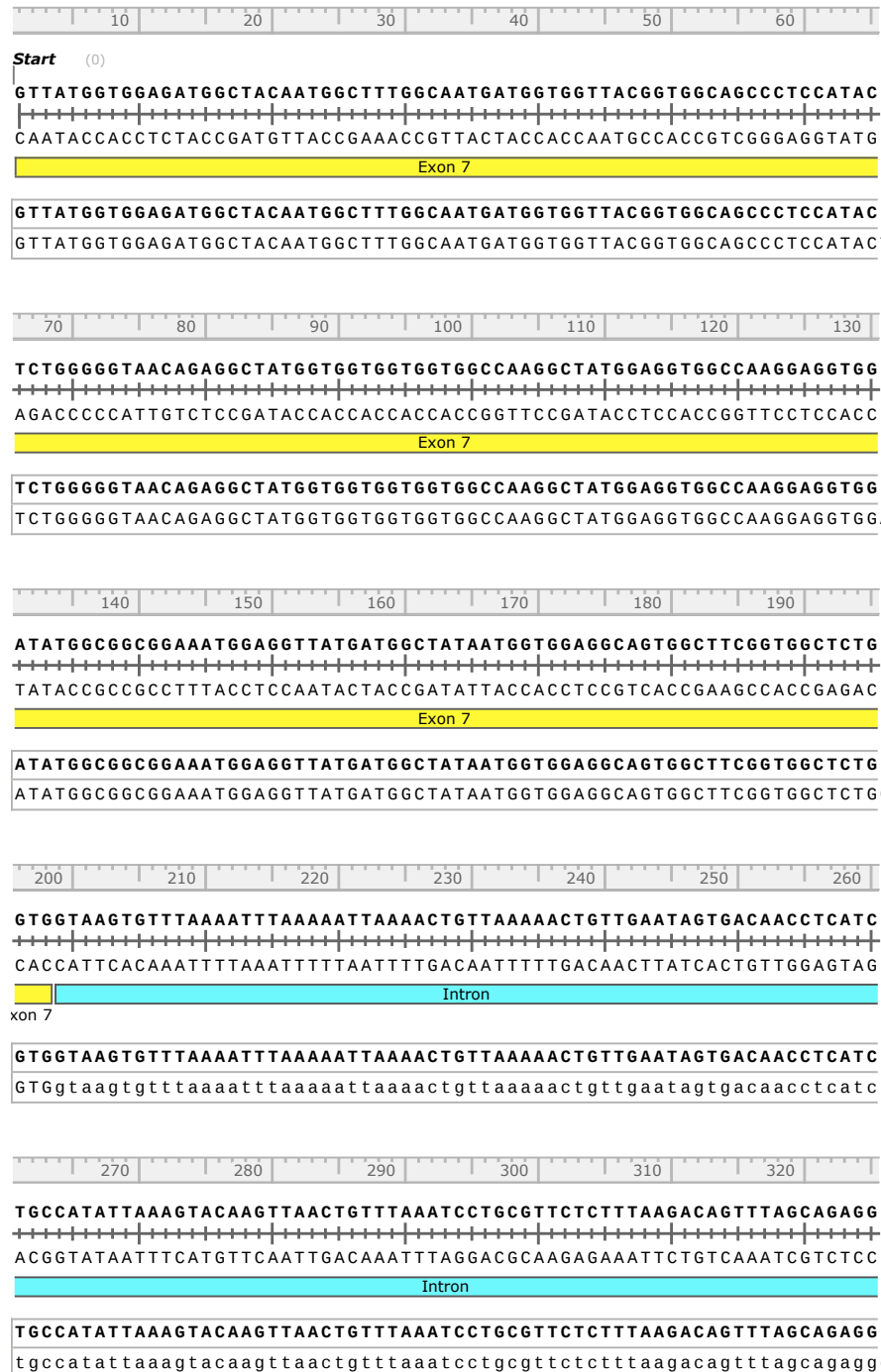
End (307)

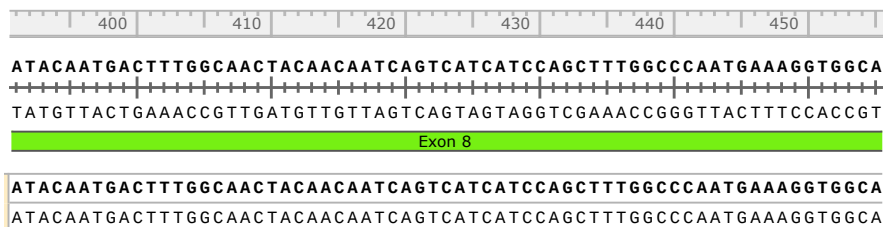
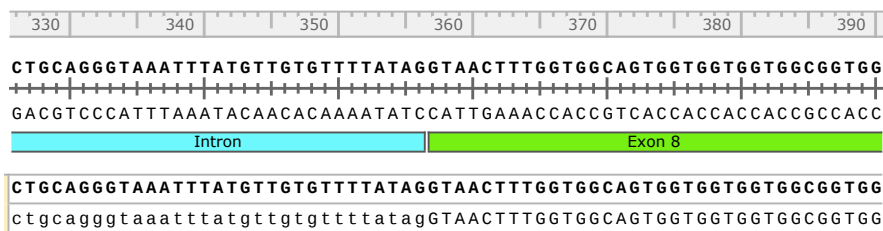
CCACCATAAC 3'
GGTGGTATTG 5'

Exon 7

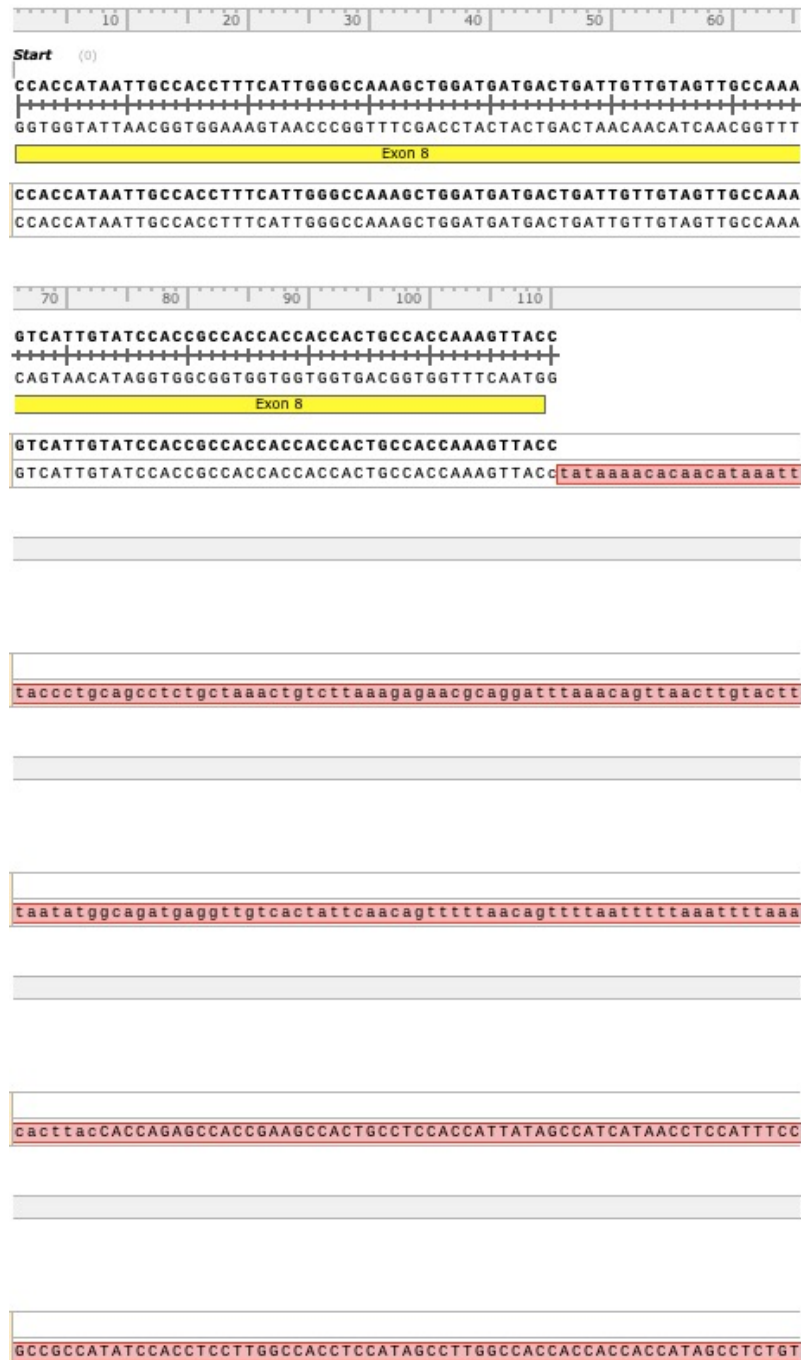
CCACCATAAC
CCACCATAAC

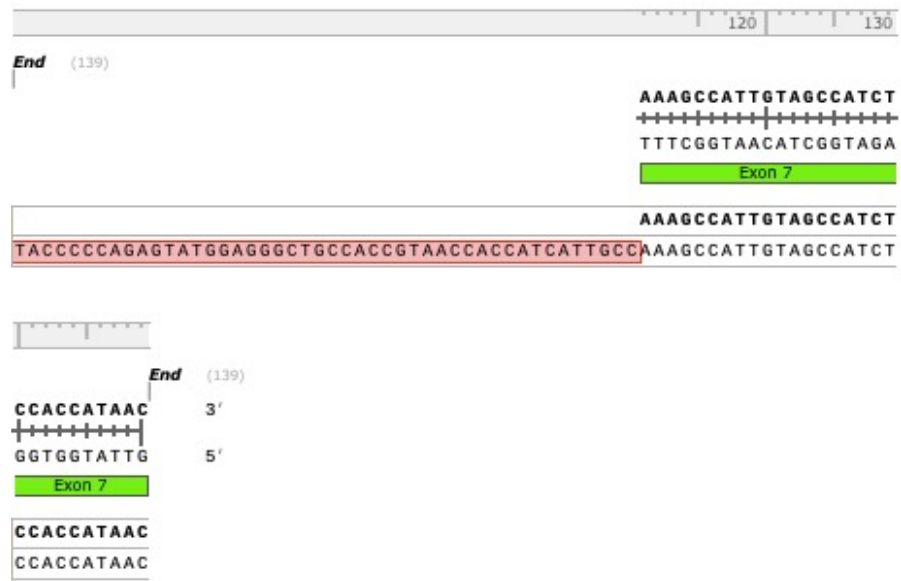
Appendix B – Alignment of sequenced *hnrnpa1* larger product to *X. tropicalis* *hnrnpa1* genomic sequence. Sequence in bold is the sequenced PCR product. Highlighted in blue is intron which is retained within the PCR product.



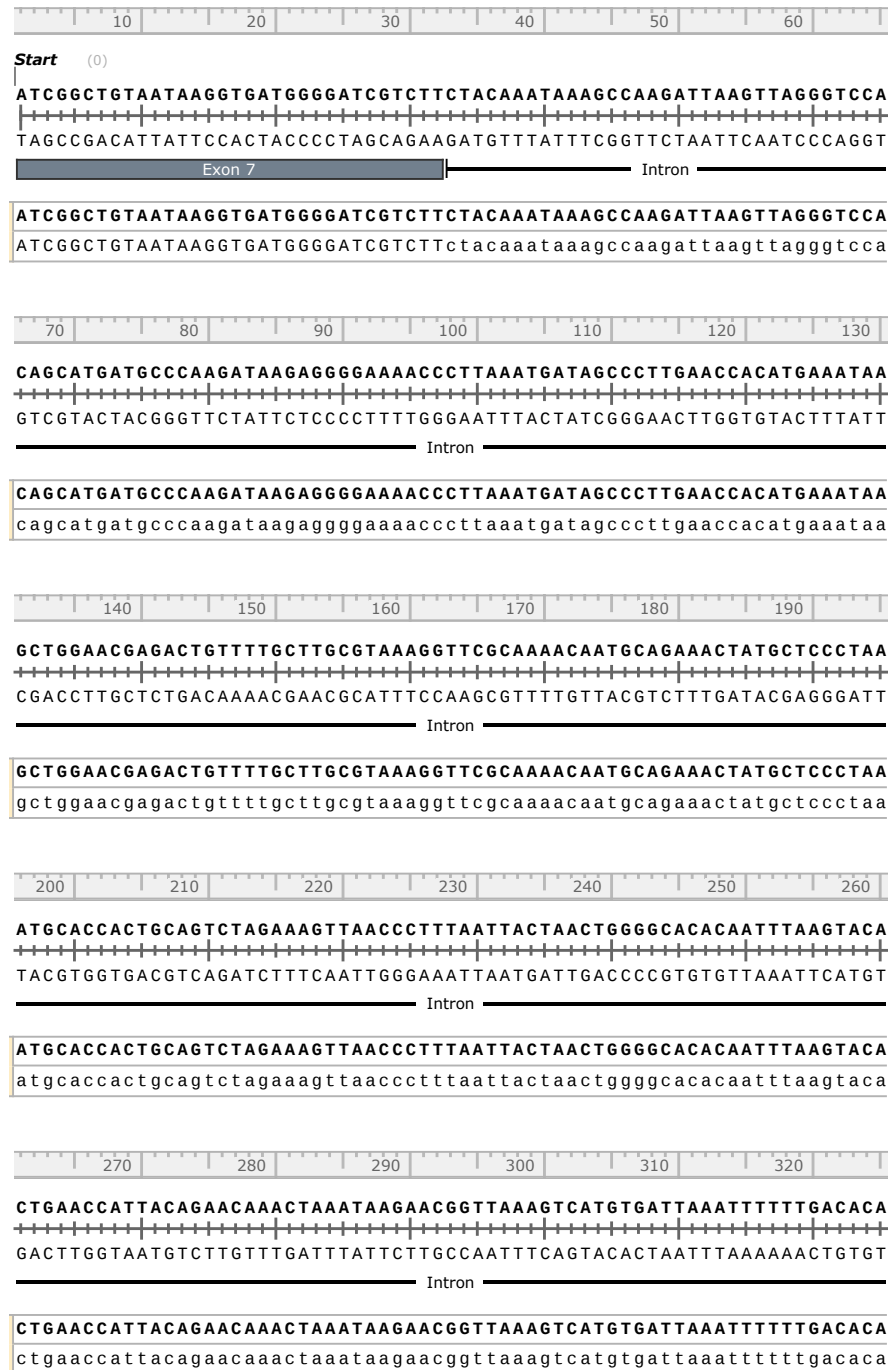


Appendix C – Alignment of sequenced smaller *hnrnpa1* product to *X. tropicalis* *hnrnpa1* genomic sequence. Sequence in bold is the sequenced PCR product. In the PCR product exon 7 is shorter as alternative splicing has spliced out much of the exon along with the intron. This can be seen in the portion of the genomic sequence highlighted in red. Lower case bases in this region are intron bases and capitals are bases from exon 7 that are also spliced out.





Appendix D – Alignment of sequenced *tra2a* larger product to *X. tropicalis tra2a* genomic sequence. Sequence in bold is the sequenced PCR product. The PCR product contains a retained intron between exons 6 and 7.



330 340 350 360 370 380 390

AAAAACAACCTTTTTTTTAAAAAGTTACCTATAGGTCATGTTGATTGTTTTTTGCTTTTGTAAGT
 +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TTTTTGTTGAAAAAAAATTTTCAATGGATATCCAGTACAACCTAACAAAAACGAAAAACATTCA

Intron

AAAAACAACCTTTTTTTTAAAAAGTTACCTATAGGTCATGTTGATTGTTTTTTGCTTTTGTAAGT
 aaaaacaactttttttt-aaaaagttacctataggtcatgttgattgttttttgcttttgtaagt

400 410 420 430 440 450

AATTGTTACTTGAAAGTTCCTAAACCTGACTGTCCTTCTCAGCTCATCTGGTCAAGTTACAGCTAA
 +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TTAACAATGAACCTTCAAGGATTTGGACTGACAGGGAAGAGTCGAGTAGACCAAGTCAATGTCGATT

Intron

AATTGTTACTTGAAAGTTCCTAAACCTGACTGTCCTTCTCAGCTCATCTGGTCAAGTTACAGCTAA
 aattgttacttgaaagttcctaaacctgactgtcccttctcagctcatctggtcagttacagctaa

460 470 480 490 500 510 520

TGCTAACAAACTCCTGCTGCACAAATATGGCAGCTCCCTCACAGAGGACCATGGGGAATAAGATA
 +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
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Intron

TGCTAACAAACTCCTGCTGCACAAATATGGCAGCTCCCTCACAGAGGACCATGGGGAATAAGATA
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530 540 550 560 570 580

GGGAATGTAAATGCATCAGGCAAATACTTATGGAAAAATTATAAATAGCATGCAAAGCCAATATT
 +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 CCCTTACATTTACGTAGTCCGTTTATGAATACCTTTTTAATATTTATCGTACGTTTCGGTTATAA

Intron

GGGAATGTAAATGCATCAGGCAAATACTTATGGAAAAATTATAAATAGCATGCAAAGCCAATATT
 ggggaatgtaaatgcatcaggcaaatacttatggaaaaattataaatagcattgcaaagccaatatt

590 600 610 620 630 640 650

ATGATATAAAAAAGGGTTTCATTTTTGGTGTCAGTGCTAACTTTCCATTACAATTTTACAGATCA
 +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TACTATATTTTCCCAAAGTAAAAACCACAGTCACAGATTGAAAGGTAATGTTAAAATGTCTAGT

Intron

ATGATATAAAAAAGGGTTTCATTTTTGGTGTCAGTGCTAACTTTCCATTACAATTTTACAGATCA
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[illegible]

aaaaacaacttttttttaaaaagttacctataggtcatgttgattgttttttgcttttgtaagta

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ggaatgtaaatgcatcaggcaaatacttatggaaaaattataaatagcatgcaaagccaatatta

tgatataaaaagggtttcatttttggtgtcagtgcttaactttccattacaattttacagatcaa



Appendix F – N1-Src Kinase is Required for Primary Neurogenesis in *Xenopus tropicalis*. Lewis, Bradley, Pizzey, Isaacs and Evans. Published in The Journal of Neuroscience in 2017. Data presented in chapter 3 of this thesis contributed to this publication

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Development/Plasticity/Repair

N1-Src Kinase Is Required for Primary Neurogenesis in *Xenopus tropicalis*

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The presence of the neuronal-specific N1-Src splice variant of the C-Src tyrosine kinase is conserved through vertebrate evolution, suggesting an important role in complex nervous systems. Alternative splicing involving an N1-Src-specific microexon leads to a 5 or 6 aa insertion into the SH3 domain of Src. A prevailing model suggests that N1-Src regulates neuronal differentiation via cytoskeletal dynamics in the growth cone. Here we investigated the role of n1-src in the early development of the amphibian *Xenopus tropicalis*, and found that n1-src expression is regulated in embryogenesis, with highest levels detected during the phases of primary and secondary neurogenesis. *In situ* hybridization analysis, using locked nucleic acid oligo probes complementary to the n1-src microexon, indicates that n1-src expression is highly enriched in the open neural plate during neurula stages and in the neural tissue of adult frogs. Given the n1-src expression pattern, we investigated a possible role for n1-src in neurogenesis. Using splice site-specific antisense morpholino oligos, we inhibited n1-src splicing, while preserving c-src expression. Differentiation of neurons in the primary nervous system is reduced in n1-src-knockdown embryos, accompanied by a severely impaired touch response in later development. These data reveal an essential role for n1-src in amphibian neural development and suggest that alternative splicing of C-Src in the developing vertebrate nervous system evolved to regulate neurogenesis.

Key words: embryo; morpholino; neurogenesis; splicing; tyrosine kinase; *Xenopus*

Significance Statement

The Src family of nonreceptor tyrosine kinases acts in signaling pathways that regulate cell migration, cell adhesion, and proliferation. Srcs are also enriched in the brain, where they play key roles in neuronal development and neurotransmission. Vertebrates have evolved a neuron-specific splice variant of C-Src, N1-Src, which differs from C-Src by just 5 or 6 aa. N1-Src is poorly understood and its high similarity to C-Src has made it difficult to delineate its function. Using antisense knockdown of the n1-src microexon, we have studied neuronal development in the *Xenopus* embryo in the absence of n1-src, while preserving c-src. Loss of n1-src causes a striking absence of primary neurogenesis, implicating n1-src in the specification of neurons early in neural development.

Introduction

The Src family of 11 nonreceptor tyrosine kinases evolved to regulate key signaling pathways involved in cell adhesion, migration, and cell fate in multicellular organisms (Thomas and Brugge, 1997). Several Src family members, including C-Src, Fyn, and Yes, are enriched in the vertebrate nervous system with roles in the developing and

mature brain and have been implicated in the pathology of neurological disorders (Grant et al., 1992; Maness, 1992; Zhao et al., 2000; Ohnishi et al., 2001; Kalia et al., 2004; Nygaard et al., 2014). Further complexity and specificity of C-Src signaling in the brain is conferred by neuronal-specific splicing to yield N1-Src or N2-Src (Brugge et al., 1985; Pyper and Bolen, 1990). The N-Src splice variants contain an additional 6 or 17 aa respectively in the SH3 domain, and are encoded by microexons situated between exons three and four of C-Src (Martinez et al., 1987). We and others have shown that N-Srcs have a higher constitutive kinase activity and an altered SH3 domain substrate specificity compared with

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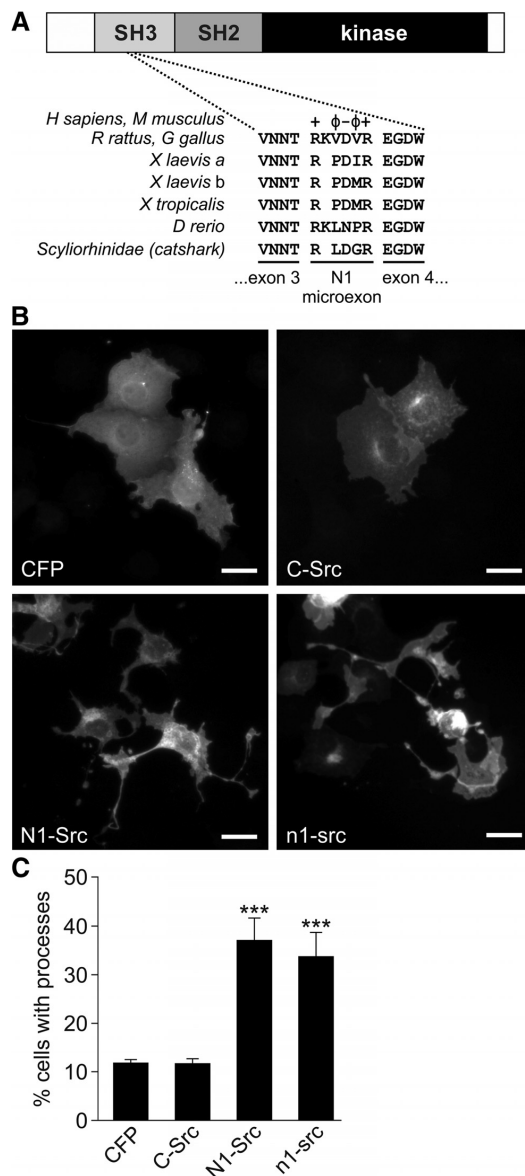


Figure 1. *Xenopus* n1-src elicits neurite-like processes in fibroblasts. **A**, Amino acid alignment of the N1-microexon in mammalian, *Xenopus*, and fish species. +, Basic; −, acidic; Φ, hydrophobic amino acid sidechains. **B**, Representative COS7 cells cotransfected for 4 d with Src-FLAG and CFP constructs. Cells were stained for Src (anti-FLAG) and CFP. **C**, Quantification of process outgrowth in COS7 cells. Each process was defined as an extension longer than one cell soma diameter and <2 μm in diameter. Data are plotted as mean ± SEM ($n = 3$ independent experiments, analyzed by Kruskal–Wallis 2-tailed ANOVA; *** $p < 0.001$; Scale bar, 10 μm).

C-Src (Dergai et al., 2010; Keenan et al., 2015). However, their *in vivo* substrates are unknown.

C-Src expression has been identified in a wide range of animal groups, including basal metazoans, such as sea sponges (Ottile et al., 1992), but its neuronal splicing to yield N1-Src only appears in the vertebrate lineage (Fig. 1A; Levy et al., 1987; Martinez et al.,

1987; Raulf et al., 1989) and N2-Src is restricted to mammals (Pyper and Bolen, 1990). Within the N1-Src microexon, there are minor differences in the length and sequence between vertebrate species. For example, a 6 aa N1-Src insert has been detected in brain tissue from the teleost fish *Xiphophorus* (Raulf et al., 1989), whereas the *c-src* locus of the diploid amphibian *Xenopus tropicalis* and the two pseudoallelic loci of allotetraploid *Xenopus laevis* contain 5 aa inserts (Collett and Steele, 1992). Identical 6 aa neuronal Src inserts are observed in N1-Src of chicks, rodents, and humans (Levy et al., 1987; Martinez et al., 1987). The appearance and conservation of a neural-restricted src isoform in the vertebrate lineage raises the intriguing possibility that n1-src function is related to the evolution and development of the complex vertebrate nervous system.

Previous studies in which N1-Src was overexpressed suggest N1-Src regulates neuronal morphology through cytoskeletal modifications affecting neurite outgrowth and axonogenesis (Worley et al., 1997; Kotani et al., 2007). However, no studies have thus far observed the development of the nervous system in the absence of N1-Src splicing. Here, we investigated n1-src function in the amphibian *Xenopus tropicalis*. We found that n1-src expression is localized to the dorsal ectoderm of the neural plate, which gives rise to the CNS during development. Using antisense morpholino oligos (AMOs), we have for the first time achieved specific inhibition of n1-src splicing in a vertebrate nervous system, without affecting *c-src* expression. The knockdown of n1-src caused abnormal touch responses in larval stage embryos, with a concomitant reduction in neuronal-specific tubulin (*tubb2a*)-positive neurons during primary neurogenesis. We propose that neuronal splicing of C-Src has evolved to be essential for vertebrate neurogenesis.

Materials and Methods

Subcloning of *Xenopus* n1-src. A plasmid encoding C-terminal FLAG-tagged *Xenopus* n1-src (pFLAG-Xn1-Src) was generated by amplifying the *Xenopus laevis* n1-src b variant open reading frame from an IMAGE clone (ID: 5572523) with the following PCR primers incorporating 5' BglII and 3' KpnI restriction sites [this codes for an n1 insertion identical to that of *Xenopus tropicalis* n1-src, as determined by examination of the *Xenopus tropicalis* genome and sequencing of relevant reverse-transcription (RT) PCR products]: forward, 5'-AGATCTCTCTAGAACCATGGGTGCCAC TAAAGCAAGCCA-3'; reverse, 5'-GGTACCGTAGATCCAAGGTG TTCCCAAGGCTGGTACTG-3'.

Digested product was ligated into pEGFP-N1 (Clontech) in which the GFP tag was replaced with a FLAG tag (pFLAG). The pCS2+ -Xn1-src-FLAG plasmid was generated by excising FLAG-tagged Xn1-src from pFLAG-Xn1-src with XbaI and ligating into XbaI-digested pCS2+. The preparation of pFLAG-C-Src and pFLAG-N1-Src was previously described (Keenan et al., 2015).

Fibroblast cell morphology assay. Ten thousand COS7 fibroblast cells were plated onto 13 mm coverslips. Twenty-four hours after plating, cells were transfected with 1 μg of plasmid DNA using Ecotransfect (Oz Biosciences) according to the manufacturer's instructions. Cells were fixed 48 h after transfection in 4% paraformaldehyde, 4% sucrose for 20 min, and then permeabilized in 0.1% Triton 1% BSA and stained with primary antibodies [mouse anti-FLAG (M2), 1:1000; rabbit anti-GFP, 1:500] in 1% BSA in PBS for 2 h at room temperature. After three washes in PBS, secondary antibodies (anti-mouse Alexa Fluor 564 and anti-rabbit Alexa Fluor 488; Invitrogen) were applied at 1:500 in 1% BSA in PBS for 1 h in the dark. Coverslips were mounted on slides using Mowial mountant (10% Mowial, 25% glycerol in 0.1 M Tris, pH 8.5) containing 1 μg/ml DAPI. Images were acquired using a 40× objective on a Nikon TE200 epifluorescence inverted microscope using a RoleraXR CCD (QImaging) camera controlled by SimplePCI Software (Hamamatsu). The percentage of COS7 cells bearing neurite-like processes was measured. Processes

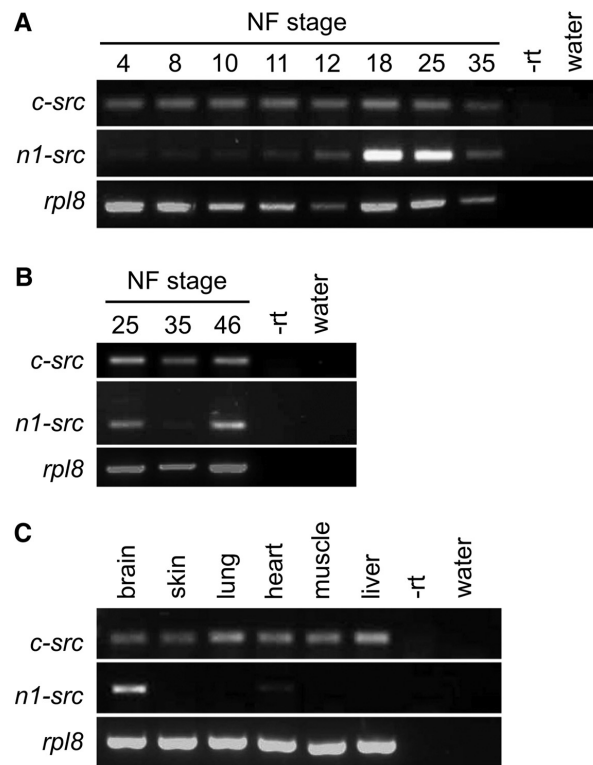


Figure 2. *n1-src* mRNA expression levels during *Xenopus tropicalis* development and in adult tissues. **A**, RT-PCR analysis of *c-src* and *n1-src* mRNA expression levels from early cleavage Stage 4 to tailbud Stage 35. *rpl8* is used as a ubiquitously expressed loading control. **B**, RT-PCR analysis of *c-src* and *n1-src* expression levels during (Stage 25) and after (Stage 35) primary neurogenesis, and during secondary neurogenesis (Stage 46). **C**, RT-PCR analysis of *c-src* and *n1-src* expression in a range of adult tissues.

were defined as being longer than the cell-soma diameter and having a width of $<2 \mu\text{m}$.

Embryological methods. All work involving animals was approved by the Biology Ethics Committee, University of York, and performed under the relevant UK Home Office legislation. *Xenopus tropicalis* embryos were produced as previously described (Khokha et al., 2005; Winterbottom et al., 2010). Embryos were microinjected at the two-cell or four-cell stage and cultured at 22°C in MRS/9 + 3% Ficoll, before transferring to MRS/20 for long-term culture. The sequences for the splice-blocking AMOs (GeneTools) are as follows: AMO a (splice acceptor), 5'-GTCA GGTCTCTATGGCACAGCATG-3'; AMO d (splice donor), 5'-GCC GCCGATGGTCACATACCTCAT-3'.

The touch response assay was conducted on Stage 28 or 41 embryos, in which the stationary animal was lightly touched on its side with the tip of a pair of forceps. Nonresponding embryos were scored as those that did not immediately swim out of the field of view in response to the touch stimulus. Videos of the locomotive phenotypes were acquired using a JVC TK-C1381 camera and processed with ArcSoft ShowBiz software.

RNA extraction and semiquantitative RT-PCR. Demembrated embryos or tissues dissected from male adult *Xenopus tropicalis* were flash-frozen on dry ice. Total RNA was extracted from tissues using Tri-Reagent and precipitated with 7.5 M LiCl/50 mM EDTA (Warrander et al., 2016). First-strand cDNA was synthesized from 1–3 μg of total RNA using random hexamer primers and Invitrogen SuperScript II Reverse Transcriptase according to the manufacturer's instructions (Warrander et al., 2016). Promega PCR Master Mix was used to amplify cDNA from the

embryos at the different developmental stages, with *rpl8* used as a loading control. Primers used to detect gene expression were as follows: *rpl8* forward, 5'-GGGCTGTC-GACTTCGCTGAA-3'; *rpl8* reverse, 5'-ATAC-GACCACCACAGCAAC-3'; *c-src* forward, 5'-ATCTCGCACCGAGACAGACT-3'; *c-src* reverse, 5'-CAGTCGCCTTCGGTTATT-3'; *n1-src* forward, 5'-ACTGTGACCTGACGCCTT-3'; *n1-src* reverse, 5'-CCTCATGTCAGG-TCTCGTGT-3'.

In situ hybridization and β -galactosidase staining. *Tubb2a* (*n-tubulin*) probe synthesis and *in situ* hybridization were carried as previously described (Winterbottom et al., 2010). A 19-mer digoxigenin end-labeled locked nucleic acid (LNA) probe was designed against the 15 base *n1-src* microexon sequence, with the addition of two bases from the flanking *c-src* exons. The probe with the following sequence was synthesized by Exiqon (Darnell et al., 2010). Locked nucleotides are indicated in bold: *n1-src* microexon probe 5'-TCCCTCATGTCAGGTCTCG-3'.

It was confirmed that no off-target sequence identities of >12 nt were present in the *Xenopus tropicalis* genome. The LNA *in situ* hybridization was undertaken as previously described (Sweetman, 2011; Warrander et al., 2016). Briefly, demembrated embryos were fixed in 0.1 M morpholinopropanesulfonic acid (MOPS), 2 mM EDTA, 1 mM MgSO_4 3.7% formaldehyde. The hybridization and washes were performed at 57°C. Embryos were hybridized for 36 h with 20 nM LNA probe preabsorbed against tailbud stage embryos. Color was developed with BM Purple (Roche) substrate until diffuse purple staining was visible, at which point embryos were washed for 12 h. The staining and washing cycle was then repeated until strong specific staining was present. For lineage tracing, β -galactosidase mRNA synthesis, embryo injection, and enzyme staining was undertaken as previously

described (Pownall et al., 1996). Both *in situ* and fixed phenotypes were imaged using a Leica MZ FLIII microscope (Leica), a SPOT 14.2 Color Mosaic camera, and SPOT Advanced software (Diagnostic Instruments).

Experimental design and statistical analysis. The fibroblast cell morphology assay was conducted in triplicate with ≥ 60 cells analyzed per condition for each independent replicate. The experimenter was blind to the plasmid transfected in each condition. Statistical analysis of the data was performed with SigmaPlot software using a Kruskal–Wallis ANOVA and Dunn *post hoc* pairwise comparisons. The *Xenopus* embryo touch response behavioral assay was conducted in triplicate with 20–65 animals analyzed per condition for each independent replicate. Statistical analysis of the percentage of nonresponding embryos was performed with a one-way ANOVA and Tukey's *post hoc* pairwise comparisons. For the *tubb2b* *in situ* hybridizations, 39–55 embryos derived from four fertilizations were unilaterally injected with control or *n1-src* AMOs. The proportion of embryos with abnormal *tubb2b* staining on the injected side compared with the contralateral internal control was statistically analyzed in R with a χ^2 test.

Results

The *Xenopus* *n1-src* splice variant promotes neurite outgrowth

We first investigated whether the activity of N1-src isoforms has been conserved during vertebrate evolution. There are minor differences in the length and sequence of the *n1-src* microexon

between mammals, amphibians, and fish. However, the distribution of charged and hydrophobic residues is conserved (Fig. 1A). Overexpression of mammalian N1-Src (but not C-Src) was previously shown to elicit morphological changes in *Xenopus* kidney epithelial cells (Worley et al., 1997) and we therefore performed a similar assay to compare the biological activity of *Xenopus* n1-src and mammalian N1-Src (Fig. 1B,C). COS7 fibroblasts were cotransfected with soluble CFP (to aid the visualization of cell morphology) and a C-terminal FLAG-tagged Src construct (C-Src, N1-Src, or n1-src) or a vector control. We and others have previously shown that C-terminal fusion tags do not affect Src activity in cells (Sandilands et al., 2004; Keenan et al., 2015). We assayed cell morphology by quantifying the percentage of cells bearing neurite-like processes. In agreement with previous findings, C-Src did not elicit process outgrowth compared with the vector control, while approximately one-third of N1-Src-transfected and n1-src-transfected cells bore processes (Fig. 1B,C), suggesting that, despite sequence differences their N1-Src insertions, activities of the amphibian and mammalian N1-Src enzymes have been highly conserved during evolution.

n1-src is expressed during neurogenesis

We next examined the temporal expression of *Xenopus* n1-src during development. Using splice variant-specific PCR primer sets, we undertook RT-PCR analysis of *c-src* and *n1-src* expression from cleavage to early larval stages (Fig. 2A). Expression of *c-src* is relatively constant throughout early development. In contrast, *n1-src* expression is highly regulated over the same period. Before the onset of transcription from the zygotic genome at blastula Stage 8, only very low levels of maternally deposited *n1-src* mRNA are detected. Zygotic *n1-src* expression begins to rise at gastrula Stage 11, reaching its highest level at neurula Stage 18, and this is maintained through early tailbud Stage 25. However, by early larval Stage 35, expression has fallen dramatically. Figure 2B indicates that *n1-src* expression increases again at Stage 46, correlating with secondary neurogenesis of motor neurons, interneurons, and sensory neurons in the closed neural tube (Schlosser et al., 2002). Therefore, *n1-src* expression is maximal during phases of neurogenesis in the nervous system.

We also examined the expression of *c-src* and *n1-src* in adult tissues and found that the highest level of *n1-src* expression is within the adult brain. Outside the brain, we detected no *n1-src* expression, except at low levels in heart muscle (Fig. 2C).

n1-src expression is enriched in the neural plate

To visualize the spatial expression pattern of n1-src in the developing embryo, we used a 19-mer LNA probe specific for the *n1-src* microexon sequence. Traditional antisense mRNA *in situ* probes are typically >150 bases in length and are unable to distinguish between the small sequence differences exhibited by the *c-src* and *n1-src* splice variants. Early-stage and late-stage neurula embryos were probed with a digoxigenin-labeled *n1-src*-specific LNA probe. In keeping with our RT analysis, we found that *n1-src* expression is highly enriched in the neural plate of neurula-stage embryos (Fig. 3). Our analysis indicates general expression of *n1-src* in cells of the neural plate at Stage 14 (Fig. 3A,B). Expression is fairly constant along the anteroposterior axis of the neural plate, with expression detected in cells of the presumptive forebrain, midbrain, and hindbrain regions, as well as the spinal cord (Fig. 3A,C,D). *n1-src* expression continues to be enriched in the neural plate as it narrows and rolls up to form the neural tube in late Stage 19 neurula embryos (Fig. 3E,F).

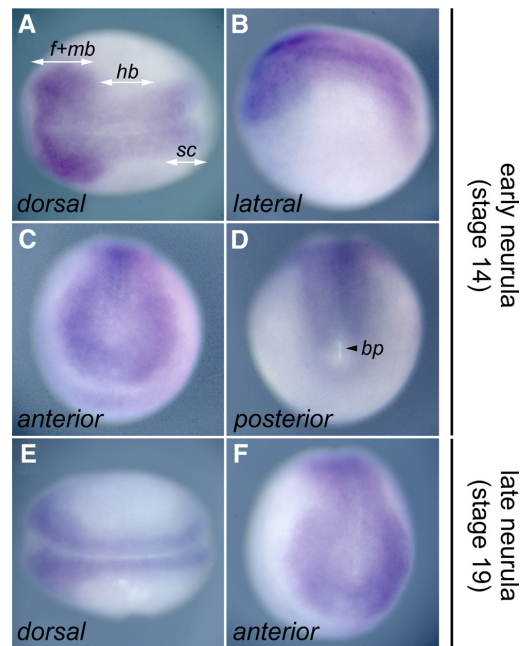


Figure 3. Expression pattern of *n1-src* during *Xenopus tropicalis* primary neurogenesis. *In situ* hybridization analysis of *n1-src* mRNA expression using a 19-mer digoxigenin end-labeled antisense probe directed against *n1-src*-specific sequence. **A–D**, Early neurula Stage 14 embryos. **E, F**, Late neurula Stage 19 embryos. **A**, Dorsal view, anterior to the left. **B**, Lateral view, anterior to the left. **C**, Anterior view, dorsal to the top. **D**, Posterior view, dorsal to the top. **E**, Dorsal view, anterior to the left. **F**, Anterior view, dorsal to the top; *f+mb*, presumptive forebrain and midbrain; *sc*, presumptive spinal cord; *bp*, blastopore.

Morpholino-mediated knockdown of *Xenopus* n1-src disrupts the touch response of embryos

MOs, which are nucleic acid analogs with a modified backbone chemistry, are able to hybridize to target RNA in a highly specific, sequence-dependent fashion. AMOs are able to knock down gene function in a number of systems (Eisen and Smith, 2008). Typical knockdown strategies use AMOs to block translation or nuclear pre-mRNA processing. AMOs targeted to splice acceptor and donor sites in pre-mRNAs have been used to block normal splicing events, leading to the formation of aberrant mRNAs containing intron sequences, thus disrupting the protein coding information normally found in the mature mRNA. AMOs have also been successfully used to induce exon skipping (Goyenvalle et al., 2010; Kang et al., 2011). Here we use this approach to block splicing involving the *n1-src*-specific microexon. Nonoverlapping AMOs targeted to the splice acceptor (AMO a) and donor (AMO d) sites of the *n1-src* microexon were designed (Fig. 4A). *n1-src* AMOs were delivered to the cells of the embryo by microinjection. In contrast to uninjected control embryos, we detected no *n1-src* expression in embryos injected with a combination of AMO a and AMO d. Furthermore, injection of AMO a or d alone also effectively blocked *n1-src* expression (Fig. 4B). Consistent with an effect on exon skipping, the expression of *c-src* was unaffected by the AMOs. We conclude that AMOs represent highly specific tools for investigating the function of the *Xenopus* n1-src isoform in early development.

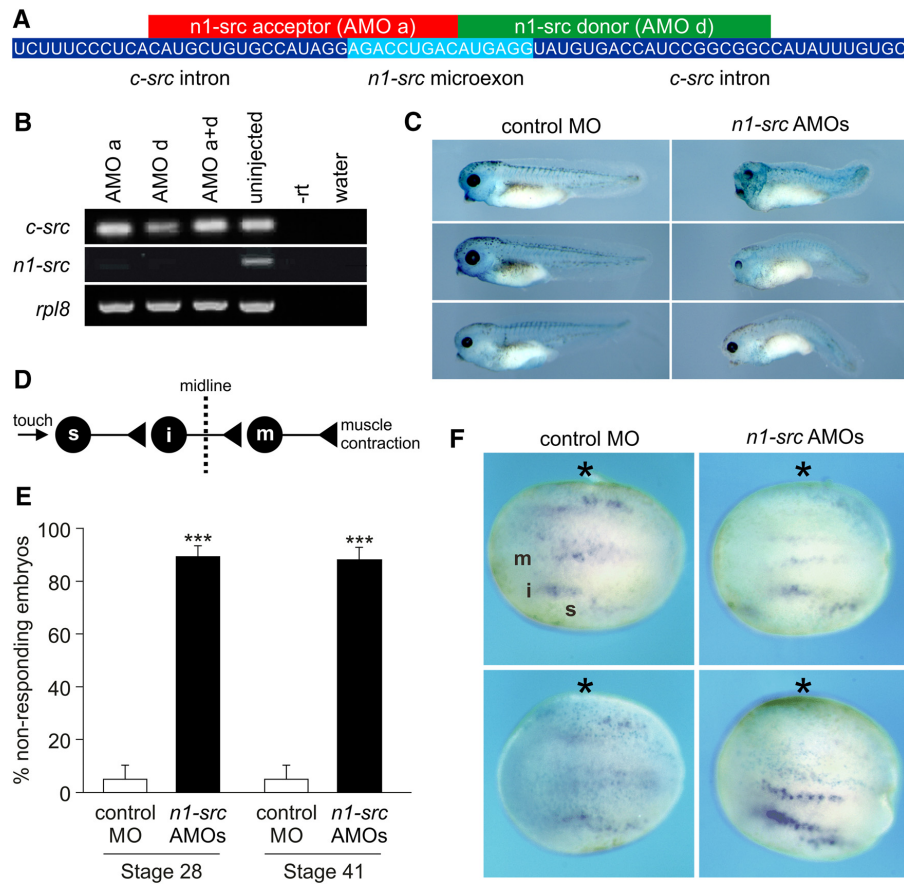


Figure 4. Abnormal touch response and primary neurogenesis in *n1-src*-knockdown embryos. **A**, Diagram showing the sequences and corresponding RNA target sequences of the splice acceptor (AMO a) and donor (AMO d) splice-blocking AMOs. **B**, RT-PCR analysis of *c-src* and *n1-src* mRNA expression at Stage 16 in control uninjected embryos and embryos injected with a total of 20 ng AMO a, AMO d, or AMO a + d. *rpl8* is used as a ubiquitously expressed loading control. –rt, No reverse transcriptase control; water, no template control. **C**, Representative phenotypes of embryos at larval Stage 41 bilaterally injected at the two-cell or four-cell stage with 10 ng total of a standard control MO or the AMO a + d combination. Embryos were coinjected with 100 pg of nuclear β -galactosidase and subsequently stained with X-gal (light blue) to demonstrate successful injection targeting. **D**, Diagram of embryo touch reflex. Touching the skin stimulates Rohon–Beard sensory neurons (s), which synapse onto commissural interneurons (i) that activate contralateral motor neurons (m), leading to muscle contraction. **E**, Quantitation of touch-response phenotype of the same embryos at larval Stage 28 and 41 bilaterally injected at the two-cell or four-cell stage with 10 ng total of a standard control MO or the AMO a + d combination. Data are plotted as mean $\pm$ SEM ($n = 3$ independent experiments, analyzed by 1-way ANOVA with Tukey's *post hoc* test, *** $p < 0.001$, control MO vs *n1-Src* AMO). **F**, *In situ* hybridization analysis of *tubb2b* expression in differentiating primary neurons of open neural plate Stage 14 embryos unilaterally injected with 5 ng total of a standard control MO or the AMO a + d combination. The injected side shows faint blue nuclear staining with the β -galactosidase lineage tracer, and is indicated with a black asterisk. Anterior is to the left. m, Motor neurons; i, interneurons; s, sensory neurons.

Injection of a standard control MO has little effect on the phenotype of larval Stage 41 embryos, whereas injection of the AMO a + d mixture leads to a mild, but highly penetrant phenotype, which is characterized by shortening and/or kinking of the tail, and variable disruption to the pigmented retina of the eye (Fig. 4C). To assess the function of the primary nervous system in *n1-src*-ablated embryos, we applied a touch stimulus to the side of larval-stage embryos, which normally elicits a dart response. The neuronal circuitry for the touch reflex (Fig. 4D; Movies 1, 3) is well characterized and comprises Rohon–Beard sensory neurons, which activate commissural interneurons that in turn synapse onto contralateral motor neurons to stimulate muscle contraction, propelling the embryo away from the stimulus (Boothby and Roberts, 1995; Li et al., 2003; Roberts et al., 2010; Fig. 4D;

Movies 1, 3). We tested the same embryos at developmental Stages 28 and 41 (before and during the onset of myelination) and the dart response was commonly abnormal or absent in *n1-src* AMO a + d-injected embryos compared with controls (Fig. 4E; one-way ANOVA, $F = 96.3$, $df = 3$, $p = 1.28 \times 10^{-6}$). These embryos instead frequently displayed an uncoordinated twitch or spasm response, indicating abnormal development of the neural circuitry necessary for the dart response (Fig. 4D; Movies 2, 4).

n1-src knockdown disrupts primary neurogenesis

To ascertain which neurons in the touch reflex are affected by *n1-src* knockdown, we next investigated the early development of the primary nervous system. During primary neurogenesis, the motor neurons, interneurons, and Rohon–Beard sensory neu-



Movie 1. Normal touch response in Stage 28 *Xenopus tropicalis* embryos. Real-time video showing the normal touch response of Stage 28 embryos injected with 10 ng of a standard control MO. Embryos right themselves and swiftly swam a short distance from the point of contact.



Movie 2. Abnormal touch response in Stage 28 *Xenopus tropicalis* embryos. Real-time video of the abnormal touch response in Stage 28 embryos injected with a total of 10 ng *n1-src* AMO a + d. Nonresponding phenotypes remain horizontal and moved slowly from the point of contact with uncoordinated twitching movements.



rons differentiate to form medial, intermediate, and lateral columns respectively on either side of the neural plate midline. These columns of differentiating neurons are separated by non-differentiating, proliferative progenitors and can be identified by expression of the neuronal-specific *tubb2b* gene (Chitnis et al., 1995). Figure 4F shows differentiating primary neurons visualized by *in situ* hybridization for *tubb2b* mRNA. Unilateral injection of AMO a + d resulted in a statistically significant reduction ($\chi^2 = 44.922$, $df = 1$, $p = 2.05 \times 10^{-11}$) of *tubb2b* expression in all three columns relative to the uninjected contralateral side (50 of 55; 90.9% abnormal) compared with embryos unilaterally injected with a control MO (8 of 39; 20.5% abnormal). Thus, we concluded that rather than regulating the development of specific subsets of neurons, *n1-src* is required for neurogenesis of all *tubb2b*-positive neurons in *Xenopus* primary nervous system development.

Discussion

The activity of amphibian and mammalian n1-src is conserved

The alternative splicing of neuronal src isoforms alters the ligand-binding specificity of the C-Src SH3 domain and the catalytic activity of its kinase domain (Brugge et al., 1985; Keenan et al., 2015). These differences are believed to underpin the reported differential activity of neuronal Srcs. The position of the N1-specific insertion into the SH3 domain of C-Src is conserved between amphibians and amniotes, and we investigated whether

the differential biological activity of n1-src isoforms has been conserved in amphibians. We find that, unlike C-Src, both *Xenopus* n1-src and mammalian N1-Src, despite different SH3 inserts (5 vs 6 aa in amphibians and mammals respectively), are able to induce neurite-like processes when transfected into COS-7 cells. In keeping with this, it has been shown previously that N1-Src overexpression in *Xenopus* A6 epithelial cells induced neurite-like processes, in contrast to the rounded phenotype of C-Src-transfected cells (Worley et al., 1997).

n1-src expression correlates with primary neurogenesis

A previous study indicated that the expression of the *n1-src* isoforms of the tetraploid amphibian *Xenopus laevis* are initiated by midneurula Stage 15 (Collett and Steele, 1992). We find that in the diploid amphibian *Xenopus tropicalis*, there is low-level maternal *n1-src* expression from the start of development, and, in contrast to the previous study, activation of zygotic *n1-src* expression is initiated as early as middle to late gastrula stages, and by early neurula stages expression is restricted to the open neural plate.

The period from late gastrula to early neurula is a key phase in the development of the primary nervous system, a simple functional nervous system, characteristic of anamniotic aquatic vertebrates, including fish and amphibians (Hartenstein, 1989). Differentiation of primary neurons enables the early development of motility, and helps embryos avoid predation in an aquatic environment. Primary neurons begin to differentiate at open neural plate



Movie 3. Normal touch response in Stage 41 *Xenopus tropicalis* embryos. Real-time video showing the normal touch response of Stage 41 embryos injected with 10 ng of a standard control MO. Embryos right themselves and swiftly swam a short distance from the point of contact.



Movie 4. Abnormal touch response in Stage 41 *Xenopus tropicalis* embryos. Real-time video of the abnormal touch response in Stage 41 embryos injected with a total of 10 ng *n1-src* AMO a + d. Nonresponding phenotypes remain horizontal and moved slowly from the point of contact with uncoordinated twitching movements.



stages in *Xenopus* embryos and primary neurogenesis continues through neurula and early tailbud stages (Schlosser et al., 2002). In keeping with our findings that *n1-src* expression is initiated during the gastrula stage, a subsequent study by Collett and Steele (1992) showed that *n1-src* expression is rapidly activated in competent gastrula dorsal ectoderm by endogenous neural-inducing signals and by the neural-inducing activity of the phorbol ester TPA in the absence of protein synthesis.

Abnormal neural development in *n1-src* knockdown embryos

We present the first analysis of the consequences of blocking the splicing events required for the expression of *n1-src* during vertebrate development. An advantage of our approach is that the morpholinos ablated *n1-src* expression while *c-src* expression was unaffected. *N1-src* knockdown caused striking behavioral, morphological, and neuronal phenotypes in the *Xenopus* embryo. At the larval stage, we found that AMO-injected embryos exhibited a severely abnormal locomotor response to touch stimuli, with many observed to twitch or spasm following the touch. The *Xenopus* touch reflex and subsequent swimming circuits involve the coordination of sensory neurons, interneurons, and motor neurons (Roberts et al., 2010). In Stage 14 embryos, and in keeping with the widespread expression of *n1-src* in the neural plate, we found that the columns of differentiating *tubb2a*-positive neurons that subsequently form the touch and swimming circuits are reduced or absent. Due to the labile nature of the morpholinos,

we predict that *n1-src* expression will slowly return, leading to a delay and perturbation in primary neurogenesis that generates the aberrant circuits observed in the larval embryo. The reduced touch response could also result from a defect in myelination, a process that begins at approximately Stage 42 (Yoshida, 1997). Furthermore, oligodendrocytes arise from the same precursors as motor neurons (Park et al., 2002). However, our observation that both Stage 28 and Stage 41 embryos exhibit the same defects rules out myelination as the sole cause of the phenotype.

Morphologically, *n1-src*-knockdown embryos exhibited a loss of retinal pigmentation and a kinked tail. The optic stalk, retina, and retinal pigmented epithelium develop from an outpocketing of the diencephalon (Fuhrmann et al., 2014). Therefore, the loss of eye pigmentation in *n1-src*-knockdown embryos may indicate a common role for *n1-src* in regulating the differentiation of cells derived from the neuroepithelium. The morphogenesis of the vertebrate main body axis involves coordinated cell movements in the axial mesoderm and the neuroepithelium (Nikolopoulou et al., 2017). Posterior axial defects have been observed in embryos in which normal convergent extension within the neuroepithelium has been inhibited through interference with components of the planar cell polarity signaling pathway (Goto and Keller, 2002). We speculate that the kinking of the posterior axis observed in *n1-src*-knockdown embryos arises through deregulation of the process of convergent extension, which drives elon-

gation and narrowing of the neuroepithelium and reflects abnormal signaling within the neuroepithelium in the absence of n1-src activity.

At present we are unable to say with any confidence where n1-src functions in the pathway leading to neuronal differentiation. However, n1-src's general neural expression is similar to that of a group of neural stabilization genes, including members of the Sox, Zic, and Iroquois families (for review, see Moody and Je, 2002). These code for transcription factors and, as is the case with n1-src, several of these genes are expressed in response to neural induction. Neural stabilization genes have multiple overlapping functions, providing a link between the signals that induce the neural plate and the hierarchy of proneural and neurogenic genes required for neuronal specification and differentiation. Thus, early expressed neural stabilization genes have roles in regulating the competence of ectodermal cells to respond to neural-inducing signals, and later expressed ones regulate the progression from neuronal progenitor to differentiated neuron (Moody and Je, 2002). Current evidence is suggestive of a role for n1-src in the process of neural stabilization. However, future studies will be required to investigate the regulatory interactions between the n1-src kinase and known components of the vertebrate neurogenic pathway.

N1-Src function and neurogenesis in higher vertebrates

Our data show that following primary neurogenesis, n1-src expression falls, but is again elevated during a second phase of neurogenesis in late larval stages. A primary nervous system is absent in amniotes, and it is the later phase of secondary neurogenesis in the closed neural tube that is more akin to the neurogenesis of amniotes, including mice and humans (Wullimann et al., 2005). A connection between neuronal differentiation and N1-Src function in amniotes is supported by an analysis of N1-Src (also termed pp60⁺) activity in the developing mouse brain, which showed a rapid increase in N1-Src activity at embryonic day (E) 12 and which peaks at E18, when increasing numbers of neuroblasts are exiting the cell cycle and differentiating (Wiestler and Walter, 1988). Furthermore, cultured neurons of the rat striatum contain little detectable N1-Src activity. However, neuronal differentiation induced by serum starvation leads to a sevenfold increase in N1-Src activity relative to C-Src (Cartwright et al., 1987). Similarly, embryonic carcinoma cells treated with retinoic acid to induce neuronal differentiation express increased levels of N1-Src (Lynch et al., 1986), and there is an increase in both N1-Src and N2-Src expression during differentiation of the neuroblastoma cell line LAN-5 (Matsunaga et al., 1993).

We present evidence for an early role for n1-src in neural development regulating the transition from neural progenitors to differentiated neurons. However, there is also evidence that N1-Src has roles regulating the cellular architecture and morphogenesis of neurons. Transgenic mice expressing N1-Src in Purkinje neurons of the cerebellum display defects in migration and dendrite morphology, which might be linked to defects in microtubule structure (Kotani et al., 2007). Conversely, in *Xenopus laevis*, overexpression of mammalian N1-Src in the optic tectum enhanced axonogenesis of retinal progenitors. Thus, n1-src is likely to have multiple roles in neural development regulating neuronal specification and morphogenesis.

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List of Abbreviations

AA	Alternative Acceptor
AD	Alternative Donor
ADP	Adenosine Di-phosphate
AKT	Protein Kinase B
ALS	Amyotrophic Lateral Sclerosis
AMO	Antisense Morpholino Oligo
AMO A+D	Antisense Morpholino Oligo Acceptor + Donor
AMPA	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid
AP	Alkaline Phosphatase
ATP	Adenosine Tri-phosphate
AVNA	Anteroventral Noradrenergic
BDNF	Brain Derived Neurotrophic Factor
BMP	Bone Morphogenic Protein
CMO	Control Morpholino Oligo
CNS	Central Nervous System
Crmp1	Collapsin Response Mediator Protein-1
CSK	C-terminal Src Kinase
DCC	Deleted in Colorectal Cancer
L1-CAM	L1 Cell Adhesion Molecule
DMEM	Dulbecco's Modified Eagle Medium
Dn-FGFR1	Dominant Negative Fibroblast Growth Factor Receptor 1
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular Signal Regulated Kinase
ES	Exon Skipping
ESE	Exon Splicing Enhancer
ESS	Exon Splicing Silencer
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FRS2	Fibroblast Growth Factor Receptor Substrate 2

FXTAS	Fragile X-Associated Tremor/Ataxia Syndrome
GR	Glucocorticoid Receptor
Grb2	Growth Factor Receptor-Bound Protein 2
GSEA	Gene Set Enrichment Analysis
AME	Analysis of Motif Enrichment
GTP	Guanosine Tri-phosphate
HCG	Human Chorionic Gonadotropin
hnRNPA1	Heterogeneous Nuclear Ribonucleoprotein A1
IR	Intron Retention
KSRP	KH-type Splicing Regulatory Protein
MAB	Maleic Acid Buffer
MAPK	Mitogen Activated Protein Kinase
MEME	Multiple Em for Motif Elicitation
MRS/2	1/2X Modified Ringers solution
MRS/20	1/20X Modified Ringers solution
MRS/9	1/9X Modified Ringers solution
MYCN	N-Myc Proto-oncogene Protein
Myt1	Myelin Transcription Factor 1
NAM/10	1/10X Normal Amphibian Medium
NAM/3	1/3X Normal Amphibian Medium
NF Stage	Nieuwkoop and Faber Stage
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
NMR	Nuclear Magnetic Resonance
NONO	Non-POU Domain-Containing Octamer-Binding Protein
nPTB	Neural Homologue of Polypyrimidine Tract Binding Protein
Nrp1	Neuropilin-1
Nrp2	Neuropilin-2
nRTK	non Receptor Tyrosine Kinase
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline + Tween
PI3K	Phosphoinositide 3-Kinase
PNS	Peripheral Nervous System
PTB	Polypyrimidine Tract Binding Protein
RA	Retinoic Acid
Raf1	RAF Proto- Oncogene Serine/Threonine-Protein Kinase
RBM45	RNA-Binding Motif Protein 45

RSV	Rous Sarcoma Virus
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
SDS PAGE	Sodium Dodecyl Sulfate Polyagarose Gel Electrophoresis
Sema3a	Semaphorin-3A
Sema3F	Semaphorin-3F
Sema6a	Semaphorin-6A
Sema6b	Semaphorin-6B
SF1	Splicing Factor 1
SF2/ASF	pre-mRNA-splicing factor SF2/Alternative Splicing Factor 1
SFK	Src Family Kinase
SFPQ	Splicing Factor, Proline And Glutamine Rich
SH1	Src Homology 1
SH2	Src Homology 2
SH3	Src Homology 3
SH4	Src Homology 4
snRNP	Small Nuclear Ribonucleoproteins
SOP	Sensory Organ Precursor
SOS	Son of Sevenless
SRSF1	Serine/Arginine-Rich Splicing factor 1
SSC	Saline-Sodium Citrate
STAT3	Signal Transducer and Activator of Transcription 3
TAE	Tris-Acetic acid-EDTA
TBE	Tris-Borate-EDTA
TDP-43	TAR DNA-Binding Protein 43
TGF- β	Transforming Growth Factor Beta
TPA	12-O-Tetradecanoylphorbol-13-Acetate
TPM	Transcripts Per Million
Tra2a	Transformer-2 Protein Homolog Alpha
U2AF	U2 Auxiliary Factor
ULBR	Unique Lipid Binding Region
Vegf	Vascular Endothelial Growth Factors

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