

Investigating the mechanisms  
directing oligodendrocyte precursor  
cell (OPC) development in the  
zebrafish hindbrain

Thesis submitted to the University of Sheffield  
for the degree of Doctor of Philosophy

by

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October 2014



# Acknowledgements

I would firstly like to thank my supervisors for the opportunity to work on this project and for their support these last four years. Thanks to Dr Jon Wood for his encouragement when the project wasn't going well and for his guidance during writing this thesis. Thanks to Prof Sudipto Roy for allowing me to join his group at the IMCB in Singapore, for teaching me about the realities of science and for the freedom to explore the project. Thanks to Dr Vincent Cunliffe for his advisory support during my project. I would like to show my gratitude to the fantastic members of the SR group for making settling into a new group and country very easy, for helping me to learn techniques and for being patient with me. A particular thanks to Dr Semil Choksi, not only for his friendship but for his endless science wisdom. He helped me so much during my time in SR lab and I cannot thank him enough.

To my wonderful friends in Singapore, we were more like a family and I'm sure we will remain friends for a long time. To the lovely Harriet, you made my first year in Singapore so much fun. Thanks for looking after me, I couldn't have done it without you! Charlie, thank you for the much needed coffee breaks and for being there during my last few weeks to help. To Pervin, Dave, Mark, AD and Ajay you guys were fantastic fun to be around. I will forever think fondly of my time there.

To the BMS babes in Sheffield, thanks for girls nights and the laughs. To Aimee, my beautiful friend, thank you for the brilliant times we had when we lived together. For the emergency G'n'T sessions and your words of encouragement during those final few months and for the email updates whilst I was in Singapore.

Thank you to Tarang, for our trips across South East Asia and for your love and reassurance. You put up with my moaning, irrational panics and always managed to make me feel better.

To my family who I cherish and couldn't imagine doing this without them. To my sister, for her friendship. To my beloved spaniels for their slobber therapy sessions. Finally to my Mum and Dad, two of the kindest people with the most wonderful hearts. Their unconditional love and support helped me to have faith in myself especially during my PhD.

# Statement of Contribution

I state that all the work presented in this thesis is my own. The *in vivo* drug screen (Chapter 6: A preliminary *in vivo* drug screen to identify compounds modulating OPC development) was performed with the Screening laboratory in the Department of Biomedical Science, University of Sheffield. Training of the drug screening protocol was provided by Dr Sarah Baxendale and Miss Celia (CJ) Holdsworth assisted with drug preparation, embryo preparation and automated *in situ* hybridisation.

# Abstract

Oligodendrocytes are the myelinating cells of the central nervous system (CNS) and are implicated in the pathobiology of many CNS diseases including multiple sclerosis and schizophrenia. Oligodendrocyte precursor cells (OPCs) are capable of migrating long distances, but the mechanisms governing the migration of OPCs to their axonal destinations are not fully understood. We sought to understand the extrinsic and intrinsic factors that contribute to the migration of a subset of OPCs in the zebrafish hindbrain. We utilized the zebrafish transgenic *tg(olig2:gfp)* line to mark OPCs and characterise their normal migration patterns. These cells normally first migrate in a ventral direction, after which they migrate away from the midline dorsolaterally to populate the hindbrain. However, how these cells initiate migration in a ventral direction is not known. We have found that removing Hedgehog signalling at the onset of their migratory phase results in a failure of OPCs to migrate in their normal ventral fashion, while increasing Hedgehog signalling results in hypermigration of these cells. This suggests that Hedgehog might not just be important for OPC fate specification, via induction of transcription factors such as *olig2*, but may also be actively involved in the development of these cells post-specification. We propose that Sonic Hedgehog acts as a chemoattractant to drive the initial ventral migration of hindbrain OPCs and furthermore provide evidence that it may function at least in part by inducing *disc1*, a schizophrenia risk factor gene, which has known roles in neural crest and neuronal migration. With several studies supporting a role of *DISC1* in neuronal migration it has been hypothesised that *DISC1* could also play a role in the process of myelination. White matter abnormalities are consistently reported in schizophrenia patients, and it is hypothesised that OPC migratory defects may cause these documented white matter abnormalities due to early genetic dysfunction. This thesis has provided evidence for a link between a well characterised schizophrenia risk factor gene, early OPC migration events and a fundamental signalling pathway.

# List of abbreviations

**AMP** Ampicillin

**bHLH** basic helix loop helix

**BMP** Bone morphogenic protein

**CNC** Cranial Neural Crest

**CNS** central nervous system

**DISC1** Disrupted in Schizophrenia-1

**DPF** Days post fertilisation

**DSB** Double strand break

**DTI** diffusor tensor imaging

**ENU** *N*-ethyl-*N*-nitrosourea

**EtOH** Ethanol

**FGF** Fibroblast growth factor

**FEZ1** Fasciculation And Elongation Protein Zeta 1

**GFP** Green fluorescent protein

**GWAS** Genome wide association studies

**HPF** hours post fertilisation

**HR** Homologous recombination

**INDELS** Insertions and deletions

**KAN** Kanamycin

**LIS1** Lissencephaly-1 Protein

**MO** Morpholino

**MS** Multiple sclerosis

**MRI** magnetic resonance imaging

**MeOH** Methanol

**MBP** Myelin basic protein

**NDEL1** NudE Neurodevelopment protein 1 like 1

**NRG1** Neuregulin-1

**NHEJ** Non-homologous end joining

**OLIG2** Oligodendrocyte lineage transcription factor

**OPC** Oligodendrocyte precursor cell

**OL** Oligodendrocyte

**PCR** Polymerase chain reaction

**PNS** Peripheral Nervous System

**pMN** motor neuron

**RT** room temperature

**SHH** Sonic Hedgehog

**SZ** schizophrenia

**SEM** standard error of the mean

**TALEN** transcription activator like endonucleases

**UTR** Untranslated region

**Y2H** Yeast two hybrid

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# Chapter 1 Introduction

## 1.1 Oligodendrocytes

### 1.1.1 Function in the adult brain

Oligodendrocytes are non-neuronal cells of the central nervous system (CNS); they are a member of the CNS glial family which also contains astrocytes, microglia and NG2 positive cells (Barres 2008, Nishiyama et al 2009) . The main function of mature oligodendrocytes is to myelinate the axons of neurons which facilitates saltatory nerve conduction, allowing for the rapid transduction of action potentials. Differentiated oligodendrocytes create a vast myelin sheath which wraps tightly around axons providing insulation, with a single oligodendrocyte having the capacity to myelinate several axons (Bunge 1968). There is also evidence which suggests that oligodendrocytes have additional roles to myelination, including providing trophic support and supplying energy to axons (Funfschilling et al 2012). Diseases of demyelination and subsequent white matter abnormalities can have devastating consequences on brain function. It is therefore critical to understand the molecular mechanisms underlying the development of myelinating oligodendrocytes from oligodendrocyte precursor cells (OPCs) in order to create successful intervention, treatment and recovery from white matter diseases.

### 1.1.2 White matter abnormalities in brain disorders

#### 1.1.2.1 Multiple Sclerosis

Multiple sclerosis (MS) is a classic disease of demyelination, where severity varies between patients. It is an auto-immune disease of the central nervous system and involves stages of demyelination and then remyelination followed by recovery (Adams et al 1989, Prineas & Graham 1981). Although remyelination is able to occur in the early stages of the disease it is often incomplete. In later stages where chronic demyelination lesions are present, remyelination fails leading to irreparable damage to the brain (Chang et al 2002). Although several studies have shown that oligodendrocyte precursor cells can enter areas of demyelination and axonal

contact can occur, remyelination does not subsequently occur (Franklin & Ffrench-Constant 2008). The molecular mechanisms underlying this deficiency in remyelination is not well understood.

#### 1.1.2.2 Schizophrenia

Although Multiple Sclerosis is probably the most studied white matter disease of the central nervous system, in recent years there has been increasing evidence showing white matter abnormalities in patients with psychiatric disorders, for example in schizophrenia (SZ) (Takahashi et al 2011). The majority of myelination occurs after birth into early adulthood and it is at this stage that some psychiatric disorders are diagnosed, in particular schizophrenia. In patients with demyelinating diseases such as MS where there are lesions in the frontal lobe, schizophrenia-like symptoms also present in some individuals (Davis et al 2003). Brain imaging techniques such as diffusor tensor imaging (DTI) have shown evidence of white matter integrity deficiencies in several brain regions including those dysfunctional in psychiatric disorders (Liddle 1996) (Foong et al 2000). Abnormalities in white matter of schizophrenia patients have also been described using Magnetic resonance imaging (MRI) (Flynn et al 2003). Studies analysing post-mortem brains of schizophrenia sufferers compared to healthy controls found differences in oligodendrocytes number and spacing suggesting that there is aberrant oligodendrocyte development in these patients (Hof et al 2003). Other evidence for white matter abnormalities includes expression differences in oligodendrocyte specific genes in schizophrenia patients. Genes associated with oligodendrocyte precursor cells, oligodendrocytes and myelin were seen to be reduced in schizophrenia patients, with these findings being replicated by several groups (Table 1) (Hakak et al 2001, Tkachev et al 2003). Another group analysed oligodendrocyte gene expression in sub-cortical regions and found that genes expressed in terminally differentiated oligodendrocytes were expressed at lower levels in sufferers compared to healthy controls (Barley et al 2009).

Gene	Expression/Function	Reference
2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP)	Has roles in process outgrowth	(Flynn et al 2003) (Hakak et al 2001)
Myelin association glycoprotein (MAG)	Myelination process	(Hakak et al 2001, Katsel et al 2005)
Myelin basic protein (MBP)	Major component of the myelin sheath	(Tkachev et al 2003)
Transferrin (TF)	Transportation of iron ions, important for oligodendrocytes during myelination	(Katsel et al 2005, Tkachev et al 2003) (Hakak et al 2001)
Claudin 11 (CLDN11)	Major component of myelin	(Tkachev et al 2003) (Katsel et al 2005)

**Table 1: Oligodendrocyte genes showing lower expression in schizophrenia patients**

These observations from patient tissue were interesting since most psychiatric research has been predominantly focused on the neuronal aspect of the disease in the past. These insights into potential defects in oligodendrocyte development or maintenance have provided the research field with the opportunity to study how non-neuronal cell types contribute to these debilitating and complex brain disorders. However it must be appreciated that although these myelin related defects have been described in human patients it is still unknown whether these defects could be a secondary result of early neuronal defects, and should be a focus of future research as non-invasive imaging techniques improve. Tackling all aspects of disease progression is critical in the development of new treatments given the present clinical setting where many of the current treatments are inconsistent in their efficacy and unsuccessful for many sufferers (Insel 2010). Proper brain connectivity requires neuronal-glia interactions, and this further highlights the requirement for understanding their individual development but also in how neuron-glia interactions are established and how defects in either of these processes may contribute to disease (Maldonado & Angulo 2014). Although there is a huge body of evidence describing white matter and oligodendrocyte defects in

psychiatric disorders, there remains little mechanistic insight into when, where and how these white matter abnormalities arise; however some evidence which will be discussed below points to a dysfunction in OPC development in the early brain (Davis et al 2003).

## 1.2 Schizophrenia

### 1.2.1 The symptoms of schizophrenia

Schizophrenia currently affects 1% of the global population and is characterised by both positive (hallucinations) and negative (social withdrawal) symptoms. There is also a large degree of cognitive dysfunction which includes working memory and attention deficits. It is a debilitating, complex and costly disease. Schizophrenia is normally diagnosed during late adolescence. Current treatment success varies between patients with some sufferers failing to respond to any available treatment. Understanding the disease pathology and how it progresses could lead to improved treatments and ideally intervention methods which would halt disease progression or reduce the chance of it developing further (Insel 2010).

### 1.2.2 High heritability of Schizophrenia

Psychiatric disorders such as schizophrenia are complicated diseases to study and model. Varying symptoms and severity between patients, differences in environmental factors and human specific behaviours create several limitations when trying to model mental illness in animals and understand the underlying mechanisms. However the discovery of a large genetic component in schizophrenia has opened up opportunities to study the disease through identification of genetic risk factors and pathways. The high heritability of the disease was recognized through family and twin studies (Sullivan et al 2003). A large study in Denmark looked at the incidence of schizophrenia in both the biological relatives and adoptive relatives of adoptees that suffered with schizophrenia (Kety 1987). Chronic schizophrenia was found to have significantly higher incidence in the biological relatives of those adoptees with schizophrenia compared to the control adoptive relatives. Another interesting study investigated the prevalence of the disease in monozygotic compared to dizygotic twins and found that monozygotic

twins had a higher concordance. However this was never 100% which showed that this disease has environmental factors to take into consideration and that risk factor genes are not completely causal (Cardno et al 1999). These results were exciting, leading to a wide effort to decipher these risk factor genes, understand their function, and how they could contribute to brain disorders when compromised. Since the discovery of the first candidate risk factors genes, the field has since identified numerous risk factor genes for schizophrenia and other brain disorders in a variety of populations worldwide. Recent Genome Wide Association Studies (GWAS) have identified over a 100 credible risk loci, many of which were not previously reported and therefore the field of schizophrenia genetics is constantly evolving with new genes, biological pathways and interactions being implicated in mental illness through these large case studies, as genome techniques become more sophisticated (Schizophrenia Working Group of the Psychiatric Genomics 2014). Understanding the function of these genes in the healthy brain, and how they work in gene networks will help us to build knowledge around the pathophysiology of the disease.

### 1.2.3 The neurodevelopmental hypothesis of schizophrenia

The underlying mechanisms of the disease are still largely unknown, however there have been several causal hypotheses suggested. One particular hypothesis however is now widely accepted and attempts to bring together genetic risk factors and dysfunction in early brain development. The neurodevelopmental hypothesis was proposed after studies suggested that schizophrenia does not arise from a healthy brain i.e. abnormalities are present early on before diagnosis. A study which followed a cohort of individuals over 45 years found that those who later developed schizophrenia were delayed at several developmental milestones (Sorensen et al 2010). Another observation is that schizophrenia is frequently diagnosed between 18-25 years of age during the final stages of brain maturation. One particular area, the prefrontal cortex is the last to mature and is a region long implicated in mental illness (Paus et al 2008). Analysis of post-mortem brains from SZ patients revealed interesting findings such as reductions in neuronal numbers, mislocalisation of neurons and brain volume changes, suggesting deficiencies in neurodevelopment



(Akbarian et al 1993) (Altshuler et al 1987). More compelling evidence to strengthen this proposal is that a large majority of the risk factor genes identified to date have important known roles in neurodevelopment.

Therefore the current neurodevelopmental hypothesis of Schizophrenia suggests that 'Subtle abnormalities during brain development will later manifest to as an adult brain disorder due to early genetic dysfunction'. This hypothesis is now widely accepted in the field, although alternative hypotheses relating to synaptic dysfunction and altered neurotransmission remain popular, especially in relation to recent GWAS findings (Schizophrenia Working Group of the Psychiatric Genomics 2014).

#### 1.2.4 Risk factor genes and genetic overlap between psychiatric disorders

Several genes have been identified from various genome studies in several populations, however no causal gene has been identified to date. Therefore schizophrenia is described as a polygenic disorder where several risk factor genes may need to have their function compromised in a variety of neurodevelopmental processes to later manifest as an adult brain disorder. It may be the case that individuals are genetically predisposed to schizophrenia and when exposed to particular environmental factors the disease is triggered in later life. Many genes have been suggested to confer risk to schizophrenia, although many since have failed to reach stringent genome wide significance but these genes have been key in identifying key biological pathways. Interestingly, it has recently been demonstrated that there are varying degrees of genetic overlap between brain disorders including schizophrenia, bipolar disorder, depression and attention deficit/hyperactivity disorders through GWAS (Cross-Disorder Group of the Psychiatric Genomics et al 2013) (Ng et al 2009). Understanding this shared genetic risk between disorders may identify common pathways for therapeutic targets and could also lead to improved diagnostics in the future.

### 1.2.5 Risk factor genes and white matter abnormalities

One important question to address is which of the identified risk factor genes play a role in oligodendrocyte development and white matter maintenance, an area known to be perturbed in schizophrenia. Since white matter abnormalities have been well documented it is expected that a proportion of these genes will have a role in oligodendrocyte function and development. Do any of these risk factor genes have a direct role in white matter development, either at a later stage during myelination or at early stages during the development of OPCs? It is plausible that dysregulation of genes involved in OPC development contributes to schizophrenia, since this disease is proposed to be neurodevelopmental in origin and many of the genes implicated are involved in neurodevelopment. There have indeed been several genes involved in oligodendrocyte development and myelination associated with schizophrenia, including but not limited to: Neuregulin-1 (*NRG1*), *ERBB4* (one of the receptors of *NRG-1*), Oligodendrocyte lineage transcription factor 2 (*OLIG2*), Myelin basic protein (*MBP*) and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (*CNP*) (Georgieva et al 2006, Owen et al 2005, Stefansson et al 2002). A novel gene, Disrupted in Schizophrenia-1 (*DISC1*), originally established to have roles exclusively in neuron development has recently been suggested to have roles in oligodendrocyte development in both cell culture, mouse and zebrafish studies (Hattori et al 2014, Katsel et al 2011, Wood et al 2009).

## 1.3 *Disrupted in schizophrenia-1 (DISC1)*

### 1.3.1 Discovery of *DISC1*

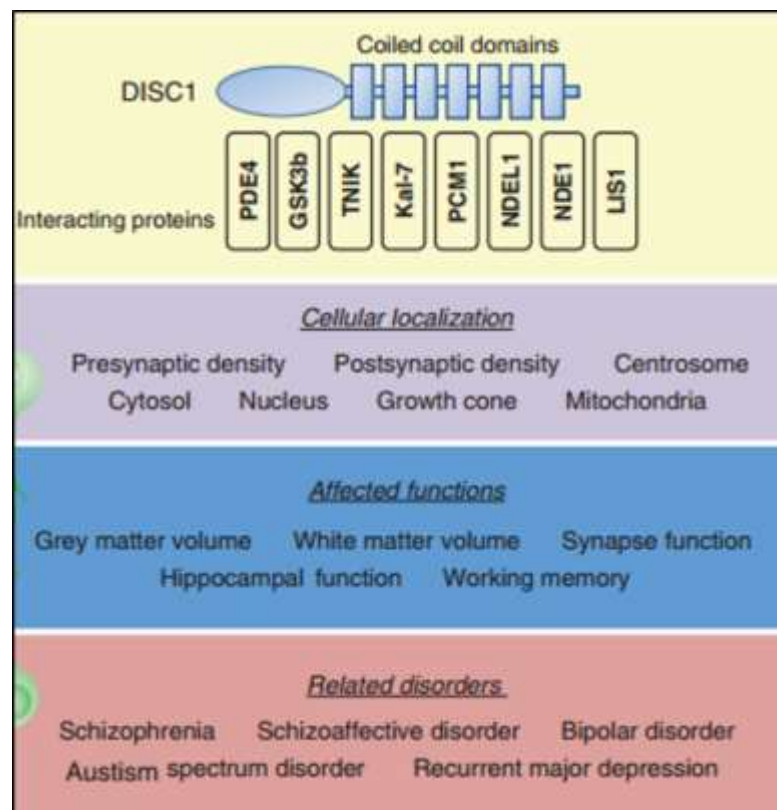
In the last ten years research has focused on dissecting the roles of proteins which are encoded by risk factor genes in nervous system development, maturation and maintenance. *DISC1* was one of the earliest schizophrenia susceptibility genes identified, with subsequent research building substantial evidence for roles in neuronal migration, neurite outgrowth and multiple cell signalling pathways. It has been widely referred to as one of the leading candidate risk factors conferring risk to schizophrenia, although recent GWAS have failed to report association with schizophrenia at genome wide significance level. However studies of *DISC1* function

have still provided fundamental insights into SZ pathophysiology and neurodevelopmental processes in general (Ripke et al 2013). The *DISC1* locus was initially described back in 1990, where a balanced t(1,11) chromosomal translocation in a Scottish family was seen to co-segregate with a high prevalence of mental illness, including SZ, bipolar disorder and major depression (St Clair et al 1990). A high percentage of family members that carried the translocation suffered from mental illness, whereas those family members that were not carriers did not suffer any mental health issues. This suggested a direct causal link between the genes disrupted by this chromosomal translocation and mental illness. The chromosomal translocation caused a breakpoint in the *DISC1* gene on chromosome 1 which was discovered through positional cloning, and the function of this gene was unknown at the time (Millar et al 2000). Even though structural genetic variants such as these are rare, these types of mutations have been exceptionally useful in understanding mechanisms of disease (Porteous et al 2014). The *DISC1* gene was cloned over a decade ago now and has been extensively studied to reveal a novel protein harbouring numerous protein interacting partners with varied roles in neurodevelopment (Millar et al 2000, Semple et al 2001). Other studies showed positive associations between *DISC1* and schizophrenia in different populations such as Finland, Iceland and Taiwan, although there were other studies which suggested a negative association (Chubb et al 2008, Ekelund et al 2001, Zhang et al 2005). Furthermore, there is also evidence to suggest that *DISC1* may also be a more general risk factor for mental illness including depression and autism (Chubb et al 2008). It is important to note that recent studies demonstrate that the *DISC1* locus does not reach genome wide significance for association to schizophrenia (Ripke et al 2013), however rare structural variants are not detected in GWAS and biological investigation of the *DISC1* protein has provided extensive evidence for roles in neurodevelopment consistent with a role in mental illness.

### 1.3.2 *DISC1* in the brain

The human *DISC1* gene spans 410 kb containing 13 exons, and encodes a full length 854 amino acid protein with an *N*-terminal globular head domain containing putative nuclear localisation signals and a *C*-terminal domain with coiled-coiled

regions. A three-dimensional crystal structure of the DISC1 protein is yet to be elucidated (Chubb et al 2008). What is particularly interesting about this protein is that it is unique, it is unlike any other protein and there is limited homology between species, with the C-terminal domain showing higher conservation (Chubb et al 2008). DISC1 shows a diverse range of tissue and cellular expression patterns. It is expressed in the adult brain (predominantly in the hippocampus and frontal cortex) but it is also expressed in the heart, kidney and liver, and has been reported to be localised to several cellular compartments including mitochondrial, nuclear, cytoplasmic and centrosomal locations (Figure 1-1).



**Figure 1-1: DISC1 biology**

The DISC1 protein has a C-terminal domain made up of coiled-coil domains which harbors the majority of binding sites for its interacting partners. The protein has been suggested to occupy a variety of cellular localizations. Mutation of DISC1 affects a variety of brain functions and is implicated in several psychiatric disorders. (Adapted from (Porteous et al 2011) license number: 3457600782345)

There are several isoforms of *DISC1* identified in humans suggesting a variety of biological functions. To date there are 22 predicted *DISC1* isoforms listed on Ensembl

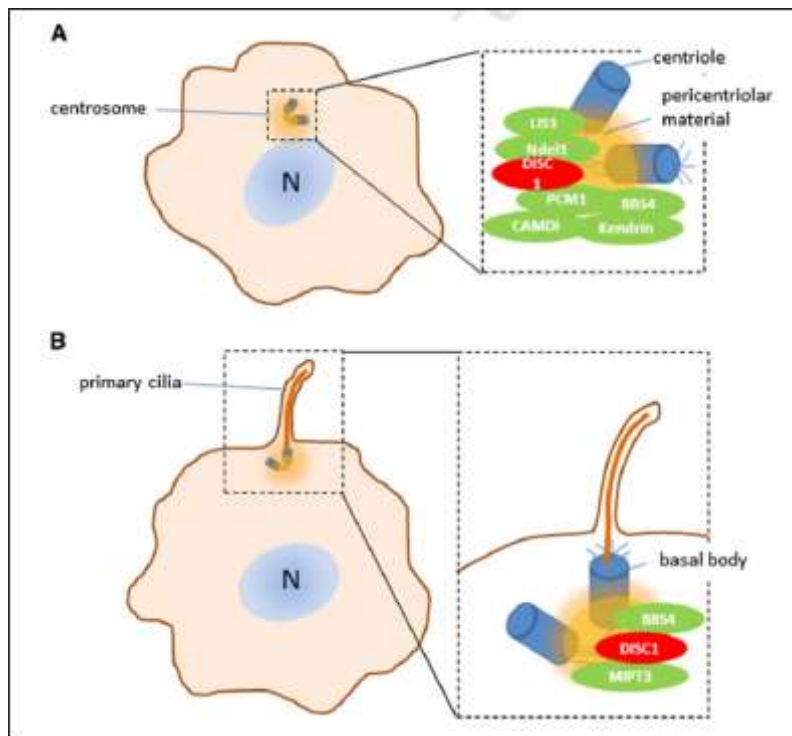
([http://www.ensembl.org/Homo\\_sapiens/Transcript/ProteinSummary?db=core;g=ENSG00000162946;r=1:231626815-232041272;t=ENST00000439617](http://www.ensembl.org/Homo_sapiens/Transcript/ProteinSummary?db=core;g=ENSG00000162946;r=1:231626815-232041272;t=ENST00000439617)). In mouse, different isoforms have been shown to be developmentally regulated, with expression peaking at stages of neurogenesis and neuronal migration during embryonic development, and later at the onset of puberty during final brain maturation (Schurov et al 2004). There are now several mouse models based on DISC1, but it must be stressed that many aspects of mental illness such as hallucinations are specific to humans. Therefore the disease can never be fully recapitulated in animal model systems. However mouse models have been shown by many to display characteristics comparable to those seen in schizophrenia patients including behavioural, anatomical and cognitive changes. For example a study screened for *Disc1* mutations in a library of *N*-ethyl-*N*-nitrosourea (ENU)-treated mice and identified two missense mutations; Q31L and L100P. These mutant mice were characterised and both showed a reduced brain volume compared to wild-type controls, with the volume decrease being most prominent in the cerebellum, cortex and thalamus. Although both mutations were associated with brain volume decrease, one mutant (L100P) showed behavioural changes reported to be similar to those in schizophrenia, whereas the other mutant (Q31L) displayed behavioural changes more akin to depression (Clapcote et al 2007). Another study using *in utero* gene transfer caused knock down of *Disc1* expression in the prefrontal cortex of mouse embryos. During adulthood these mice elicited schizophrenia-like behaviours due to circuitry abnormalities caused by aberrant neurodevelopment including abnormalities in dopaminergic maturation (Niwa et al 2010).

Much of what is known about the function of DISC1 in neurodevelopment has come from yeast two hybrid (Y2H) studies that have implicated DISC1 in several aspects of neurodevelopment (Figure 1-1). Several of the protein binding sites for these functions have been shown to be present in the C-terminal end of the protein, which is lost in the original Scottish family (Porteous et al 2011).

### 1.3.3 Interacting partners implicated in neuronal migration

To date, several interacting partners of DISC1 have been identified through yeast and mammalian two hybrid experiments (Camargo et al 2007). A large proportion of these were cytoskeletal proteins with roles in neuronal migration and neurite outgrowth for example NDEL1, LIS1 and FEZ1. LIS1 is a critical protein for neuronal migration; mutations in the gene encoding it cause lissencephaly, a condition that results from severe neuronal migration defects. NDEL1 (also referred to as NUDEL) was originally characterised through its interaction with LIS1 and dynein and has been shown to be a cytoskeletal protein important for coupling the centrosome to the nucleus (nucleokinesis) during cell migration (Niethammer et al 2000). The complex formed between DISC1 and NDEL1 is developmentally regulated in the mouse brain with a peak at E17 and during early postnatal development. Interestingly relative protein co-localisation between LIS1, NDEL1 and DISC1 was observed in the developing mouse cerebral cortex and hippocampus (Brandon et al 2004). DISC1 was also shown to localise to the centrosome, through co-localisation with Y-tubulin, which suggested that interactions with proteins such as NDEL1 could be required for its centrosomal recruitment (Morris et al 2003). It has been subsequently shown from these initial studies that DISC1 forms complexes with NDEL1 and through this complex can also interact with LIS1 and that it also has important roles in neuronal migration (Figure 1-2A). Another study identified a decrease in NDEL1, LIS1 and FEZ1 in the hippocampus of individuals with schizophrenia during post-mortem brain analysis (Lipska et al 2006). FEZ1 is a protein that is important for axon outgrowth, another process relying heavily on the cytoskeleton, and has similarly been shown to interact with DISC1 through Y2H experiments. DISC1 has also been shown to interact with microtubules and microtubule-associated proteins, namely MAP1A and MIPT3 (Morris et al 2003). These interactions with centrosomal and microtubule-associated proteins have led to the proposal that DISC1 could have functions in creating stable protein networks important for neuronal migration during brain development. In mutation cases where the domains harbouring the major binding sites are affected, such as in the original chromosomal translocation identified in the Scottish family, this could

result in destabilisation of protein complexes and aberrant migration during development (Millar et al 2000).



**Figure 1-2: The DISC1 protein at the centrosome and primary cilia**

The DISC1 protein has been reported to localize to the centrosome and interact with centrosomal proteins implicated in neuronal migration (A). The DISC1 protein has also been reported to localize to the basal body of primary cilia where it interacts with other basal body proteins(B). Taken from (Wang & Brandon 2011) License number: 3458260976001.

Several studies have since supported a role for DISC1 in neuronal migration, key studies will be discussed here. RNA interference based knockdown of *Disc1* in the embryonic mouse brain via *in utero* electroporation resulted in neuronal migration delays. At E18.5 there was a significant increase in the distance between the centrosome and the nucleus which does suggest there is mis-positioning of the centrosome in the absence of DISC1, thereby presenting a possible role for DISC1 in centrosomal/nuclear positioning (Kamiya et al 2005). The same group later showed an interaction between PCM1, BBS4 and DISC1. Pericentriolar material 1 (PCM1) has itself been proposed as a genetic risk factor for schizophrenia (Blouin et al 1998), whereas BBS4 is mutated in Bardet-Biedl syndrome, a ciliopathy where patients display neuropsychiatric symptoms. DISC1 and BBS4 were shown to recruit PCM1 to the centrosome through complex formation. Disruption of this complex

caused defects in neuronal migration (Kamiya et al 2008). An interaction between DISC1 and amyloid precursor protein (APP), a protein central to the pathogenesis of Alzheimer's disease, has been reported to be involved in cortical precursor neuronal migration. Overexpression of DISC1 could rescue an APP knockdown migration defect phenotype *in vivo*, while APP knockdown *in vitro* caused a redistribution of DISC1 (Young-Pearse et al 2010). A more recent study demonstrated a role for Disc1 in cortical interneuron migration. Suppression of Disc1 resulted in delayed cortical interneuron migration and additionally these cells showed abnormal morphology with longer branches indicative of reduced nucleokinesis and less branching suggesting cells were unable to sense their external environment (Steinecke et al 2012).

Taken together these studies have provided a substantial body of evidence that DISC1 is important for neuronal migration in brain development. Mutations in *DISC1* could therefore contribute to schizophrenia pathology via migratory defects which cause mis-positioning of neurons and lead to brain connectivity abnormalities.

#### 1.3.4 Primary cilia and *DISC1*

Primary cilia are finger-like projections of the plasma membrane and are present on most cell types in the body including neuronal cells. Their function has been repeatedly shown to be critical for cellular development and homeostasis with many human disorders resulting from mutations in genes important for primary and motile ciliogenesis (Goetz & Anderson 2010). A feature of some ciliopathies is brain deformities which suggest that cilia have important roles in neurodevelopment (Lee & Gleeson 2011). Cilia are now appreciated as signalling hubs of the cell, important for transducing a wide variety of signalling pathways including the SHH signalling pathway. Interestingly, there has been a handful of studies linking *DISC1* to primary cilia both directly using immunofluorescence and through known interacting partners of *DISC1* including *TRAF3IP1* (also known as MIP-T3) (Figure 1-2B). DISC1-GFP was shown to localise to the basal body of cilia when transfected into cultured NIH3T3 cells. Furthermore, knockdown of *DISC1* through siRNA reduced primary cilia number suggesting that *DISC1* could have



functions in regulating the formation of cilia (Marley & von Zastrow 2010). Another study showed that mice mutated in *Traf3ip1*, which encodes a DISC1-interacting partner, were devoid of cilia and had disrupted SHH signalling. Homozygous mutant embryos had neural patterning defects, confirming the requirement for functional cilia and SHH signalling in dorso-ventral neural tube development (Berbari et al 2011). Although DISC1 has not as yet been shown to localise to neuronal cilia this data is still very interesting. Recent studies have shown primary cilia to have a role in directing migration of interneurons. Migrating interneurons were found to display primary cilia expressing receptors for guidance cues including ErbB4, Robo1/2 and CXCR4/7 (Higginbotham et al 2012). Another paper showed that primary cilia on neurons undergoing migration were located to the leading process. The authors also discovered that chemical inhibition of SHH signalling caused altered leading process dynamics which suggests that during migration primary cilia could be transducing SHH which drives migration of neurons in the required direction (Baudoin et al 2012).

One could therefore hypothesise that DISC1 functions through or responds to signals transduced by primary cilia during migration of CNS cell types. Positional cues provided by the external environment are sensed by primary cilia, then in turn cytoskeletal proteins enriched in the basal body or cilia axoneme respond to these signals and function to drive migration accordingly.

### 1.3.5 Evidence for a role of DISC1 in OL development

Although the majority of studies about *DISC1* biology and function are focused on its role in neuronal development and maintenance there have been recent studies which have also linked *DISC1* to oligodendrocyte development. However further investigation is required to understand the role of *DISC1* in this process. A study utilising a mouse model expressing an inducible human mutant dominant negative form of DISC1 exclusively in the forebrain analysed for oligodendrocyte gene expression changes. It was found that the human mutant *DISC1* caused significant changes in the expression of oligodendrocyte related genes in mice, including *Olig2*, *Sox10*, *Mag*, *Plp*, *Pdgfra*, *Cnp1* and *Qki* all of which were up-regulated (Katsel et al 2011). Furthermore, this study found increased expression of *Nrg-1* and its

receptors. NRG-1 signalling is important in oligodendrocyte development and there is also evidence suggesting it to be a genetic risk factor for schizophrenia (Vartanian et al 1999) (Li et al 2006). This suggests that *DISC1* could regulate or work in conjunction with *NRG1* during oligodendrocyte development. Another study showed that in *Nrg1* knockout mice, *Disc1* expression was reduced during neurodevelopment. The study also showed that *DISC1* was expressed in a variety of brain cell types including neurons, astrocytes, oligodendrocytes and microglia *in vitro* (Seshadri et al 2010). Expression in oligodendrocytes was restricted to cell bodies and expression in OPCs was not addressed. Cells were co-stained with an anti-*DISC1* mExon3 antibody and cell specific antibodies, however the specificity of the anti-*DISC1* mExon3 has not been extensively scrutinised.

A recent *in vitro* study using primary rat oligodendrocyte precursor cells suggested that *DISC1* was a negative regulator of oligodendrocyte differentiation, with knockdown via RNA interference resulting in a higher percentage of mature oligodendrocytes. When overexpressed, *DISC1* caused an increase in the number of progenitors. It was also suggested that *Sox10* and *Nkx2.2* had functions in oligodendrocyte development downstream of *DISC1* since their expression was decreased when *DISC1* was overexpressed, and increased upon *DISC1* knockdown. The study did not give an indication as to an effect of OPC migration when *DISC1* expression was altered (Hattori et al 2014).

The same group recently identified a role for a *DISC1* interaction partner in regulating oligodendrocyte differentiation. *DISC1*-binding zinc finger protein (DBZ) was shown to be specifically expressed in the CNS, including the oligodendrocyte lineage. DBZ knockout mice had an increased number of immature oligodendrocytes suggesting that it acts in oligodendrocyte differentiation (Shimizu et al 2014). When compared to *DISC1* which was suggested by the previous study to promote the oligodendrocyte precursor cell state, it could perhaps be hypothesised that DBZ binds to *DISC1* during later stages of oligodendrocyte differentiation and inhibits its progenitor promoting function. However this has not been shown and is merely speculative.

Furthermore, a CNS exon array carried out in neurons, astrocytes, OPCs and oligodendrocytes showed DISC1 to be most highly expressed in OPCs, further encouraging a fundamental role for DISC1 in OPCs (Cahoy et al 2008).

## 1.4 Oligodendrocyte development

### 1.4.1 Specification of oligodendrocyte precursor cells

Oligodendrocyte precursor cells are produced from the same pool of progenitors that first produce motor neurons (pMN) in the ventral neural tube (Pringle et al 1996). A discrete population of OPCs is specified in the ventral neural tube and this initial specification is governed by opposing extracellular signals. Sonic hedgehog (SHH), a protein secreted by the notochord, which sits below the neural tube, and then floor plate of the ventral neural tube, is crucial for orchestrating the early patterning of the dorso-ventral CNS. The opposing signal is bone morphogenic protein (BMP), which acts dorsally, and works to repress the long range SHH signal thereby setting up a morphogen gradient of SHH (Mehler et al 1997). Another dorsal signal which acts to repress SHH is Wnt/beta-catenin (Robertson et al 2004). SHH specifies discrete sections of the D/V neural tube through regulation of transcription factors to create different progenitor regions. Ablation of ventral SHH results in a failure to produce oligodendrocytes (Orentas et al 1999). OPCs are born in one of these discrete progenitor regions and then subsequently migrate to their axonal destinations creating a widespread distribution of oligodendrocytes throughout the nervous system. This is not the only population of oligodendrocyte precursor cells in the developing CNS, it should be noted that there is another population which is specified by a SHH-independent pathway in the dorsal telencephalon (Cai et al 2005, Fogarty et al 2005)

### 1.4.2 Early gene expression in OPC specification

The basic helix-loop-helix transcription factors *OLIG1* and *OLIG2* act downstream of SHH signalling to specify the pMN domain which gives rise to first MNs and then OLs (Lu et al 2000, Takebayashi et al 2000, Zhou et al 2000). *OLIG2* is critical for the development of these cells (Zhou & Anderson 2002) which then subsequently express *NKX2.2* once specification of motor neurons is complete. *Olig2* knockout

mice fail to give rise to oligodendrocytes in the majority of the CNS (Zhou & Anderson 2002), however studies have suggested that *Nkx2.2* expression is not critical for early specification of OPCs but involved in their maturation during differentiation (Qi et al 2001). Another transcription factor reported to have a required role in OPC specification is *Ascl1/mash1*. Mice lacking *Ascl1* have drastically reduced numbers of OPCs early on but these are recovered at later stages (Sugimori et al 2008). Studies in the zebrafish have identified a role for Delta-Notch signalling in maintaining some *olig2+* precursors once pMNs are specified (Park & Appel 2003). Early specification of OPCs is also dependent on the growth factor neuregulin-1 (NRG1). In spinal cord explants lacking *Nrg1*, oligodendrocytes fail to develop (Vartanian et al 1999). NRG1 also acts a chemoattractant during OPC migration.

#### 1.4.3 Proliferation of OPCs

Once the initial specification of OPCs has been established through expression of the transcription factors described above, OPCs undergo proliferation both before and after migration. These waves of proliferation are dependent on various growth factors which also act to promote the survival of OPCs. Platelet derived growth factor A (PDGF-A) is an important growth factor for oligodendrocyte proliferation. Loss of *PDGF-A* in a knock out mouse leads to a decrease in OPC numbers and hypomyelination (Fruttiger et al 1999), whereas overexpression of this growth factor increases OPC number (Calver et al 1998). Other growth factors such as bFGF (McKinnon et al 1991) and CXCL2 are also involved in OPC proliferation and development (Robinson et al 1998).

#### 1.4.4 Migration of OPCs to their axonal targets

Once OPCs have proliferated they need to exit their defined progenitor domains and migrate to populate the CNS, which requires a wide variety of external factors to create defined maps for OPCs to respond and migrate in the specified direction. The capacity of OPCs to migrate long distances has been known for some time from initial studies using the optic nerve (Small et al 1987) (Ono et al 1997). Growth factors such as PDGF-AA and FGF-2 are responsible for directing OPCs to

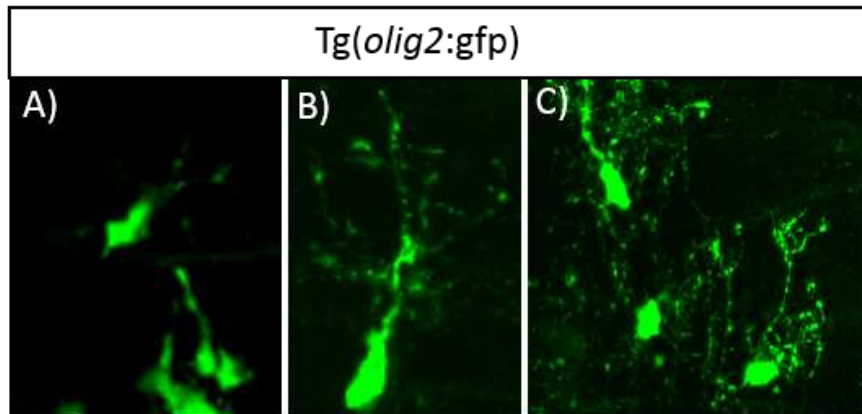
destinations far away from their birth place by inducing cell motility, but also by acting as chemoattractants (Bribian et al 2006). Other chemoattractants include netrin-1 and semaphorins 3A and 3F (Spassky et al 2002). SHH has also been proposed to act as a chemoattractant in the optic nerve (Merchan et al 2007). Chemorepellents are also required during migration to ensure OPCs do not travel past their destinations and these include chemokine (C-X-C motif) ligand 1 (CXCL1) and some members of the semaphorin family (Tsai et al 2002). Communication with ECM proteins fine-tunes the direction of OPC migration. Adhesion molecules have the ability to control the speed and direction by which OPCs migrate and these are present on the surfaces of surrounding cells, including axons and astrocytes (de Castro & Bribian 2005). Some of these adhesion molecules are also thought to act as stop signals for migrating OPCs, which will therefore act as a cue to OPCs that they have reached their desired axonal destination. OPCs also repel each other using contact inhibition, thus distributing themselves more uniformly with no two cells being too close together. All these factors help migrating OPCs to communicate with and respond correctly to their external environment.

As discussed earlier, migration defects in neuronal cells have been commonly described in schizophrenia patients with many of the risk factor genes identified having functional roles in the cytoskeleton and neuronal migration. At this point it can be proposed that an area of oligodendrocyte biology that may be defective in SZ brains is OPC migration. If OPCs lack the ability to migrate at full capacity it would mean that at least a proportion of those destined for areas far away from the birth place may never reach their correct axons leading to defective myelination of those neurons. This could subsequently result in brain connectivity alterations, potentially leading to cognitive defects and psychosis.

#### 1.4.5 Transition from OPC state to differentiated oligodendrocytes

After proliferation and migration, OPCs will start to up-regulate genes required for their terminal differentiation. It is at this point that cells will start to lose their capacity for migration/proliferation and undergo drastic morphological changes.

Early OPCs have a bipolar simple morphology (Figure 1-3A) but as development progresses and the cells start to differentiate they become more complex and extend long fine processes characteristic of pre-myelinating oligodendrocytes (Figure 1-3C).



**Figure 1-3: Morphologies of oligodendrocytes during development**

During early development oligodendrocyte precursor cells have a simple bipolar morphology (A). During migration OPCs extend long processes into the surrounding environment (B). Once at their destination, differentiated oligodendrocytes extend fine processes to wrap and myelinate axons (C). OPCs are shown from the zebrafish *tg(olig2:gfp)* line

There are two opposing actions occurring during early differentiation; factors that promote differentiation and factors that function to inhibit differentiation. Those factors that act to inhibit the differentiation of OPCs include G protein-coupled receptor 17 (GPR17) (Chen et al 2009), members of the Notch signalling pathway (Kondo & Raff 2000, Wang et al 1998) and Leucine-rich repeat and immunoglobulin domain-containing-1 (LINGO1). Insulin-like growth factor 1 (IGF-1) is a promoter of OL differentiation; in mice lacking *Igf-1* there is a decrease in myelin and mature OLs (Ye et al 2002). There are also a number of transcription factors which are expressed during differentiation. As mentioned previously *Mash1/Ascl1* and *Nkx2.2* also have roles in OL differentiation (Sugimori et al 2008) (Qi et al 2001). *OLIG2* is expressed throughout OPC development and has also been suggested to have a role in OPC differentiation. One recent study showed that it is responsible for recruiting BRG-1, a chromatin-remodelling enzyme, to key gene regulatory elements during differentiation (Yu et al 2013).

### 1.4.6 Myelination

The final stage of OL development is the myelination of axons in the CNS and it has been recently demonstrated that OLs have only a short window of time in which to make contact and myelinate axons (Czopka et al 2013). A number of studies have identified signalling systems that assist OLs in sensing their axonal environments, allowing for process extension and subsequent myelination. Although *OLIG1* is well known as an OL lineage specification transcription factor, its role in myelination was not shown until later. In *Olig1* knock out mice, although OPCs form there is a failure in myelination although the underlying mechanism has not yet been elucidated (Xin et al 2005). In order to myelinate axons, oligodendrocytes are required to synthesise specific proteins including Proteolipid Protein (PLP) and Myelin Basic Protein (MBP) (Campagnoni 1988). As previously discussed, in humans the majority of myelination occurs post-natally up until early adulthood. The majority of studies have used the murine model to investigate oligodendrocyte development, however other models systems including the zebrafish has also provided valuable information.

## 1.5 The zebrafish (*Danio rerio*) as a model system of myelination

### 1.5.1 Advantages of the zebrafish

The zebrafish embryo offers many advantages making it an excellent model system to study vertebrate development, but it has also found favour in modelling human diseases. The zebrafish genome has approximately 70% of human genes, making it a valuable genetic tool in understanding human genetic function in development, maintenance and disease (Howe et al 2013). Fish are relatively inexpensive to maintain compared to mice and rats. They also have a short generation time, produce 100-200 embryos per clutch and are externally fertilised. Being externally fertilised means that they can be easily manipulated at early stages of development. Another advantage is that their embryos are also transparent during early development, allowing for easier visualisation of dynamic processes such as cell migration. There are many transgenic lines available in which different cell

types of the brain are labelled via expression of fluorescent proteins including the *tg(olig2:gfp)* line which was used during this project for analysing OPCs (Park et al 2002). This simple visualisation of OPCs means that we could more efficiently analyse OPC migration in a temporal manner.

### 1.5.2 Myelination studies in the zebrafish

Although the majority of oligodendrocyte and myelination studies discussed above were based on mouse/rat and cell culture studies, more recently the zebrafish has been exploited as a model system for studying many aspects of Schwann cell and oligodendrocyte myelination. However this discussion will describe zebrafish oligodendrocyte myelination studies only (Levavasseur et al 1998) (Brosamle & Halpern 2002, Park et al 2002). Since fish have a greater capacity to regenerate the CNS, understanding their genetic and developmental differences is of great interest (Stuermer et al 1992). One of the first studies to address myelination in the zebrafish was by Halpern et al, whereby the orthologues of key myelin genes were investigated in zebrafish larvae and myelination was characterised using *in situ* hybridisation and electron microscopy. Expression of *plp*, *mbp* and *p0* was observed in the zebrafish embryo as early as 2 dpf, and in larvae myelinated axons were detected at 7 dpf, making them a rapid tool for understanding myelination with conservation of critical myelin genes (Brosamle & Halpern 2002). Similarly other groups have identified the zebrafish as a useful model system and have further investigated the development and genetics of OPC myelination during CNS development. One such study focused on dissecting the genes required for zebrafish myelination using ENU mutagenesis and described a number of mutations affecting *mbp* expression, and therefore myelination, in the CNS, PNS (Peripheral nervous system) or both. Three mutations were found to affect CNS myelination and were named *mol<sup>st20</sup>*, *st47* and *st51*. These mutants had altered *shh* and/or *olig2* expression abnormalities, suggesting that they interfered with early stages of oligodendrocyte development, most likely during OPC specification. A further three mutations were found to affect both CNS and PNS myelination, showing genetic overlap between Schwann cell and oligodendrocyte myelination as with higher vertebrate systems (Pogoda et al 2006). Other groups focused on using *in vivo* live



imaging of transgenic zebrafish lines specifically marking oligodendrocytes to observe their migration, distribution and axonal contact behaviours. Using the *tg(nkx2.2:megfp)* and *tg(olig2:gfp)* lines, live embryos were mounted and oligodendrocytes imaged over various time periods where they showed dynamic behaviours. Oligodendrocytes were observed extending filopodium-like processes with continuous extension and retraction into the surrounding environment before settling into their final location, and the migratory path and final position seemed to rely on contact between OPCs. Interestingly, when OPCs were ablated, neighbouring OPCs moved into the ablated area to allow for evenly dispersed oligodendrocytes and the authors suggest that this retraction and extension of processes acts as a surveillance mechanisms to ensure a uniformly distributed population of oligodendrocytes prior to axonal wrapping (Kirby et al 2006). In mice and human systems, OPCs can replace ablated areas showing evolutionary similarities, and suggesting that OPCs may compete for space.

Oligodendrocytes that associate with large axons are limited in how many axons they can myelinate. If they associate with small axons, they capable of myelinating several axons but how this relationship is established is unknown. Whether axons play a role in governing how many axons an oligodendrocyte can myelinate was addressed using the zebrafish. In the zebrafish, the first axon to be myelinated is the Mauthner axon which is very large in size. Addition of more Mauthner axons can regulate myelination; oligodendrocytes that would normally only myelinate a single Mauthner axon in this case could myelinate a larger number and this was not at the expense of other axons. This suggested that axons can regulate oligodendrocyte myelination (Almeida et al 2011). Several other studies have sought to investigate the role of single genes in aspects of zebrafish myelination including *kif1b* and *tuba8l3a* (puma) which were shown to have roles in mRNA localisation of *mbp* and *lingo-1*, which was identified as a negative regulator of oligodendrocyte differentiation (Lyons et al 2009) (Larson et al 2010) (Yin & Hu 2014). Another study showed additional roles in for *sox10*, not only to promote myelin gene expression but also in the survival of oligodendrocytes prior to axonal wrapping (Takada et al 2010).

A very recent genetic screen identified a mutation in the *hmgcs1* gene in the zebrafish, which caused aberrations in OPC migration whereby cells migrated past their axon targets and therefore failed to myelinate. These OPCs also failed to express any myelin genes. This gene has roles in the cholesterol biosynthetic pathway which also synthesises isoprenoids and it is known that myelin requires high levels of cholesterol. This data showed that cholesterol biosynthetic pathway products had roles in OPC migration and the expression of myelin genes which was a novel observation (Mathews et al 2014).

### 1.5.3 Using the zebrafish as a model system for schizophrenia

Although the zebrafish is a very powerful developmental biology tool it is unable to recapitulate certain behaviours that are characteristic of psychiatric disorders. Mouse models with behavioural changes of relevance to schizophrenia have also been developed, but even these higher vertebrate models possess limitations since many of the symptoms in schizophrenia (such as hallucinations) are self-reported and assumed to be human specific (Pletnikov et al 2008) (Hikida et al 2007). Nonetheless, zebrafish are still a powerful system for investigating the underlying genetics and molecular mechanisms controlling neurodevelopmental processes since their nervous system is less complex than that of higher vertebrates but share many common features and signalling pathways.

### 1.5.4 Zebrafish *disc1*

As previously mentioned, there is high genetic similarity between the zebrafish genome and the human genome. The *DISC1* gene is conserved between species, although there is no reported homolog in *Drosophila* (*D.melanogaster*) and worms (*C.elegans*), which suggests that its functional roles may lie exclusively in complex brain development such as myelination (Chubb et al 2008). There have been three different transcripts of zebrafish *disc1* identified thus far; all three transcripts maintain the same protein coding length, only differing in their untranslated regions (UTR). Since there have been several human isoforms identified it is plausible that more zebrafish *disc1* isoforms are present and this requires further

investigation. With regards to sequence conservation with human *DISC1*, the C-terminal end is more highly conserved compared to the N-terminal end which is poorly conserved. The C-terminal end is 35% identical to the human protein and shares 57% similarity. As with the C-terminal portion of the human *DISC1* gene it is predicted to form multiple coiled-coil domains. Its location within the genome also shows similarity to human *DISC1*, with the genes *TSNAX* and *EGLN1* and their fish orthologues being positioned directly upstream to *DISC1* in both humans and zebrafish (Wood et al 2009). When semi-quantitative RT-PCR of *disc1* was used to investigate *disc1* expression throughout development, it was found to be dynamically regulated (Drerup et al 2009). Expression was high at 3 hours post-fertilisation, this suggests that there is maternal *disc1* mRNA that is deposited during early development. Expression was subsequently down-regulated between 12 and 48 hpf, after which *disc1* started to be expressed again from 48 hpf and subsequently increased. At 120 hpf, expression of *disc1* was still present. Since there was no expression identified using this method between 12-48 hpf during which time the body axis is established and the nervous system specified, it suggests that *disc1* does not have early roles but functions later during nervous system development (Drerup et al 2009). However expression of the *disc1* mRNA transcript was detected through *in situ* hybridisation at 8 somites, 16 somites and 24 hpf. Since the expression pattern at these early time points appeared specific it would suggest that the RT-PCR results are inaccurate. These results do suggest that *disc1* is maternally expressed during early development and shows specific expression in different tissue regions during the developmental process.

#### 1.5.5 Zebrafish *disc1* in brain development

To date there has been only a handful of studies investigating the role of *disc1* in zebrafish development, and those studies that have been performed suggest important roles for *disc1* in the development of a variety of cell types as summarised in the table below.

Publication	<i>disc1</i> function identified
(Drerup et al 2009)	Role in cranial neural crest development through suppression of the transcription factors <i>sox10</i> and <i>foxd3</i>
(De Rienzo et al 2011)	Role of <i>disc1</i> in brain development through regulation of the $\beta$ -catenin-mediated Wnt pathway
(Wood et al 2009)	Role of <i>disc1</i> in the development of hindbrain OPCs.

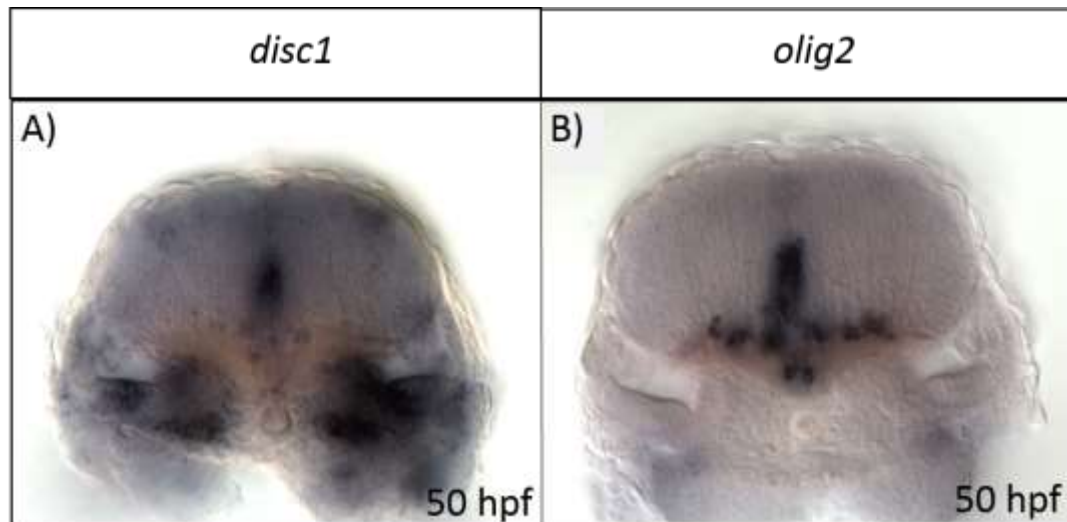
**Table 2: Studies identifying roles for *disc1* in zebrafish development**

A role for *disc1* in cranial neural crest (CNC) development has been characterised in the zebrafish through morpholino knockdown analysis of *disc1* in developing embryos. Cranial neural crest cells, like oligodendrocyte precursor cells, have the capacity to migrate long distances from their origin. CNC cells give rise to numerous tissues including pigment cells, the peripheral nervous system (PNS) and jaw cartilage. The migration direction and final location is important for their subsequent differentiation into the correct cell type through exposure to the appropriate signals. It was found that a loss of the Disc1 protein in developing embryos resulted in aberrant directional migration of CNC cells however their motility was not affected. It was also observed that in *disc1* morphants, the expression of *sox10* and *foxd3* was increased and these transcription factors have known roles in CNC cell migration and differentiation. The authors suggested that *disc1* is responsible for *sox10* and *foxd3* repression in CNC cells. Another hypothesis could be that CNC cells lose their ability to respond to positional information and therefore migrate in aberrant directions due to loss of *disc1*. It is interesting that CNC cells were reported to not have altered motility, as it might be expected that upon knockdown of a cytoskeletal scaffold protein with known roles in neuronal migration, the cells would have altered motility however only the direction of migration in this case was compromised. The same group also reported lower jaw cartilage defects in *disc1* morphants (Drerup et al 2009). CNC cells give rise to the jaw cartilage and this is therefore consistent with aberrant CNC migration.

Another group analysed the zebrafish *disc1*<sup>fh291</sup> mutant identified through a TILLING screen. This mutation leads to a premature stop codon in exon 2 and therefore is predicted to give rise to a prematurely terminated protein product. Although

heterozygotes developed normally, homozygotes presented with brain abnormalities at 24 hpf including small ventricles. Mutants also presented with a bent tail. This was phenocopied in *disc1* morphant embryos, and the phenotype could be rescued with injection of human Disc1 RNA. The authors suggest that zebrafish *disc1* functions through  $\beta$ -catenin-mediated Wnt signalling (De Rienzo et al 2011). This group did not describe any lower jaw cartilage cranio-facial defects that have been recognised by two other groups working with *disc1* morphants, suggesting inconsistencies between studies (Drerup et al 2009, Wood et al 2009).

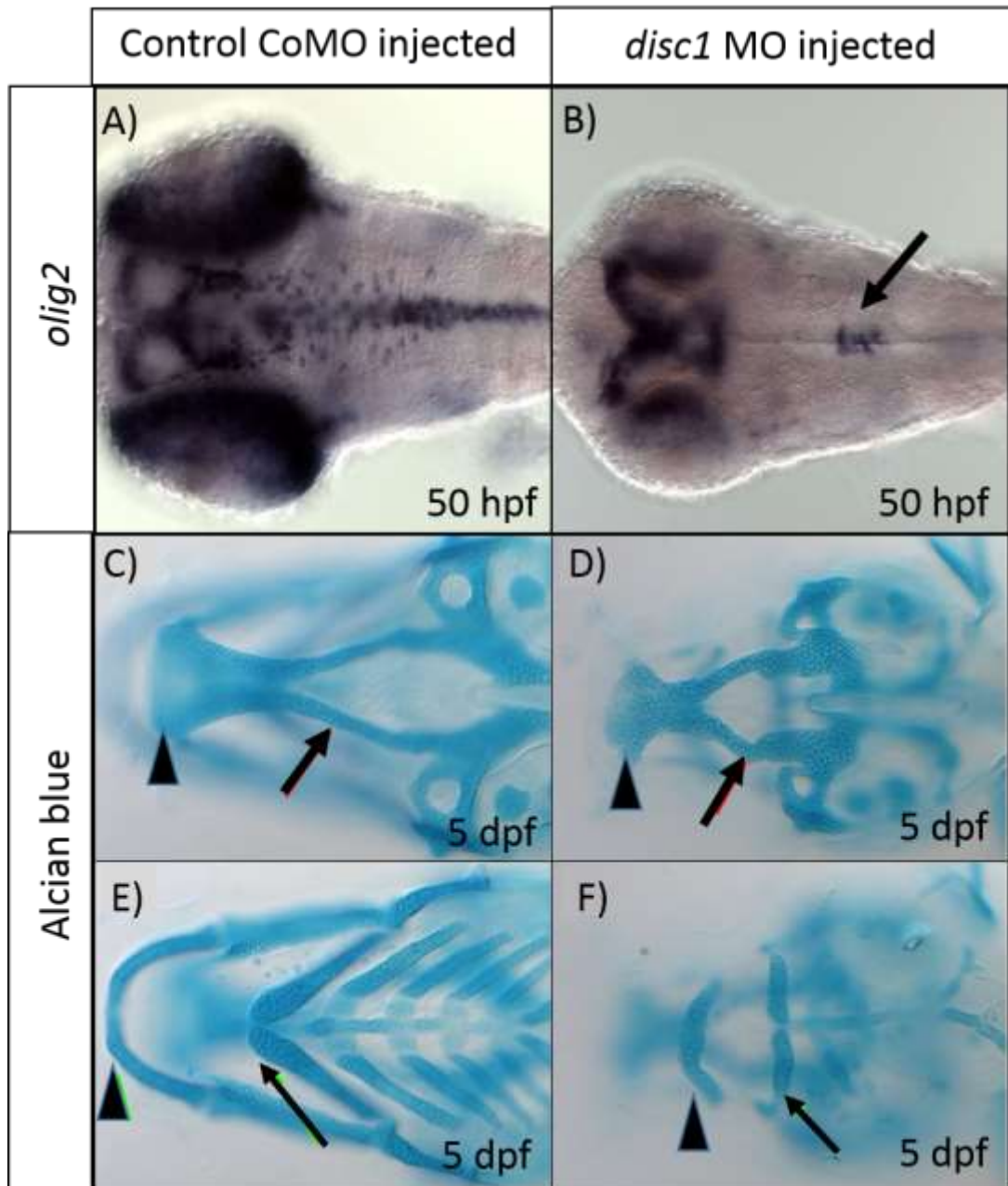
Finally, a role for *disc1* in zebrafish OPC development was described in a study performed in our group at the University of Sheffield, where the author suggested that *disc1* could have active roles in OPC migration (Wood et al 2009). Using *in situ* hybridisation, the expression pattern of *disc1* during development was analysed. Expression at 24 hpf was observed in presumptive neural crest and the otic vesicles and whereas at 50 hpf, expression was found in the developing lower jaw cartilage, otic vesicles and in a strip of midline expression in the hindbrain. This defined strip of expression was very similar to the expression of *olig2* which was intriguing, as it suggested a potential link between *disc1* and oligodendrocyte development (Figure 1-4).



**Figure 1-4: Overlapping expression patterns of *disc1* and *olig2* in the midline of the zebrafish hindbrain**

The expression pattern of *disc1* mRNA (A) in the midline of the zebrafish hindbrain was similar to the expression pattern of *olig2*, a genetic marker of the oligodendrocyte lineage (B) which suggested that *disc1* may have a role in oligodendrocyte development. (Wood et al 2009)

To determine whether *disc1* functions during OPC development, two morpholinos (MO) were designed, a translation blocking and a splice blocking morpholino which were injected into embryos at the one cell stage. Embryos were then allowed to develop until 50 hpf (during the time where *disc1* was normally expressed) and compared against control morpholino-injected embryos. In control embryos, OPCs in the hindbrain had migrated away from their birthplace in rhombomeres 5 and 6 both along the anterior-posterior axis and laterally to populate the hindbrain. However in *disc1* morphants, OPCs were specified but remained restricted to their birthplace and failed to expand to populate the hindbrain (Figure 1-5 A and B). This suggested that *disc1* was not required for the initial specification of OPCs but has functions in later stages of development, perhaps a direct role in migration. Cartilage analysis using Alcian blue staining showed lower jaw defects similar to those documented by Drerup et al (Figure 1-5 E and F).

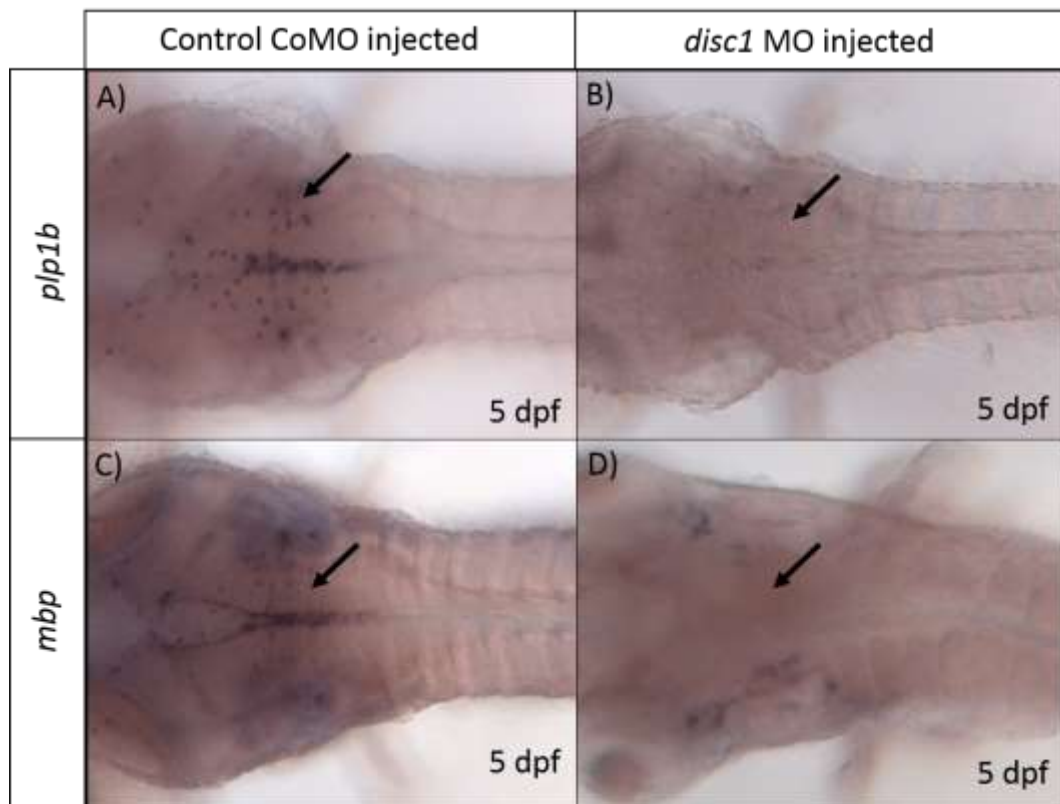


**Figure 1-5: OPC development and lower cartilage defects in *disc1* morphants**

In control embryos, *olig2*<sup>+</sup> positive OPCs migrate from rhombomeres 5 and 6 into the surrounding hindbrain (A). When *disc1* was knocked down using morpholino injections, OPCs were restricted to their birthplace and failed to migrate into the surrounding hindbrain (arrow in B). The *disc1* morphants also displayed lower jaw cartilage defects, although the ethmoid plate and the trabeculae developed normally but were smaller (compare arrows in C with D). The meckels and ceratohyal cartilage were abnormal and other branchial cartilages were absent (Compare E with F). (Wood et al 2009)

At 5 dpf, *disc1* morphants were analysed for genetic markers of differentiated oligodendrocytes and it was observed that expression of OL markers was lost in the

morphants compared to control-injected embryos (Figure 1-6). If OPCs were unable to reach their axonal targets within the hindbrain, then the cells might not be exposed to appropriate signals to initiate differentiation. Whether and how *disc1* contributes to the migration of OPCs was not formally addressed however it could be hypothesised that it works as a cytoskeletal scaffold protein, required to create cytoskeletal protein complexes to drive the physical movement of cells from their birthplace into the surrounding environment.



**Figure 1-6: Expression analysis for *plp1b* and *mbp* show a loss of differentiated oligodendrocytes in *disc1* morphants.**

At 5 dpf, control embryos show expression of *mbp* and *plp1b*, which are markers of oligodendrocyte differentiation and myelination (A and C). In *disc1* morphants there was no expression of these genes suggesting a loss of differentiated oligodendrocytes in the hindbrain (B and D). (Wood et al 2009)

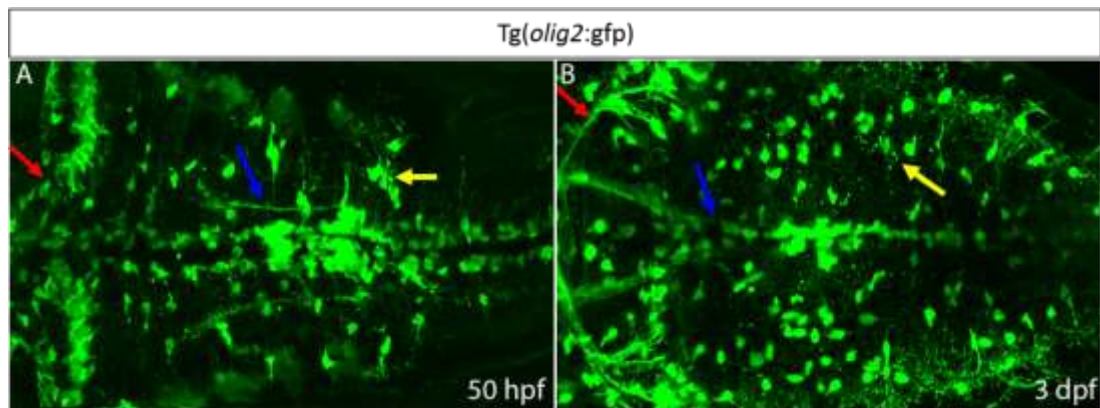
Zebrafish oligodendrocyte hindbrain development has not been well characterised in comparison to zebrafish spinal cord OPC development and so these findings also presented an opportunity to characterise the mechanisms involved in hindbrain OPC development and migration. A handful of studies has investigated the development of this subset of cells but further work was required to identify



similarities and differences between the development of hindbrain OPCs compared with spinal cord OPCs. Further investigation was also required to address evolutionary differences in OPC development between zebrafish and higher vertebrates.

### 1.5.6 OPC development in the zebrafish hindbrain

A study characterising the development of *olig2*-positive cells in the hindbrain was published soon after our group published evidence of a role for *disc1* in OPC hindbrain development (Zannino & Appel 2009). The transcription factor *olig2* is not only a marker of the oligodendrocyte lineage in the zebrafish spinal cord, but also of motor neurons in a common pool of precursors identified as pMN (Takebayashi et al 2000) (Lu et al 2002). This common pool of *olig2* expressing pMNs first gives rise to motor neurons after which oligodendrocytes are specified. In the hindbrain, both motor neurons and OPCs are specified in rhombomeres suggesting that they could arise from a common pool of *olig2*-positive precursor cells as in the spinal cord. The study aimed to address whether *olig2*-positive progenitors in rhombomeres 5 and 6 are a common pool for motor neurons and oligodendrocytes. The group showed that it was the case and these *olig2*-positive cells give rise to abducens motor neurons and some hindbrain OPCs (Zannino & Appel 2009). Furthermore this study demonstrated that the OPCs specified in this region actively migrate away from the birthplace using live imaging techniques. Morphologically, motor neurons and oligodendrocytes can be readily distinguished therefore OPCs can be exclusively analysed using the *tg(olig2:gfp)* line. OPCs undergoing migration contain a cell body with a single or two processes (Figure 1-7 yellow arrows). Whereas abducens motor neurons extend long thin axonal processes anteriorly on either side of the midline (Figure 1-7 blue arrow). Cells remaining in r5 and r6 will contain the cell bodies of abducens motor neurons, neuroepithelial cells and OPCs that have not started to migrate (Figure 1-7).



**Figure 1-7: The *tg(olig2:gfp)* line expresses GFP in many cell types of the hindbrain.** A population of cerebellar neurons is labelled with GFP (red arrows). The abducens motor neurons (blue arrows) are also labelled with GFP and extend long processes anteriorly. OPCs (yellow arrows) also express GFP and have a bipolar morphology during early migration (yellow arrow in A) and then extend fine process indicative of differentiation and myelination events (yellow arrow in B).

Another group recently studied the role of FGF signalling on OPC development in the zebrafish hindbrain and their data suggests that FGF signalling works in conjunction with Shh signalling to control *olig2* expression. Upon chemical inhibition of FGF signalling using SU5402 at 20 hpf before normal specification of the cell type, *olig2* expression was lost in the hindbrain but normally expressed in the spinal cord and midbrain. Similarly *sox10* expression was also reduced in the hindbrain. The authors found no increase in apoptotic cells in the hindbrain of SU5402 treated embryos which suggested that inhibiting FGF signalling does not cause cell death but specifically causes a loss of *olig2* expression and it is likely that cells remain in their progenitor state (Esain et al 2010). The study also showed that in the Shh signalling pathway mutant *smoothened*, *sox10* and *olig2* expression was lost as expected but FGF signalling was unaffected. This provided evidence of an independent role for FGF-signalling in the control of *olig2* expression. Although these studies have provided valuable information regarding the development of OPCs and other cells in the hindbrain further work is necessary to complete the characterisation of hindbrain OPCs.

## 1.6 Hypothesis

Given the established roles of DISC1 in neuronal migration in rodents, it was an interesting observation that in the zebrafish CNS, *disc1* appears to be predominately expressed in oligodendrocyte precursors. Although there has been a recent publication suggesting a direct link between Disc1 and oligodendrocyte differentiation in the mouse, its role in oligodendrocyte development has not been explored in depth (Katsel et al 2011). The zebrafish provides another system in which *disc1* function in non-neuronal brain development can be studied.

Investigating how *disc1* functions in normal OPC development could potentially provide fundamental insight into the oligodendrocyte and white matter abnormalities that have been well documented in schizophrenia and therefore further investigation is warranted. Furthermore, the development of hindbrain OPCs in the zebrafish has not been thoroughly investigated, so addressing the mechanisms controlling their general development in more detail may also provide useful contributions to the field of neurobiology.

The starting hypothesis for this project was:

‘The schizophrenia risk factor, *disc1*, drives OPC migration in the hindbrain of the zebrafish embryo, through regulating cytoskeletal complexes’

## 1.7 Project objectives

This project aimed to characterise general OPC development in the hindbrain over an extended time course, specifically focusing on initial process extension and migration direction. The second aim was to confirm *disc1* expression in OPCs and attempt to elucidate its cellular localisation in OPCs to investigate protein recruitment during migration and to develop a candidate list of interacting partners also involved in OPC migration through similar cellular localisations. Finally we sought to identify novel signalling pathways with roles in OPC development using a medium-throughput *in vivo* drug screen.

# Chapter 2 Material and Methods

## 2.1 Zebrafish husbandry

### 2.1.1 Zebrafish line maintenance

The LWT, AB and Nacre wild-type zebrafish strains were raised and maintained at the Bateson Centre, BMS aquaria, University of Sheffield and the Institute of Molecular and Cell Biology IMCB in an AVA approved zebrafish aquarium, Singapore.

The Tg(*olig2:gfp*) transgenic line (Shin et al 2003), *ptch1/ptch2* (Koudijs et al 2008), *iguana<sup>ts294e</sup>* (Brand et al 1996) and *smu<sup>b641</sup>* (Varga et al 2001) mutant zebrafish lines were maintained at the Bateson Centre, BMS aquaria, University of Sheffield and the Institute of Molecular and Cell Biology, Singapore.

The Tg(*shha:gfp*) transgenic line (Ertzer et al 2007) was maintained at the Institute of Molecular and Cell Biology, Singapore.

Adult Zebrafish were maintained on a 14 hour light/10 hour dark cycle according to standard protocols at 28 °C and were mated using spawning tanks and through pair mating in individual cross tanks (Nüsslein-Volhard & Dahm 2002).

### 2.1.2 Embryo harvesting

Eggs were collected using a plastic tea strainer and transferred to 90mm petri dishes (Sterilin, Newport, UK) in batches of 50 and allowed to develop to the desired developmental stage using standard staging protocols (Kimmel et al 1995). Embryos were raised at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>, 0.0001% methylene blue).

## 2.2 General Molecular Cloning

### 2.2.1 Polymerase chain reaction (PCR)

#### 2.2.1.1 Standard PCR

Each 20 µl PCR reaction contained 10ng of template DNA, 1 µl of Advantage 2 polymerase mix (Clontech), 2 µl 10X buffer, 10pmol/µl of each forward (FWD) and

reverse (RVS) primer and 0.2 mM dNTP mix. Reaction mixtures were kept on ice until they were placed in a thermal cycler (Bio-Rad, DNA Engine Peltier Thermal Cycler). The cycling reaction was performed with the following conditions: template denaturation 95°C for 1 minute followed by 27-30 amplification cycles at 95°C for 30 seconds, 53-61°C for 30 seconds (dependent on primer annealing temperature) and then 68°C for 1 minute per 1 kb of product. Finally, reactions were incubated at 68°C for 10 minutes for proof-reading, after which samples were kept at 4°C.

#### 2.2.1.2 PCR for cloning

Each 50 µl reaction contained 50-100ng plasmid DNA or 1 µl of a cDNA synthesis reaction, 1 µl of expand high fidelity enzyme mix (Roche), 5 µl of 10X expand high fidelity buffer containing 15 mM MgCl<sub>2</sub>, 10 pmol/µl of each FWD and RVS primer and 0.2mM dNTP mix. The cycling reaction was performed with the following conditions: 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 53-61°C for 30 seconds (dependent on primer annealing temperature) and then 72°C for 1 minute per 1kb of product. Finally the reactions were incubated at 72°C for 10 minutes, after which samples were held at 4°C.

#### 2.2.2 Purification of PCR products

PCR-amplified DNA was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. DNA was eluted in 30 -50 µl nuclease free sterile water.

#### 2.2.3 Gel extraction

DNA fragments were electrophoresed in a 1% agarose gel and subsequently extracted using the QIAquick Gel Extraction Kit (Qiagen) according the manufacturer's protocol. DNA was eluted in 30-50 µl nuclease free sterile water.

#### 2.2.4 Restriction endonuclease digestion

Restriction endonuclease reactions were performed in 20–50 µl volumes using 1 unit of the appropriate restriction enzyme for every 1 µg of DNA using the required buffer in sterile water. The reaction was incubated at the temperature specified by

the manufacturer (New England Biolab) for 2-4 hours. DNA was then purified using the PCR purification or gel extraction protocols outlined previously (Section 2.2.2 and 2.2.3).

## 2.2.5 Cloning

### 2.2.5.1 Standard cloning

DNA was amplified through PCR using modified primers containing the required endonuclease restriction sites, then the product was purified and digested using the appropriate restriction enzymes. At the same time approximately 50 ng of the required vector was also digested with the same restriction endonucleases. Both the vector and PCR product were gel extracted and purified. The vector was then treated with Antarctic Phosphatase (New England Biolabs) to prevent recircularisation. A ligation reaction was then set up containing approximately a 3:1 molar ratio of PCR product to vector in a final volume of 10µl containing T4 DNA ligase and ligase buffer (Roche). The ligation reaction was left for 1 hour at room temperature and then 1-2µl was transformed into chemically competent *E.coli* (see section 2.2.6). The next day, single colonies were prepped, grown in liquid culture and plasmid DNA purified using the QIAprep Spin Miniprep Kit (Qiagen). To screen for positive inserts plasmid DNA was digested with the same restriction endonucleases as with the ligation reaction, and fragments were then electrophoresed and examined for 2 bands containing the correct sized vector and insert bands.

### 2.2.5.2 TOPO cloning

DNA was amplified by PCR (section 2.2.1.2) and purified (section 2.2.2), Taq polymerase leaves terminal deoxyadenosine (A) overhangs on the 3' end of the PCR product which is utilised by the TOPO® TA Cloning® system (Life Technologies). PCR products were gel extracted and a ligation reaction set up containing 0.5-4 µl of PCR product, 1 µl of pCR™II-TOPO® vector or pCR™2.1-TOPO® vector, 1 µl of salt solution made up to 6 µl with nuclease free water. The reaction was left at room temperature for 15 minutes and then kept on ice prior to bacterial transformation. 1-2 µl of the reaction was transformed into *E.coli* which was spread on LB-agar

plates containing the relevant antibiotic for selection that had previously been spread with 50µl X-gal for blue white colony screening. White colonies were selected and screened for positive inserts as outlined in the previous section (2.2.5.1).

## 2.2.6 Transformation of competent cells

For each single transformation, 1 vial containing 50-100 µl of chemically competent cells *E.coli* DH5α was used. The cells were placed on ice to thaw, after which 10-20 ng of DNA was added and mixed gently with the cells which were then kept on ice for 30 minutes. The cells were then heat-shocked at 42.2°C for 1 minute and returned to ice for 10 minutes. After that 250 µl of LB media (without antibiotic) was added and the cells were placed in a 37°C shaking incubator for 1 hour. 50-100 µl of culture was then spread on a pre-warmed agar plate containing the required antibiotic. Plates were then incubated overnight at 37°C. The next day individual colonies were picked and grown in LB media containing the required antibiotic overnight in a 37°C shaking incubator. The next day, plasmid DNA was isolated using the QIAprep Spin Miniprep Kit or the QIAGEN Plasmid Midi Kit (Qiagen) and eluted in 50-500µl nuclease-free sterile water.

## 2.2.7 RNA extraction

### 2.2.7.1 Trizol method

500 µl of Trizol reagent (Life Technologies) was added to 30 dechorionated embryos and the embryos were lysed by passing through a hypodermic needle to disrupt the tissue. A further 500 µl of Trizol was then added followed by incubation for 5 minutes at room temperature. After this, 200 µl of chloroform was added, and the tube was shaken vigorously for 15 seconds and then incubated for 3 minutes at room temperature. Subsequently the tube was centrifuged at 12,000g at 4°C for 15 minutes; the upper aqueous phase was transferred to a fresh tube and the RNA was precipitated by mixing with 500 µl of isopropyl alcohol. The sample was incubated for 10 minutes at room temperature and then centrifuged at 12,000g for 10 minutes at 4°C to pellet the RNA. The RNA pellet was then washed with 1ml of cold 75% ethanol. The pellet was air-dried and dissolved in 20 µl of DEPC-treated water.

#### 2.2.7.2 RNA extraction using the RNeasy Mini Kit

As an alternative to the Trizol RNA extraction protocol, RNA was extracted using the Qiagen RNeasy Mini Kit according to the manufacture's protocol.

#### 2.2.8 First strand cDNA synthesis

2-3µg of RNA was added to a 10µl reaction containing 0.05ng of oligo(dT)<sub>20</sub> and 10ng of dNTP mix. The reaction was mixed and incubated for 5 minutes at 65°C, after which it was cooled on ice for 1 minute. The following was then added to the reaction: 2 µl of 0.1M DDT, 2 µl 10x reverse transcriptase reaction buffer, 40 units of RNaseOUT and 200 units of Superscript III reverse transcriptase (Life Technologies, Invitrogen), then the samples were mixed by pipetting and incubated for 60 minutes at 50°C. The reaction was then terminated at 85°C for 5 minutes, after which 1 µl RNaseH was added and incubated for 20 minutes at 37°C to destroy the RNA. The cDNA samples were then stored at -20°C.

#### 2.2.9 Quantitative Polymerase Chain Reaction (qPCR)

12.5 ng of cDNA was added to a 10 µl reaction containing 0.5 µl of 20X PrimeTime Assay (Integrate DNA technologies (IDT)) primers, 5µl of 2X Brilliant III ultra-fast qPCR master mix (Aligent) and 3.5µl of nuclease free water in 96 well plates. Quantitative PCR was performed using the BioRad C1000 touch thermal cycler CFX96 Real time system. Prime time Mini qPCR assay primers were designed using the IDT real time PCR tool and did not require prior optimisation (<http://eu.idtdna.com/Scitools/Applications/RealTimePCR/>). All reactions were denatured at 95°C for 3 minutes followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds. Data was exported and analysed on the Bio-Rad CFX Manager, after which data were exported onto Microsoft Excel and processed.

#### 2.2.10 Digoxigenin- labelled RNA probe synthesis

Digoxigenin (DIG)-labelled anti-sense RNA probes were generated through *in vitro* transcription. At least 1 µg of linearized plasmid DNA was required as the template in a 20µl reaction which also contained 2 µl 10X DIG labelling mix, 2 µl 10X reaction buffer, 1 µl of RNasin (RNase Inhibitor) and 2 µl of RNA polymerase (T7,T3 or SP6)



in nuclease free water. The reaction was then incubated for a minimum of 2 hours at 37°C, then 1 µl of RNase free DNase was added and returned to 37°C for 15 minutes to destroy the template DNA. One microliter of 0.5M EDTA (pH8.0) was added and mixed gently to stop the reaction, then 2.5 µl 4M LiCl and 75µl of cold EtOH followed by gentle mixing. This solution was kept at -20°C overnight to precipitate the RNA. The next day the tube was centrifuged at 12,000 rpm for 30 minutes at 4°C, the supernatant was carefully discarded and the pellet was washed in 1ml of 70% EtOH and centrifuged again for 10 minutes at 12,000 rpm at 4°C. The supernatant was carefully removed to leave the RNA pellet which was air dried and dissolved in 20 µl DEPC-treated water. DIG-labelled RNA probes were stored at -80°C in aqueous solution or at -20°C if diluted in hybridisation A solution.

### 2.3 Microinjection

For microinjection, adult pairs were set up using barriers in a pair mating tank. The barriers kept the females and males apart thereby controlling when they mated. This meant that fresh 1 cell stage embryos could be obtained throughout the duration of microinjection allowing for maximum numbers of embryos to be injected. Glass capillaries (Harvard apparatus, GC120T-10) were individually placed in a flaming/Brown p-97 micropipette puller which used a heated filament to pull a single glass capillary into two microinjection needles. The needle was filled with the appropriate solution using Eppendorf microloader pipette tips, and the needle end was blunted using sharp forceps. The microinjector was calibrated using a graticule, after which embryos were harvested and brushed individually in wells of a 1.5% agarose mould. Embryos were aligned to have the cell away from the injection manipulator so that the needle entered the embryo through the yolk and injected into the cell to minimise damage. Embryos were injected in rows of 25 and batches of 100 in the first 30 minutes of development, after which they were placed in fresh egg water and left to develop at 28°C. Un-injected embryos were kept as controls for survival and fresh embryos were collected for each injection round.

### 2.3.1 Morpholino antisense oligonucleotides

Morpholinos were designed and manufactured by Gene Tools, LLC (Philomath, OR, USA). They were provided in lyophilised form and resuspended with sterile water into a 1mM stock solution (except for p53 MO which was prepared as a 2mM stock). Sequences were as follows:

disc1aug: 5'-CCTGACCATTCTGCGAACATCATG-3'

olig2aug: 5'-CGTTCAGTGCGCTCTCAGCTTCTCG-3'

p53MO: 5'-GCGCCATTGCTTTGCAAGAATTG-3'

CoMO (human beta globin): 5'-CCTCTTACCTCAGTTACAATTTATA 3'

### 2.3.2 Capped mRNA

Capped mRNA was synthesised using mMMESSAGE mMACHINE® kit (Ambion) according to the manufacturer's instructions and was injected at a concentration of 25-100 ng/μl in DEPC-treated water.

### 2.3.3 Plasmid DNA

Plasmid DNA containing I-SceI sites was linearized in a 5 μl reaction containing 750 ng of DNA, 0.5 μl of I-SceI buffer and 0.5 μl of I-SceI enzyme. The reaction was incubated for 30 minutes at room temperature before being injected at a concentration of 25-50 ng/μl.

### 2.3.4 TALEN mRNA and donor DNA

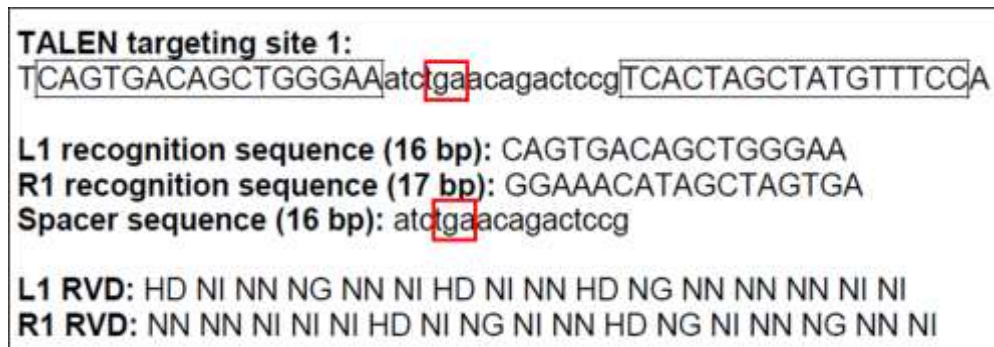
TALEN mRNA and donor DNA was co-injected into embryos. TALEN mRNA was injected at a concentration of 150 ng/μl (per each arm) and while linearized donor DNA was injected at 50 ng/μl.

## 2.4 TALEN-based genome editing

### 2.4.1 TALEN arm synthesis

TALEN left and right arm plasmids were designed and synthesised by ZGENEBIO, Taiwan. The *disc1* gene was targeted around the TGA STOP codon at the 3' end of the gene. A 16bp TALEN left arm was synthesised on the 5' side of the TGA stop in

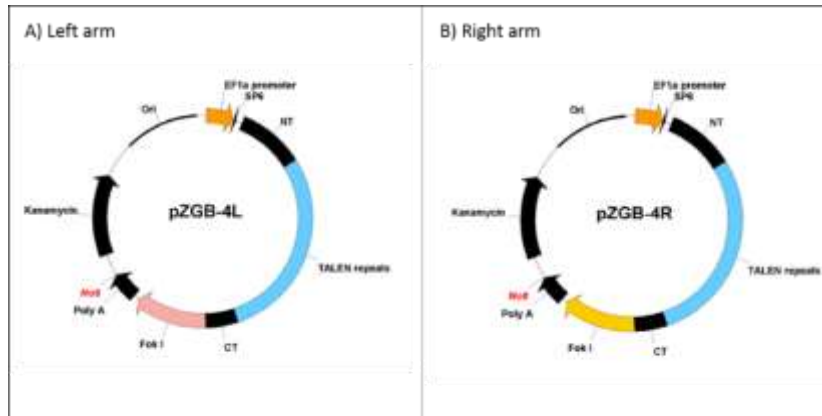
exon 14 and a 17bp TALEN right arm was synthesised on the 3' side of the TGA stop in the 3' UTR. The spacer sequence of 16bp between the two arms was the target region where double strand breaks (DSB) should be induced (Figure 2-1).



**Figure 2-1: Sequence of the TALEN targeting site in the 3' disc1 gene**

The TALEN target site is situated in the 3' end of the gene, the spacer sequence contains the stop codon of the *disc1* gene (shown in the red box). Repeat variable diresidues (RVD) are responsible for the recognition of a specific nucleotide.

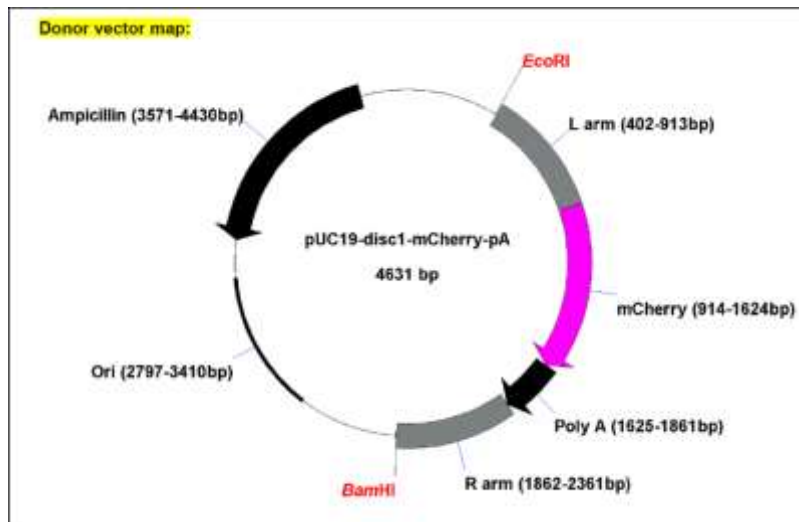
The TALEN arm plasmids were sent spotted on filter paper and were retransformed into *E.coli* and propagated on LB-kanamycin plates (Figure 2-2).



**Figure 2-2: TALEN arm vector map information**

### 2.4.2 Donor DNA synthesis

The donor DNA was designed and synthesised by ZGENEBIO, Taiwan. Left and right 500bp homology arms were designed around a mCherry and poly A tail sequence (Figure 2-3). Donor DNA was sent on filter paper and retransformed on an ampicillin plate. DNA was linearized with *EcoR1* and *BamHI* for 2 hours, then gel extracted and purified using a QIAquick Gel Extraction Kit (Qiagen).



**Figure 2-3: Donor DNA vector map information**

The donor DNA vector contained a mCherry sequence with a polyA tail. On either side of the fluorescent sequence was the left and right 500 bp *disc1* homology arms (labelled as L arm and R arm).

### 2.4.3 TALEN mRNA synthesis

The left and right TALEN arms were linearized using *Not1* (section 2.2.4) and purified (section 2.2.2). This linearized purified DNA, was used as a template for an *in vitro* transcription reaction (section 2.3.2) using SP6 polymerase. Capped mRNA was synthesised using the mMESSAGE mMACHINE<sup>®</sup> kit (Ambion) according to the manufacturers protocol. Aliquots were stored at -80°C.

### 2.4.4 Screening for DSBs

Wildtype AB embryos were injected with the left and right TALEN mRNAs at a range of concentrations and DNA was extracted from batches of 30 pooled embryos harvested between 24-48 hpf using 20 0µl of DNA extraction buffer. A 453 bp region surrounding the STOP codon of *disc1* was amplified. 10 µl of the PCR product was added to a 20 µl reaction containing 1 µl of T7 endonuclease and 2 µl 10X NEB2 buffer (New England Biolabs) which was then incubated at 37°C for 1 hour. Samples were electrophoresed on a 3% agarose/TBE gel; if samples had 2 products, the same genomic DNA was re-amplified using PCR and cloned using the TOPO<sup>®</sup> TA Cloning<sup>®</sup> system (Life Technologies). The TOPO reaction was transformed into *E.coli* and positive white colonies selected for sequencing. Sequences were aligned with

the wild type *disc1* sequence

([http://www.ensembl.org/Danio\\_rerio/Gene/Summary?db=core;g=ENSDARG0000021895;r=13:50035365-50123165](http://www.ensembl.org/Danio_rerio/Gene/Summary?db=core;g=ENSDARG0000021895;r=13:50035365-50123165)) using FinchTV (Geospiza) and DNASTAR-

lasergene SeqMan Pro to analyse the rate of INDELS induced by the TALEN mRNA.

## 2.5 Antibody development

Zebrafish Disc1 monoclonal antibodies were developed by Abmart using their SEAL technology against the peptide EDNLPVQSRD (residues 119-128). In total 6 different antibodies were developed against the same peptide. Ascites fluid was provided in lyophilised form and was resuspended in 50% glycerol and stored at -20°C.

Antibody reference name	Concentration (mg/ml)	Volume (ul)
2D24	36.5	100
2I13	15.6	500
6D7	19.8	500
6G10	26.4	500
7A10	18.0	100
7F14	20.8	500

**Table 3: Antibodies developed against zebrafish disc1**

Antibodies were tested by western blot and immunofluorescence analysis using various concentrations and conditions to analyse the efficiency of the antibodies to detect endogenous and over-expressed Disc1.

## 2.6 Cryostat sectioning

Embryos were first fixed in 4% PFA overnight at 4°C and then mounted in a 1.5% low melting point (LMP) agarose and 5% sucrose solution. Once the agarose had set, embryos in agarose were placed in a 30% sucrose solution overnight at 4°C. Samples were then frozen in optimal cutting temperature (OCT) compound (Agar Scientific) and then sectioned on a cryostat taking 20 to 40 µM sections. The sections were then baked onto slides for 2 hours at 37°C and stored at -20°C.

## 2.7 5-bromo-2-deoxyuridine (BrdU) pulse labelling

Embryos were transferred from 5, 10 and 15% DMSO in E3 medium before being incubated for 20 minute on ice in E3 medium containing 2 mg/ml BrdU in 15% DMSO. Embryos were then returned to E3 medium for 20 minutes after which embryos were fixed in 4% PFA overnight at 4°C. To detect BrdU incorporation embryos were dehydrated in 100% MeOH and were stored at -20°C for at least 1 hour. Embryos were then rehydrated through a PBS/MeOH series after which they were washed in PBS twice for 5 minutes. Embryos were then permeabilised in 10 µg/ml Proteinase K for a period of time dependent on their development stage (24 hpf for 10 minutes, 36 hpf for 15 minutes, 48 hpf for 20 minutes). Embryos were rinsed in glycine (2 mg/ml in PBS) twice and were then washed in PBS for 5X5 minutes. The embryos were then re-fixed in 4% PFA for 20 minutes, then washed for 5X 5 minutes in water and were then incubated in 2N HCL made from concentrated HCL for 1 hour. Embryos were then washed for 6X15 minutes PBXT and were then blocked in 10% normal goat serum (NGS) for 1 hour. Embryos were then incubated in 1:200 primary BrdU antibody (Sigma) in 5% NGS in PBXT overnight and the following day were washed for 6-15 minutes in PBXT before being incubated overnight in secondary antibody in 2% NGS in PBXT. The next day embryos were washed for 6X15 minutes in PBXT before being re-fixed in 4% PFA prior to dissection and imaging (Lyons et al 2005).

## 2.8 *In situ* hybridisation

### 2.8.1 Whole mount chromogenic *in situ* hybridisation

Embryos were fixed overnight at 4 °C in 4% PFA and then washed in PBST for 3x 10 minutes before being transferred into 100% Methanol (MeOH). Embryos were then stored overnight at -20°C but can be stored in 100% MeOH at -20 °C indefinitely until use. Embryos were rehydrated in 50% MeOH in PBS which was followed by 5X 5 minute washes in PBST. Embryos were then permeabilised in 10 µg/ml proteinase K for a period of time dependent on their developmental stage (<15 somites for 1-2 minutes, 15-25 somites for 5 minutes, 24 hpf for 10 minutes, 36 hpf for 15 minutes, 48 hpf for 20 minutes and 72 hours for 40 minutes). Embryos were then re-fixed in

4 % PFA for 20 minutes followed by 5X 5 minute washes with PBST. Embryos were then rinsed in pre-heated hybridisation buffer at 70°C and then placed in fresh hybridisation buffer at 70°C for >3 hours. Embryos were then hybridised overnight at 70°C in the appropriate DIG labelled RNA probe. Embryos were washed at 70°C in 50:50 hybridisation solution: 2XSSC for 20 minutes, 2XSSC for 20 minutes, followed by 2X 60 minute washes in 0.2XSSC. Embryos were then washed at room temperature (RT) for 10 minutes in 50:50 0.2XSSC: PBT followed by a 10 minute wash in PBT. Embryos were blocked in PBT for 3 hours at RT and then incubated overnight at 4°C in 1:2000 anti-DIG alkaline phosphatase (Roche). The following day embryos were washed for 6X 20 minutes in PBST and then equilibrated in AP staining buffer without NBT/BCIP for 3x 15 minutes. Embryos were then stained in the dark in AP staining buffer containing 3.5 µl/ml BCIP and 4.5 µl/ml NBT until stained to the desired level of intensity and were then rinsed in AP staining buffer lacking NBT/BCIP before being washed in PBST for 3X 5 minutes. Embryos were then fixed in 4% PFA overnight at 4°C before being transferred through a glycerol/PBS series and stored in 80% glycerol/20% PBS. Mutant and sibling embryos were treated the same during *in situ* hybridisation and stained in staining buffer for the same length of time.

### 2.8.2 TSA fluorescent *in situ* hybridisation

Fluorescent *in situ* hybridisation was carried out using the Tyramide Signal Amplification (TSA™) system from Perkin Elmer. Day 1 of *in situ* hybridisation was performed as previously described (section 2.8.1). One day 2, the 2XSSC washes were also the same, however after these washes embryos were placed in 50:50 2XSSC: PBST for 10 minutes and afterwards for 10 minutes in PBT. Embryos were then blocked for 3 hours at RT in Maleate buffer blocking reagent before being incubated in 1:500 anti-DIG POD (Roche) overnight at 4°C. The next day, embryos were rinsed in PBST and then washed for 6X 15 minutes in PBST. Embryos were then equilibrated in TNT for 3X 5 minutes. Embryos were then stained with 1:200 TSA™ DNP (HRP) in amplification diluent for 1 hour. The reaction was stopped by washing the embryos 3x 10 minutes in TNT. Embryos were then blocked in Maleate buffer blocking reagent for 3 hours after which they were incubated in anti-DNP

POD at 1:1000 (Perkin Elmer) overnight at 4°C. Embryos were then rinsed in PBST and then washed for 6X 15 minutes in PBST. Embryos were equilibrated in TNT for 3X 5 minutes before being incubated in 1:200 TSA™ Plus Cyanine 3 in amplification dilutant. The reaction was stopped by washing embryos in TNT for 3X 5 minute. Embryos were then washed for 4X 15 minutes in PBST and then transferred into 4% PFA for 30 minutes. Finally, embryos were washed in PBST for 5x5 minutes after which embryos were ran through a glycerol series and stored in 80% glycerol/20% PBS.

### 2.8.3 Antibody staining after *in situ* hybridisation

The *in situ* hybridisation stain was developed as normal and then embryos were washed in PBST and fixed for 20 minutes in 4% PFA at RT. Embryos were then washed for 5x5 minutes in PBST and then incubated in PBDT blocking solution for 1 hour at RT, followed by incubation overnight at 4°C in primary antibody diluted in PBDT. The following day, embryos were washed for 4X 30 minutes in PBDT followed by incubation in the secondary antibody for 2 hours at RT. Embryos were washed for 6X 15 minutes in PBDT, then ran through a glycerol series before being stored in 80% glycerol in PBS.



## 2.8.4 *In situ* Hybridisation RNA probes

Probe name	Gene	RE/polymerase	Reference
<i>disc1</i>	<i>Disrupted in schizophrenia-1</i>	<i>Spe1/T7</i>	Wood et al 2009
<i>olig2</i>	<i>Oligodendrocyte transcription factor-2</i>	<i>Sma1/T7</i>	IMAGE: BC06559
<i>sox10</i>	<i>SRY-box 10</i>	<i>Sal1/T7</i>	IMAGE:7168235 (Dutton et al 2001)
<i>nrg-1</i>	<i>Neuregulin-1</i>	<i>Ecor1/T7</i>	IMAGE: 8339257
<i>hhp</i>	<i>Hedgehog interacting protein</i>	<i>Not1/T7</i>	(Koudijs et al 2005)
<i>fabp7a</i>	<i>Brain fatty acid binding protein</i>	<i>Not1/SP6</i>	This thesis
<i>pafah1b1b</i> (previous name <i>Lis1a</i> )	<i>platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit b</i>	<i>Not1/SP6</i>	This thesis
<i>pafah1b1a</i> (previous name <i>lis1b</i> )	<i>platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit a</i>	<i>Not1/SP6</i>	This thesis
<i>ndel1a</i>	<i>nudE nuclear distribution gene E homolog like 1 (A. nidulans) A</i>	<i>Not1/SP6</i>	This thesis
<i>ndel1b</i>	<i>nudE nuclear distribution gene E homolog like 1 (A. nidulans) B</i>	<i>Not1/SP6</i>	This thesis

**Table 4: Riboprobes used for *in situ* hybridisation**

## 2.9 Immunohistochemistry

### 2.9.1 Whole mount Immunohistochemistry

Embryos were fixed for 2 hours at RT, then washed for 5X 5 minutes in PBXT. (Alternatively, embryos were fixed for either 2 hours or overnight at 4°C, washed for 5x 5 minutes in PBS and stored in 100% MeOH at least overnight at -20°C. Embryos were then rehydrated in serial dilutions of MeOH in PBS (75%, 50% and 25% MeOH) and washed 5x 5 minutes in PBXT). Embryos over 2 dpf then underwent acetone cracking. Embryos were placed in 100% acetone and incubated at -20°C for 20-30 minutes dependent on their developmental stage, after which they were washed 5X 5 minutes in PBXT and then blocked in PBDT blocking solution for >1 hour at RT. Embryos were then incubated overnight at 4°C in primary antibody diluted in PBDT, followed by 4X 30 minute washes the next day. Embryos were then incubated in secondary antibody diluted in PBDT for 2 hours at RT or overnight at 4°C. Embryos were washed for 4X 30 minutes in PBDT and transferred into 80% glycerol/20% PBS.

### 2.9.2 Immunohistochemistry on sections

Slides were defrosted at RT for >30 minutes after which sections on the slide were circled with a PAP pen (Abcam) and allowed to dry for >30 minutes. Sections were rinsed twice in PBDT and then washed for 3X 5 minutes in PBDT. Slides were blocked for 1 hour at RT in PBDT blocking buffer after which 500 µl of primary antibody diluted in PBDT was pipetted onto each slide and incubated for 1-2 hours in a humidified chamber at RT. Slides were then washed for 5x 5 minutes in PBDT and then 500 µl of secondary antibody diluted in PBDT was pipetted onto each slide which was then incubated for 1 hour at RT in a humidified chamber in the dark. Slides were washed for 5X 5 minutes in PBDT in the dark, after which 2 drops of Vectashield (Vector Laboratories) were added to each slide prior to being covered with a coverslip. Each coverslip was held in place by clear nail polish that was applied around the edges.

### 2.9.3 Primary Antibodies

Epitope/antibody	Staining dilution	Supplier/reference
Rabbit Arl13b	1:500	(Duldulao et al 2009)
Mouse mCherry	1:500	Living Colours
Rabbit GFP	1:1000	Torrey Pines
Mouse acetylated tubulin	1:500	Sigma
Mouse gamma tubulin	1:500	Sigma
Mouse zrf-1	1:1000	ZIRC
Mouse BrdU	1:200	Sigma

**Table 5: Primary antibody information used in immunohistochemistry**

### 2.9.4 Secondary Antibodies

Alexa fluor Secondary antibody	Concentration	Supplier/reference
Alexa fluor 488 donkey anti-rabbit	1:500	Abcam
Alexa fluor 488 donkey anti-mouse	1:500	Abcam
Alexa fluor 555 donkey anti-rabbit	1:500	Abcam
Alexa fluor 555 donkey anti-mouse	1:500	Abcam

**Table 6: Secondary antibody information used in immunohistochemistry**

## 2.10 Mounting and imaging

Embryos stored in 80% glycerol/20% PBS were transferred into a cavity dish and dissected under a Leica dissecting microscope using fine needles. Windows were created in electrical tape stuck on microscope slides, where a central square was cut and the specimen placed in the centre in 80% glycerol/20% PBS for light microscopy or Vectashield for fluorescence imaging. The number of layers of electrical tape used was dependent on the thickness of the specimen to be imaged. A coverslip was placed over the embryo prior to imaging.

For light microscopy, images were obtained using a Leica DM2500 compound microscope fitted with a Leica DFC-420C digital camera, or a Carl Zeiss AXIO Zeiss Imager M2 and AXIO Vision 4.7.2 software. An Olympus FV-1000 confocal microscope was used to capture confocal images utilising the Olympus FluoView FV-1000 ASW 1.6 software. Images were then compiled into composite figures using ImageJ (National institutes of Health) and Adobe Photoshop CS6.0 software.

## 2.11 Cyclopamine treatments

Dechorionated embryos were treated in E3 containing the appropriate concentration of cyclopamine (20, 40 or 60  $\mu$ M) diluted from a stock concentration of 20 mM in DMSO (LC Laboratories). Embryos were treated from 34 hpf or 38 hpf and kept in cyclopamine solution until the desired time point. Embryos were then fixed in 4% PFA.

## 2.12 Bead implantation assay

### 2.12.1 Shh protein preparation

Recombinant mouse Sonic Hedgehog (Shh) N-terminus (R&D systems) was reconstituted to 25  $\mu$ g/ml in 0.1% BSA in PBS, 20  $\mu$ l aliquots were prepared and stored at -80°C.

### 2.12.2 Bead preparation

CM Affi-Gel<sup>®</sup> Blue Gel agarose beads (Bio-Rad) were washed in PBS and the smallest beads collected for implantation. Ten agarose beads were placed in a petri dish lid after which excess PBS was removed and beads were soaked in 15  $\mu$ l of 25  $\mu$ g/ml recombinant mouse Shh N-terminus overnight at 4°C.

### 2.12.3 Implantation

Tg(*olig2:gfp*) embryos at 34 hpf were dechorionated and then anaesthetized with tricaine. Embryos were placed on 1.5% LMP-agarose and a small incision was made into the dorsal hindbrain using minute pins (Fine Science Tools). The minute pin was used to pick up a single agarose bead and the bead was inserted into the hindbrain

where the incision was made. Embryos were then placed in fresh egg water, left to develop until 50 hpf and were fixed in 4% PFA.

## 2.13 Drug screening assay

### 2.13.1 Control drug assay

Embryos were exposed at different developmental stages to positive control compounds EGFR-inhibitor I and EGFR-inhibitor II (Calbiochem) the former of which has been previously shown to affect OPC development (Wood et al 2009). Embryos were harvested and allowed to develop up to 26, 28, 30 or 32 hpf before being exposed to either 50  $\mu$ M EGFR-inhibitor I or EGFR-inhibitor II separately in 1% DMSO in 5 ml of E3 medium. Embryos were incubated at 28°C and fixed in fish fix at 50 hpf.

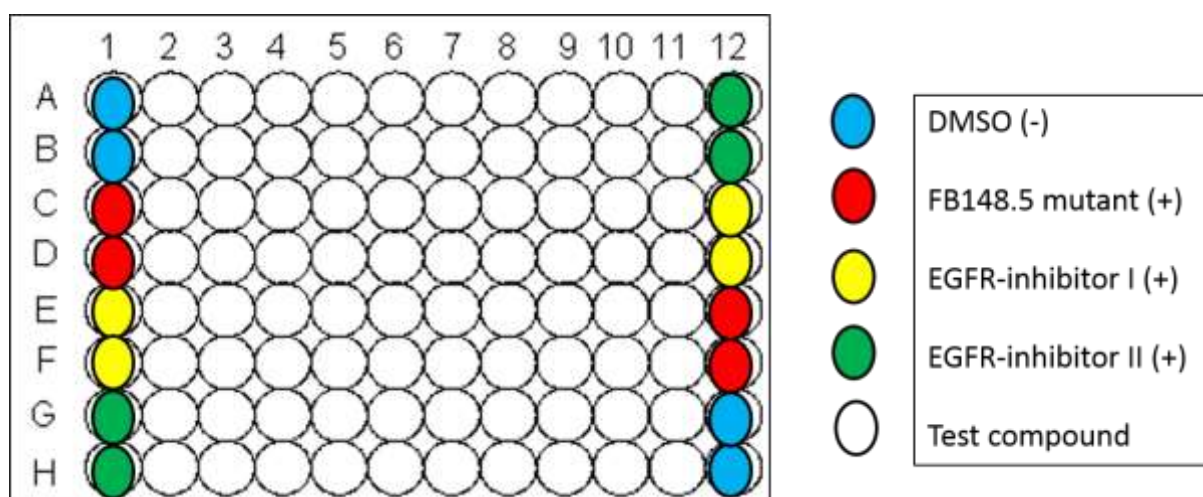
### 2.13.2 Embryo and plate preparation

Nacre wild-type embryos were treated with 10 mg/ml of Pronase (Sigma) at 24 hpf for 15 minutes at 28°C to remove their chorions, and transferred into fresh E3 medium. The embryos were left to recover for an hour and were then transferred into 96 well mesh bottom plates, with 3 embryos per well. The embryos were then put into E3 medium containing the chemical compounds (in DMSO) at 28 hpf and kept overnight in a 28°C incubator. At 50/51 hpf the embryos were fixed in Fish Fix (25 ml per plate) overnight at 4°C. The next day the embryos were bleached (30 ml per plate) for 20 minutes to remove pigment in the eyes and head and were then transferred into 50:50 PBS: MeOH before being stored in 100% MeOH at -20°C. Dependent on the yield of embryos, approximately 2 to 4 plates were screened each week.

### 2.13.3 Spectrum library drug preparation

The drug screen utilised the Spectrum library (Microsource delivery system), copy plate number 6, which contained a collection of 2000 compounds at 2.5 mM. The drug plates were in a 96 well format; 247.5  $\mu$ l of E3 Medium (lacking methylene blue) and 2.5  $\mu$ l of compound were added to each well, to give a final test concentration of 25  $\mu$ M. Rows 1 and 12 contained control samples and rows 2-11

contained test compounds from the Spectrum library 2. The mesh bottom plates containing the embryos were placed into the plastic bottom plates holding the compound and E3 solution, thereby exposing the embryos to the drugs. A total of 25 plates were used in the primary drug screen (Figure 2-4).



**Figure 2-4: Set up of screening plate**

Rows 1 and 12 contained controls, blue well represent the DMSO controls. The red well contained F8148.5 mutants which act as a positive control for decreased migration. The yellow and green EGFR-inhibitor I and II which also act as a positive control for decreased migration. Rows 2-11 contained the spectrum library compounds (25  $\mu$ M of the compound in each well). A total of 25 plates were used to test the entire spectrum library all of which were prepared the exact same.

#### 2.13.4 Automated *in situ* hybridisation staining

Embryos were kept in the mesh bottom plates throughout the *in situ* hybridisation staining process (see chapter 2.6.1). All solutions were made up manually but the washes were carried out using the Biolane robot using a standard *in situ* hybridisation protocol. Four 96 well plates could be processed at one time using the Biolane robot. All washes and blocking was performed in the Biolane robot, however the incubation in the DIG-*olig2* riboprobe diluted in hybridisation A solution and staining in AP buffer containing NCIP/BCIP was done manually. Once the desired level of staining had been reached embryos were fixed in fish fix and stored at 4°C. The embryos were transferred to 80% glycerol/20% PBS prior to imaging.



**Figure 2-5: Biolane Robot for automated an *olig2 in situ* hybridisation**

The Biolane robot was used during the drug screen to analyse for OPC migration defects. The Biolane robot could stain 4 plates at a time (two plates on the left hand side with the blue tubes and two plates on the right hand side with the red tubes). Solutions were made up manually and attached to the Biolane robot which was programmed to carry out the automated *in situ* hybridization over 3 days.

## 2.14 Solutions and buffers

### 2.14.1 General buffers and solutions

E3 medium	15 mM NaCl, 0.5 mM KCl, 1 mM CaCl <sub>2</sub> , 1 mM MgSO <sub>4</sub> , 1.5 mM KH <sub>2</sub> PO <sub>4</sub> , 0.05 mM Na <sub>2</sub> HPO <sub>4</sub> , 0.7 mM NaHCO <sub>3</sub> and 0.0001% Methylene Blue per litre
Fish Fix	0.1 M Phosphate buffer, 0.12 mM CaCl <sub>2</sub> , 4% sucrose (w/v), 4% paraformaldehyde (PFA) powder (w/v)
Phosphate Buffered Saline (PBS)	1 PBS tablet in 200 mls H <sub>2</sub> O (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na <sub>2</sub> HPO <sub>4</sub> , 1.47 mM KH <sub>2</sub> PO <sub>4</sub> , final pH 7.4)
Bleaching Solution	3% H <sub>2</sub> O <sub>2</sub> , 0.5% KOH in PBS
Diethylpyrocarbonate (DEPC) treated water	0.1% DEPC (1 ml in 1l H <sub>2</sub> O) incubated at 37°C overnight then autoclaved 15 minutes at 121°C
PBST	PBS with 0.1% Tween20 (v/v)
DNA extraction buffer	10mM Tris pH 8, 2mM EDTA, 0.2% Triton X-100, 200µg/ml Proteinase K

**Table 7: Recipe information for general buffers and solutions**



### 2.14.2 Buffers for *in situ* hybridisation

Hybridisation A Solution	50% formamide, 5XSSC, 500 µg/ml tRNA, 50 µg/ml Heparin, 0.1% tween20, pH 6.0 with 1 M Citric Acid (0.46 ml per 50 ml) tRNA and Heparin omitted in hybB solution
PBT	PBST, 2%sheep serum, 0.2% BSA
AP Staining Buffer	100mM Tris-HCL pH 9.5, 50mM MgCl <sub>2</sub> , 100mM NaCl, 0.1% Tween20 (v/v)
AP Staining solution	100mM Tris-HCL pH 9.5, 50mM MgCl <sub>2</sub> , 100mM NaCl, 0.1% Tween20 (v/v), 4.5 ul/ml NBT, 3.5ul/ml BCIP

**Table 8: Recipe information for *in situ* hybridisation buffers**

### 2.14.3 Buffers for TSA fluorescent *in situ* hybridisation

Maleate Buffer (MB)	150mM maleic acid, 100mM NaCl, pH 7.5. Stored at 4°C
Maleate buffer blocking reagent	2% blocking reagent (Roche) in 1xMB
TNT	100mM Tris pH7.5, 150mM NaCl, 0.05% Tween20

**Table 9: Recipe information for TSA fluorescent *in situ* hybridisation buffers**

### 2.14.4 Buffers for Immunohistochemistry

PBXT	PBS plus 0.1% Triton X-100
PBDT	PBS, 1% DMSO, 1% BSA, 0.5% Triton-X 100
Blocking solution	PBDT + 2% Sheep serum

**Table 10: Recipe information for immunohistochemistry buffers**

## 2.15 Primers

Primers were designed using Primer3 v0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/>)

### 2.15.1 Primers to screen for TALEN induced mutations

Primer Name	Primer sequence	Primer detail
FWD. <i>disc1</i> .DSB.exon14	GGGGAAAACATATTCAGGGTGGTCTAAT	453bp region around <i>disc1</i> stop codon
RVS. <i>disc1</i> .DBS.3'UTR	GCAGCTTCACTTGTGCGCATCA	

**Table 11: Primer sequences used in TALEN induced mutation screening**

### 2.15.2 Primers to screen for homologous recombination events

Primer Name	Primer sequence	Primer detail
FWD <i>disc1</i> .intron14.F2	GACGGTTCACCAGCTGATTCTCTTC	FWD primer in intron 14 of <i>disc1</i> , upstream of left homology arm. 1238bp region
RVSmcherryR2	CTCCATGTGCACCTGAAGCGC	

**Table 12: Primer sequences for homologous recombination screening**

### 2.15.3 Primers for cDNA templates for *in situ* hybridisation probes

Primer name	Primer sequence
FWD.ndel1a	GCTCAATATTGTGGGCGATT
RVS.ndel1a	TGACCAAAACCAGTGTTCCA
FWD.fabp7a	GATGCATTTTGTGCCACTTG
RVS.fabp7a	ACAAAGGCAGGCCTCAATAA
FWD.lis1a	ACGAGCTAAACCGAGCGATA
RVS.lis1a	AGGTGTTGTGAAGGGTCCTG

**Table 13: Primer sequences for cDNA amplification**

### 2.15.4 Prime Time Mini qPCR Assay primers for qPCR (IDT)

Primers were designed using IDT real time PCR tool

Probe sequence: 5'/56-FAM/CGTTTCGCT/ZEN/CTCGCTGACCTTCT/3IABkFQ/-3'

Primer name	Primer sequence
FOR.Disc1	CGAGTGCTGAGTTTGTCCATC
REV.Disc1	ATTCCTGATACGGCTGTGAG
FOR. Ef1a	GGCTGGTGTGGTGAATTTG
REV.Ef1a	TTCTTGGCTATAATTGGGCTCC

**Table 14: Primer sequences for qPCR**

# Chapter 3 Oligodendrocyte precursor cell migration and proliferation analysis in the hindbrain of the zebrafish embryo

Understanding the external signalling pathways which initiate migration of oligodendrocyte precursor cells (OPCs), and the downstream targets of these signalling pathways will provide valuable information regarding OPC hindbrain development.

## 3.1 Time course of OPC development in the zebrafish hindbrain

To better characterise OPC development in the early zebrafish hindbrain, OPCs were analysed using a genetic marker of their lineage concentrating on proliferation and migration events which occurred between developmental stages 24 hours post fertilisation (hpf) and 3 days post fertilisation (dpf).

### 3.1.1 Time course of OPC development in the zebrafish hindbrain using *in situ* hybridisation

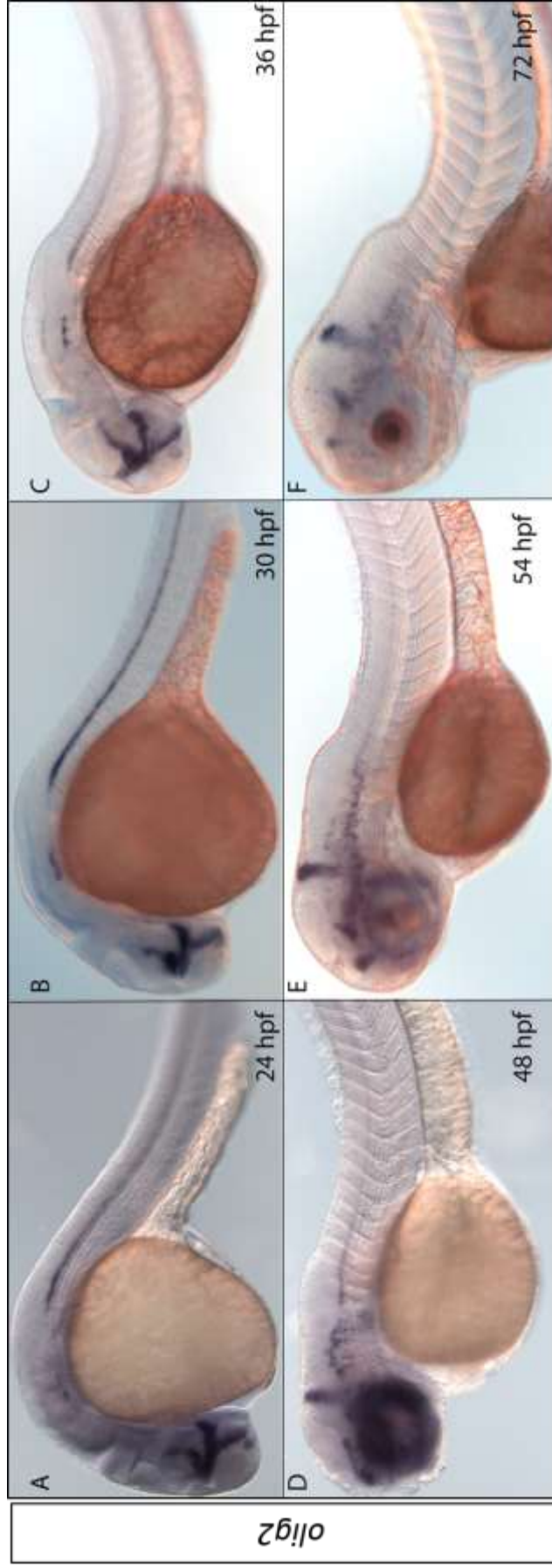
Using *in situ* hybridisation to detect expression of the transcription factor *olig2*, a well-established genetic marker of the oligodendrocyte lineage, the expression pattern of this gene was analysed over a developmental time course to determine how OPCs populated the hindbrain. Of particular interest was: I) when and where were *olig2*-positive cells first specified in the hindbrain; II) when did *olig2*-positive cells first undergo proliferation prior to migration; III) when did *olig2*-positive cells start to migrate away from their birthplace; IV) in which direction did *olig2*-positive cells first migrate and what were their subsequent directions of migration; V) when did cells complete migration and what was the final pattern of *olig2*-positive cell expression, and finally; VI) when did *olig2* positive cells start to change their shape from simple to more complex morphology to indicate the beginning of differentiation events.

A DIG-labelled anti-sense *olig2* riboprobe was transcribed and used to detect *olig2* mRNA expression using chromogenic detection methods in AB strain wild-type embryos. The transcription factor *olig2*, also marks neuroepithelium, motor neurons and OPCs and therefore we also relied on morphological changes to determine their cellular specification. These embryos were fixed at 24 hpf, 30 hpf, 36 hpf, 48 hpf, 54 hpf and 72 hpf for analysis over two days of development. Embryos were mounted so as to view both dorsal and lateral orientations which were compared to determine early events in OPC development in the hindbrain. Dorsal views of the zebrafish embryo head showed that *olig2*-positive cells were first specified at 24 hpf in distinct patches of cells in rhombomeres 5 and 6 with expression of *olig2* in these areas increasing at 30 hpf suggesting that a round of cell proliferation had occurred (Figure 3-1 comparing panels A to B). At 36 hpf *olig2*-positive cells were present in three distinct patches of expression in rhombomeres 5, 6 and 7 (Figure 3-1 C). By 48 hpf *olig2*-positive cells had delaminated away from r5/6 and expanded along the anterior-posterior axis and subsequently started to migrate laterally away from the midline (Figure 3-1 D). At 54 hpf OPCs had migrated long distances away from the midline to populate the majority of the hindbrain, at this point they showed a uniform distribution on either side of midline (Figure 3-1 E). This uniform distribution was very consistent between wild type embryos suggesting that migration is a highly regulated process. Finally at 72 hpf OPCs had reached their destination within the hindbrain and *olig2* expression seemed to have been down-regulated (Figure 3-1 F). At this point it would be expected that OPCs will have begun pre-differentiation, and morphological changes will have started to occur as OPCs seek axonal targets.

Lateral views of *olig2* positive cell development showed similar changes in *olig2*-positive cell expression as with the dorsal views, however this orientation showed more clearly how OPCs moved in a ventral direction (Figure 3-2 comparing panels C to D). OPCs always migrated from the midline in a ventral direction and did not display any movement upwards from the midline into dorsal territories (Figure 3-2 D-E).



**Figure 3-1: Dorsal views of *olig2* expression in the zebrafish embryo hindbrain over a developmental time course**  
*Initial olig2* positive cells are first specified in the hindbrain at 24hpf in rhombomeres 5 and 6 (A). *olig2* positive cells undergo proliferation prior to migration (B and C). *olig2* positive cells migrate along the anterior-posterior axis (D) and then laterally to populate the hindbrain (E). At 3 dpf *olig2* expression seems to be down-regulated, indicative of early differentiation events (F). All images are dorsal views. Anterior to the left and posterior to the right.



**Figure 3-2: Lateral views of *olig2* expression in the zebrafish embryo hindbrain over a developmental time course**

*Olig2* positive cells are first specified at 24 hpf in rhombomeres 5 and 6 (A). At 36 hpf *olig2* positive cells are seen in rhombomeres 5,6 and 7 (B) and by 48 hpf these cells have migrated ventrally and along the anterior-posterior axis (D). Cells continued to migrate after 54 hpf (E) and were positioned uniformly in the hindbrain by 72 hpf (F). OPCs remain restricted to ventral hindbrain during their development. Only *olig2*-positive cells that had migrated away from r5/6 were considered as early OPCs.

### 3.1.2 Time course of OPC development utilizing the tg(*olig2:gfp*) line

Although the *olig2* expression analysis using *in situ* hybridisation was informative as to the development of OPCs in the hindbrain, no conclusions could be drawn as to the direction that OPCs first extend their processes and therefore their initial path of early migration. This was important to address, as deciphering where processes were first extending would provide essential information as to the location of early extracellular signalling, such as a chemoattractant required to initiate migration. It also failed to address any morphological changes over development as staining for cytoplasmic mRNA is insufficient for labelling the fine processes extended by OPCs. This analysis of process extension was achieved using the tg(*olig2:gfp*) line (Figure 3-3). This transgenic reporter line, uses *olig2* regulatory elements to drive the expression of green fluorescent protein (GFP) so that development of the OPC lineage can be tracked using GFP expression as a read out (Shin et al 2003). As discussed in section 1.5.6, this transgenic marker also labels cerebellar neurons, the pMN domain and neuroepithelial cells which have been marked with arrows in (Figure 1-7) and only morphologically distinguishable *olig2*-positive OPCs were included in the analysis studies. Embryos were fixed at the same time points as with the previous time course and immunofluorescence staining using an anti-GFP antibody was performed to enhance the GFP signal. Embryos were mounted dorsally to view the process extension pattern of GFP-positive cells throughout the whole hindbrain. Transverse sections were taken through the hindbrain to visualise how OPCs extended processes along the dorso-ventral axis. These two views of OPC development provided a clear picture of process extension patterns both at initial stages of migration and during migration. Dorsal images showed the same pattern of expression as with the *olig2 in situ* hybridisation analysis but the images provided more detailed information regarding cell morphology changes throughout this developmental time period. Early on during specification and migration during 24-48hpf developmental stages, GFP-*olig2* positive cells were of relatively simple morphology at 24-36 hpf in r5/6 and were a combination of neuroepithelium, early motor neurons and early OPCs (Figure 3-3 A-C). After 36 hpf GFP-*olig2* positive cells



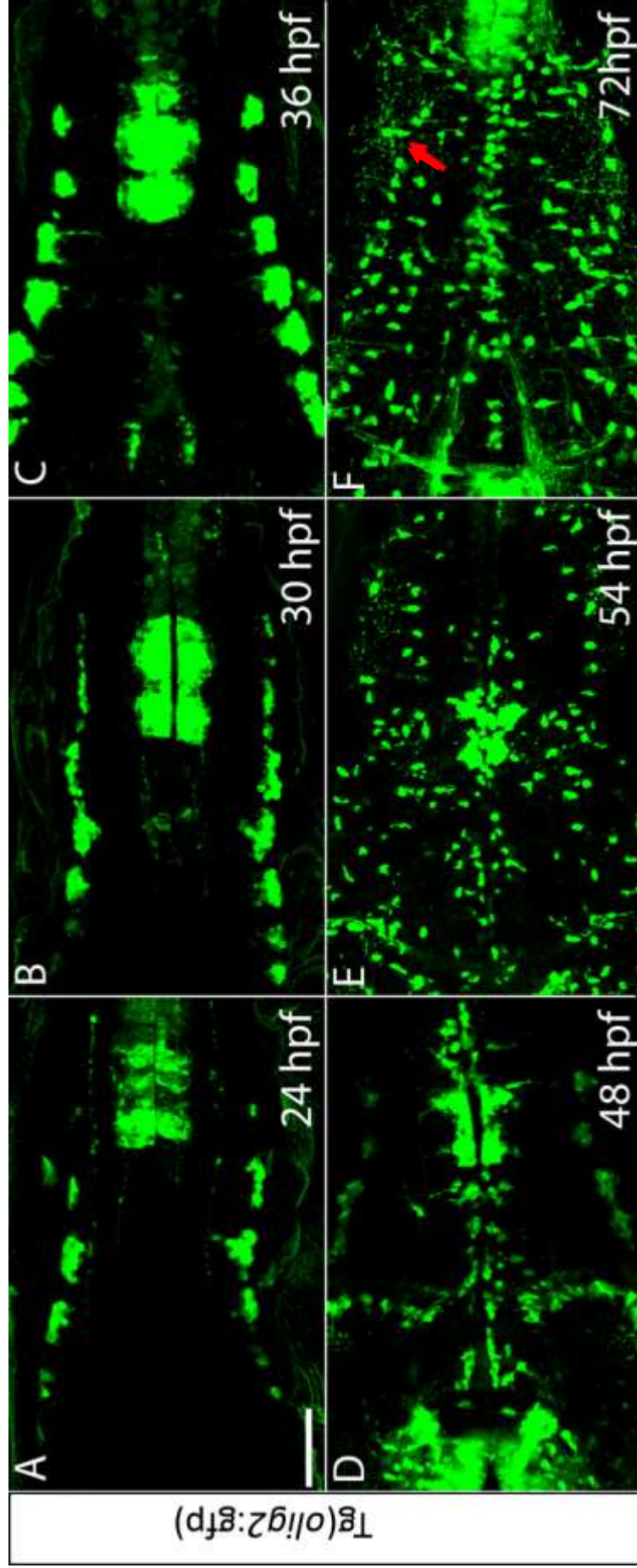
that had delaminated from their birthplace and started migration way from the midline had morphological characteristics of early OPCs. They were bipolar and extended few processes during this migratory phase (Figure 3-3 C-E). At 72hpf OPCs cells extended from the midline were likely to have reached their axonal targets and more substantial morphological changes in OPCs were observed; OPCs changed from a simple bipolar morphology to cells extending multiple fine processes indicative of early differentiation events (Figure 3-3 F red arrow). This line also allowed for the visualisation of single cells which meant that migration analysis could be performed on dorsal sections. Those *olig2* positive cells in the hindbrain that had migrated away from r5/6 were counted during the different developmental stages. At 24 hpf and 30 hpf there were no GFP *olig2*-positive cells that had extended away from r5/6, however at 36 hpf there was some cellular migration along the posterior midline ( $4\pm 3$ ,  $n=10$ ). By 48 hpf there were several cells that had migrated away from r5/6 along the midline and out laterally ( $52\pm 7$ ,  $n=10$ ) suggesting extensive migration between these time points. It is also likely that proliferation of the pMN domain in r5/6 has occurred, however due to the high density of cells in r5/6 this could not be accurately quantified. At 54 hpf there had been more lateral movement of GFP *olig2*-positive cells ( $120\pm 20$ ,  $n=10$ ) and finally at 72 hpf cells were uniformly distributed and populated the entire ventral hindbrain ( $150\pm 20$ ,  $n=10$ ). As migration progressed, the cell counts suggested that proliferation had taken place within r5/6 during these developmental stages allowing for close and uniform distribution of OPCs in the ventral hindbrain.

Transverse sections showed that OPCs initially extended processes in a ventral direction rather than a dorsal direction during early migration, which suggested that there may be a chemoattractant located in a lower ventral position to newly specified OPCs in the midline. Furthermore OPCs were restricted to the ventral portion of the hindbrain and this can be observed in transverse sections (Figure 3-4) but also lateral sections where it can be clearly seen that the dorsal area of the hindbrain are devoid of OPCs (Figure 3-2). It was hypothesised that OPCs responded to this ventral chemoattractant(s) and initially migrated in this direction before migrating laterally to populate the lower hindbrain (Figure 3-4). It is reasonable to

assume that a ventral chemoattractant exists since hindbrain OPCs are restricted to the ventral region during development.

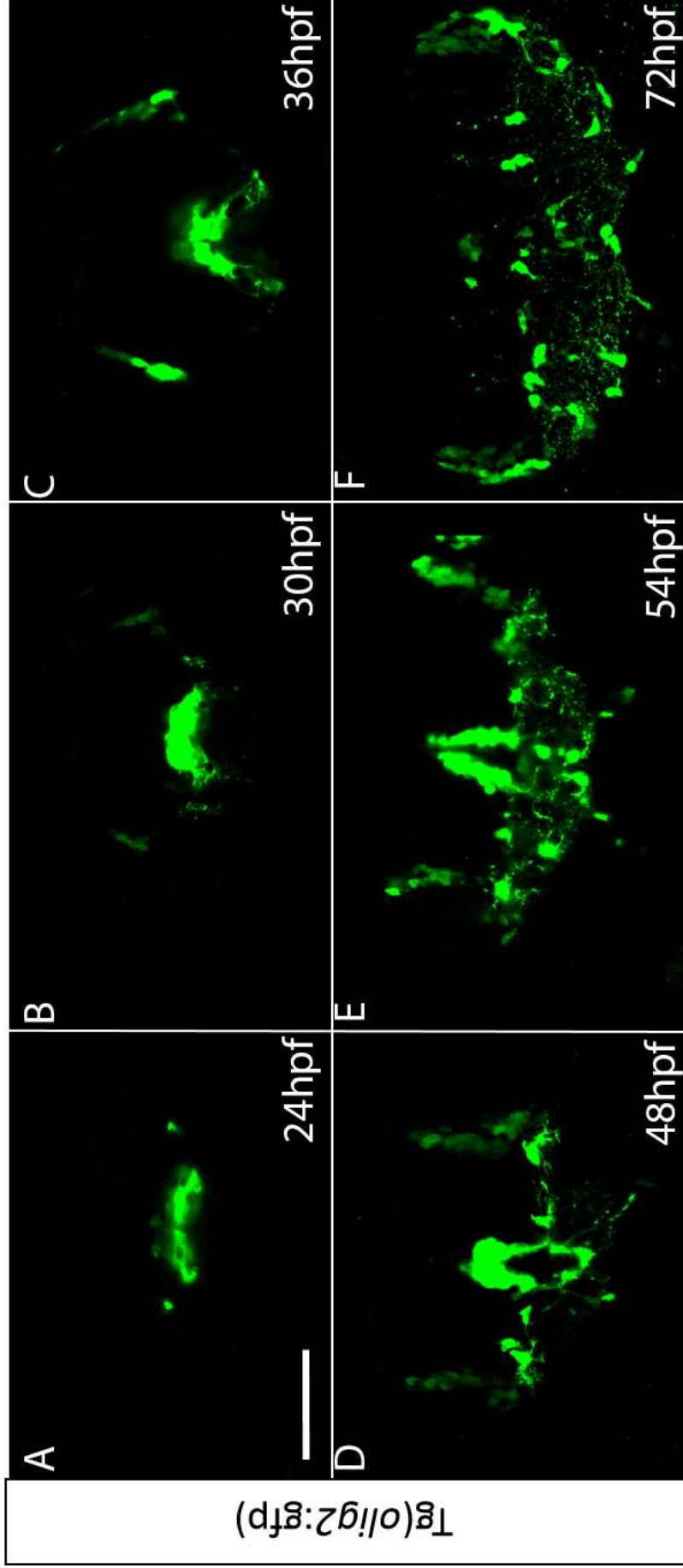
This early analysis of *olig2*-positive OPC development addressed several points; I) *olig2* positive OPCs were first specified at 24 hpf in rhombomeres 5 and 6 of the hindbrain, at this point OPCs were indistinguishable from neuroepithelium and pMN cells which are also marked by *olig2* and present in r5/6; II) *olig2*-positive cells underwent presumptive early rounds of proliferation up until 36 hpf; III) OPCs that had delaminated from r5/6 extended ventral processes during very early migration; IV) then *olig2*-positive OPCs migrated along the anterior-posterior axis before migrating into the lateral domains of the hindbrain; V) by 3 dpf *olig2*-positive OPC cells had migrated to populate the ventral half of the hindbrain and started to undergo morphological changes, extending several fine processes thereby revealing differentiation events. OPCs first extended processes ventrally and remained ventral during migration which led to the proposal that a ventral chemoattractant is required during the early stages of the initiation of migration, and perhaps during later stages as well to ensure that OPCs remain restricted to the ventral hindbrain.

The aim of this analysis was to observe early OPC development with particular focus on early migration events. One question that arose from this analysis was: what extracellular signal in the ventral portion of the hindbrain, was controlling the initial ventral process extension?



**Figure 3-3: Dorsal views of GFP expression in the tg(olig2:gfp) zebrafish embryo hindbrain over a developmental time course**

The tg(*olig2:gfp*) line showed morphological changes and early development of olig2-positive cells during development. During early specification and proliferation (A-C) and migratory stages (D-E) *olig2*-positive cells were simple in morphology during delamination away from the pMN domain in r5/r6 whereas later in development during/after migration *olig2*-positive OPCs became more complex and began to extend several fine processes suggesting they have taken on an oligodendrocyte fate and early differentiation events were occurring (F). Red arrow in F shows an *olig2*-positive OPC extending processes into surrounding environment. Scale bar 75  $\mu$ M.

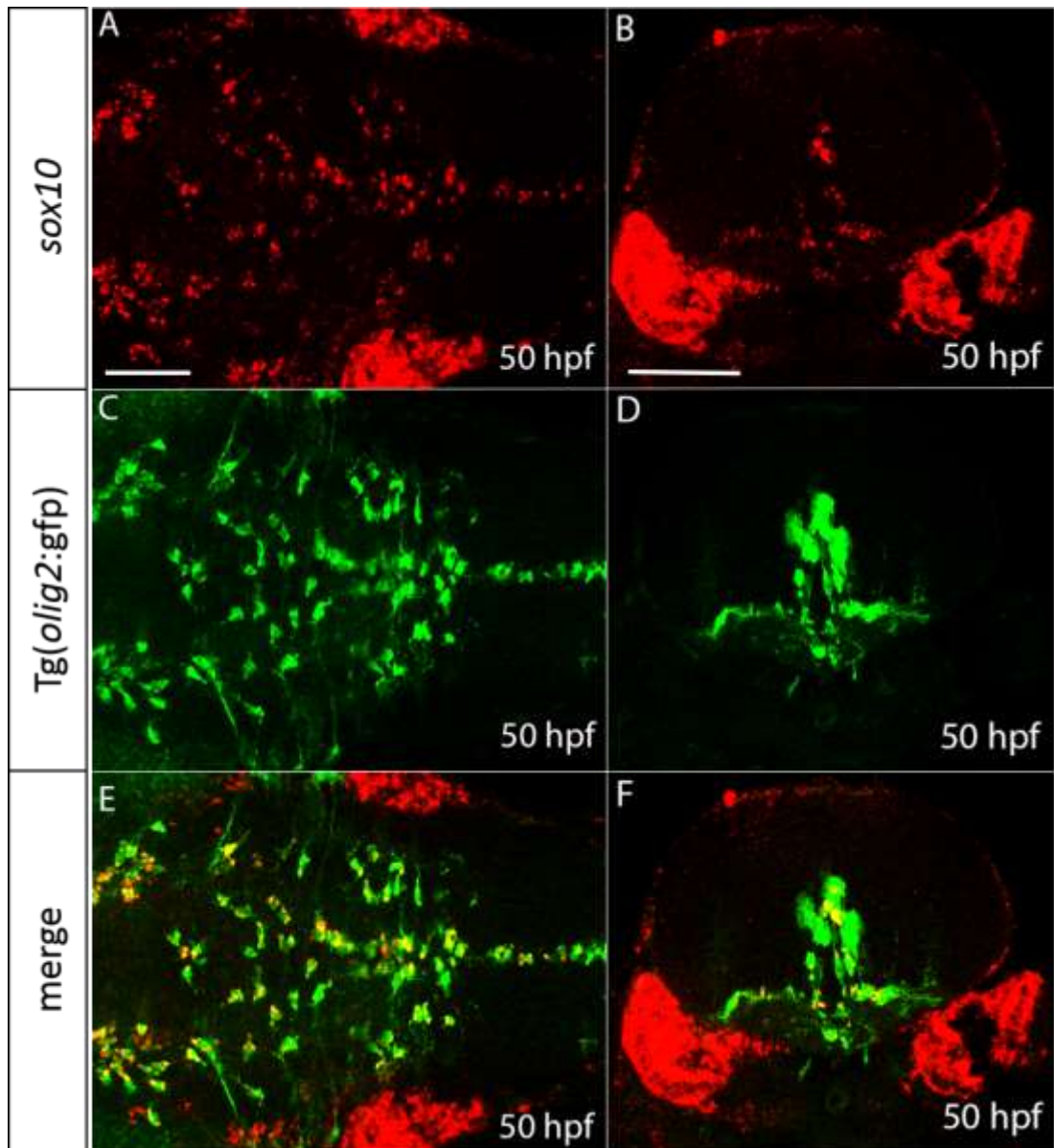


**Figure 3-4: Transverse view of GFP expression in the tg(olig2:gfp) hindbrain over a developmental time course**

Transverse sections of the hindbrain showed that OPCs initially extend processes in a ventral as opposed to a dorsal direction way from r5/6 (B-D). Cells then extended processes laterally and migrated to populate the ventral hindbrain (E-F). At 54 hpf, *olig2*-positive OPCs had populated the ventral portion of the hindbrain and extended fine processes into the surrounding environment (E-F). All images are transverse. Dorsal at the top and Ventral at the bottom. Scale bar 75  $\mu$ M.

### 3.2 Expression of *sox10* in migrating *olig2*-positive cells identifies bona fide oligodendrocyte precursor cells

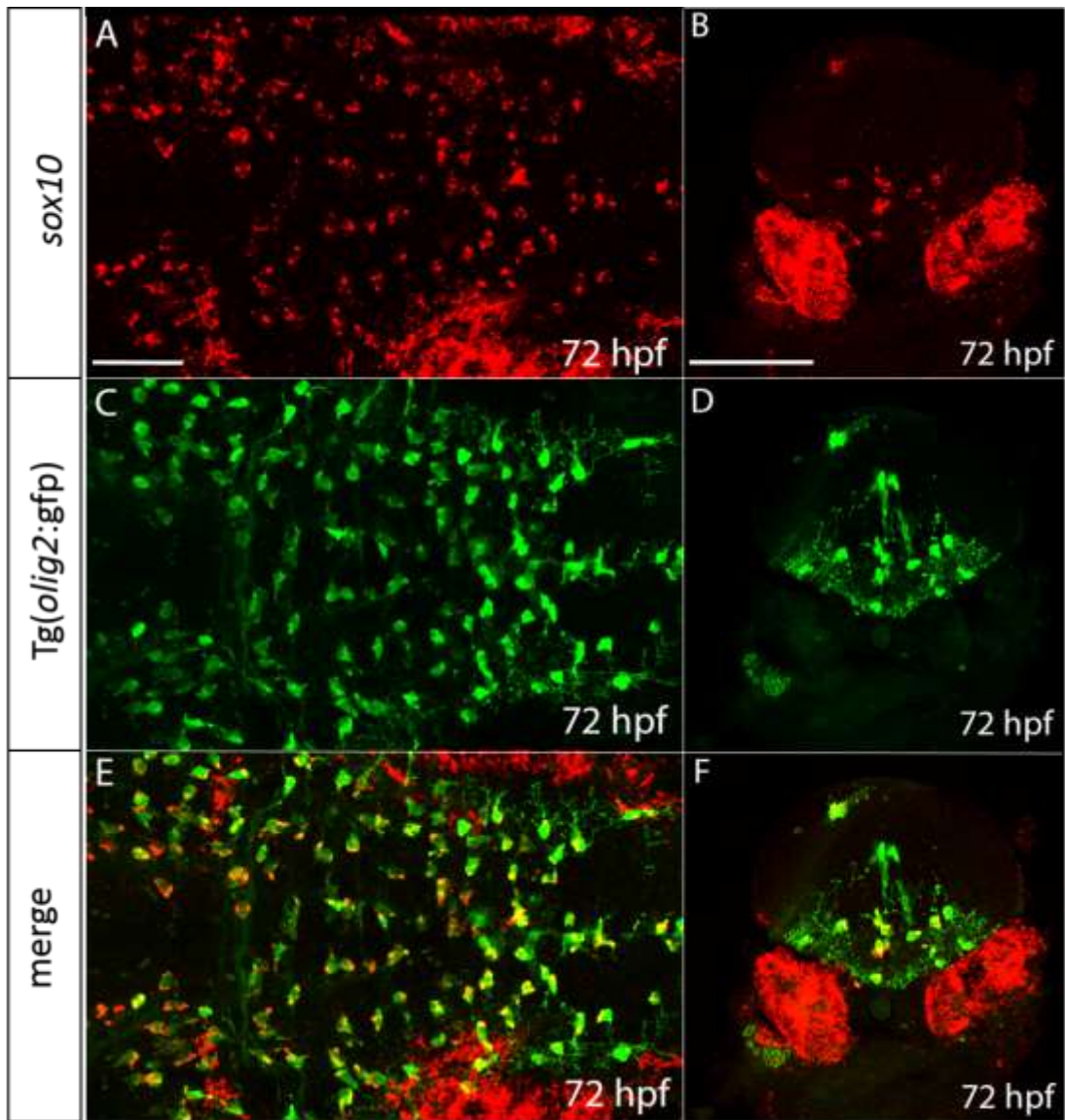
Before identifying the extracellular signalling pathways that initiate migration it was important to confirm that the *olig2*-positive cells that were migrating away from the birthplace were OPCs since *olig2* also marks neuroepithelial cells and pMNs in rhombomeres 5 and 6 (Zannino & Appel 2009). To confirm this, fluorescent *in situ* hybridisation in *tg(olig2:gfp)* embryos was performed using a *sox10* riboprobe during the migratory phase (50 hpf) and at 72 hpf when OPCs had populated the ventral hindbrain. The *sox10* transcription factor is a specific marker of OPCs and oligodendrocytes in the CNS, however it is also expressed in neural crest and otic cells (Kuhlbrodt et al 1998) (Stolt et al 2002). A combination of *olig2* and *sox10* expression however in a single cell marks OPCs. Expression of *sox10* was confirmed in migrating GFP *olig2*-positive cells at 50 hpf (Figure 3-5 E and F) and at 72 hpf when GFP *olig2*-positive cells had populated the hindbrain (Figure 3-6 E and F). These stained embryos showed that the previous analysis of GFP positive cell specification and migration in the hindbrain was labelling OPCs and investigation of extracellular signals directing *olig2/sox10* positive cell migration could now confidently be pursued.



**Figure 3-5: The expression pattern of *sox10* overlaps with migrating GFP *olig2*-positive cells in the hindbrain of 50 hpf *tg(olig2:gfp)* embryos.**

*sox10* expression was seen to overlap with expression of GFP *olig2*-positive cells in the hindbrain confirming their identity as OPCs in dorsal sections (A, C and E). In transverse sections through rhombomeres 5 and 6 (A, B and F), not all GFP *olig2*-positive cells were labelled with *sox10* in the midline suggesting these are neuroepithelial or abducens motor neurons (F). Scale bar 100  $\mu$ M.





**Figure 3-6: The expression pattern of *sox10* in the hindbrain overlaps with GFP *olig2*-positive cells that have populated the hindbrain by 72 hpf in *tg(olig2:gfp)* embryos.** The expression pattern of GFP *olig2* positive cells overlapped with *sox10* mRNA in OPCs that had populated the hindbrain in dorsal sections (A,C and E), however some GFP *olig2* positive cells in the midline in a transverse section of rhombomere 5 showed top midline cells did not express *sox10*, these cell types were likely to be the abducens motor neurons (B, D and F). Scale bar 100  $\mu$ M.

### 3.3 Identifying the extracellular ventral chemoattractant Sonic Hedgehog (Shh)

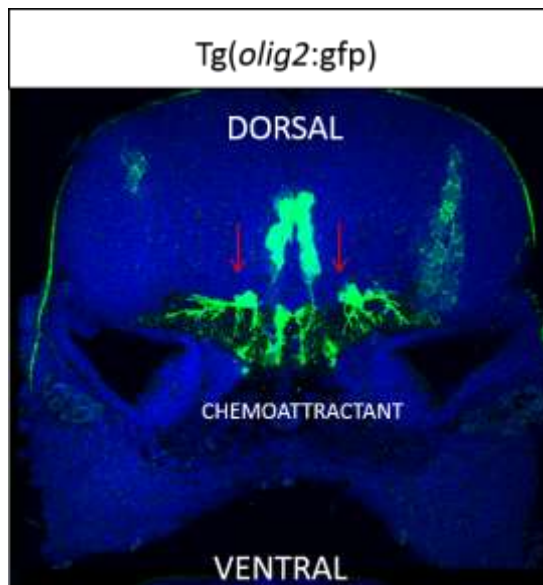
Early OPCs in the zebrafish hindbrain always initially extended processes and migrated in a ventral manner, no dorsal process extension was ever observed during early migration which suggested that there is a ventral chemoattractant signalling beneath OPCs to encourage their downward movement (Figure 3-7).

#### 3.3.1 Analysis of Shh signalling during early OPC migration

There are already known signalling pathways (Section 1.4 for details) required for proper OPC development in mouse and other animal systems which provided a candidate list of potential chemoattractants controlling early ventral OPC migration. The candidate chemoattractant signal was hypothesised to have a ventral neural tube location and one particular signalling pathway with already known roles in OPC development is the Hedgehog (Hh) pathway. The hedgehog pathway is a conserved signalling pathway, however there are some differences between the mammalian Hh pathway and zebrafish Hh pathway. There are three known classes of Hh genes in vertebrates named; Sonic (*SHH*), Indian (*IHH*) and Desert hedgehog (*DHH*). Most vertebrates have one member of each gene however in zebrafish there are 5 hedgehog genes identified thus far. Zebrafish have two sonic hedgehog genes, *shh* and *twhh* (also referred to as *shhb*) (Ekker et al 1995, Krauss et al 1993). Zebrafish also have two Indian hedgehog genes *ihha* and *ihhb*, finally there is one desert hedgehog gene (*dhh*) (Avaron et al 2006, Currie & Ingham 1996). Although the roles of Shh in zebrafish oligodendrocyte development has been most extensively studied, there have also been documented roles for *ihhb* in neural tube oligodendrocyte specification independent from *shh* (Chung et al 2013). In mammals the Hh ligand binds to the Ptc receptor, which in the absence of a Hh ligand acts to repress the smoothed receptor (Smo), causing the release of Smo from Ptc. The smoothed receptor then accumulates on primary cilia and causes a downstream signaling pathway and a transcriptional response by Gli proteins. Zebrafish possess only one *smo* gene however there are two *ptch* genes; *ptch1* and *ptch2* (Koudijs et al 2008). The Shh signalling pathway is fundamental in patterning



the dorso-ventral axis of the neural tube through creation of a morphogen gradient which starts in the notochord and floor plate (Section 1.4.1), and although *shh* signalling has been suggested to have roles in OPC migration in the optic nerve, its role in the migration of hindbrain OPCs in the zebrafish has not been explored. Therefore a potential role for *shh* in hindbrain OPC migration was investigated further.

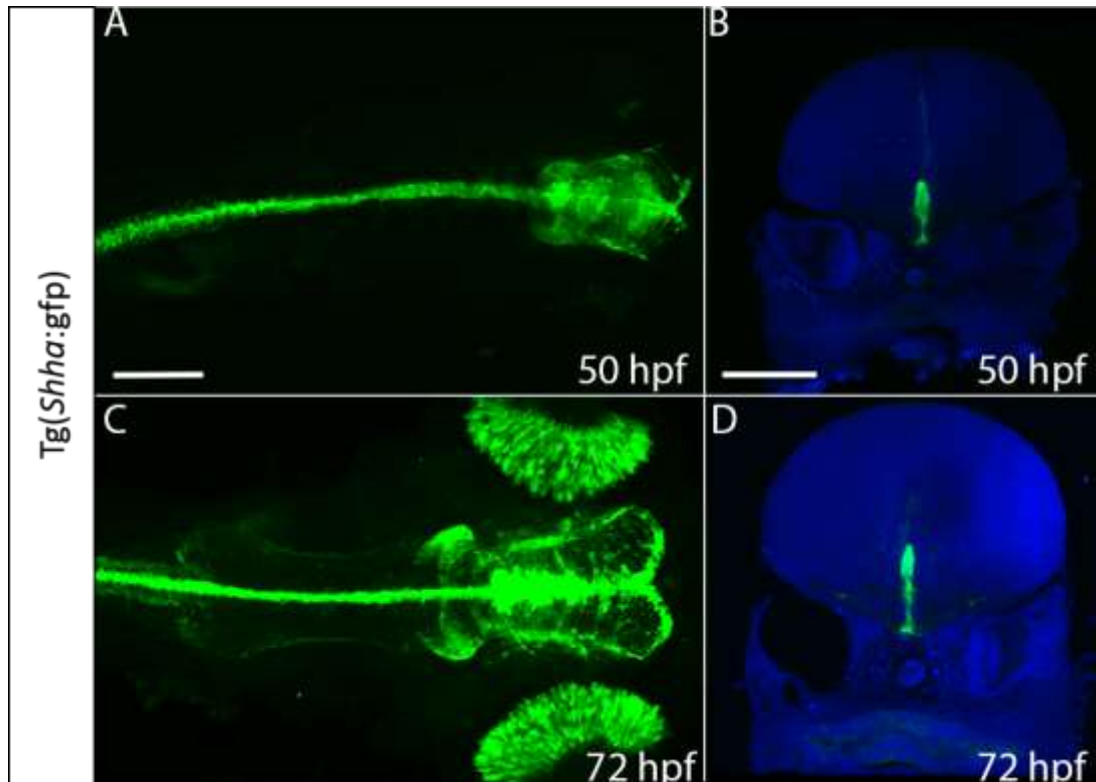


**Figure 3-7: OPCs extending processes in a ventral direction only**

A transverse section taken through the hindbrain at 50 hpf during active migration showed OPCs exclusively extending initial ventral processes. Processes started to move laterally after initial ventral migration to populate the ventral hindbrain. No dorsal process extension was ever observed during initial migration which proposed a potential ventral chemoattractant is required for directing this downward movement. Red arrows demonstrate the direction of initial extension of OPC processes during early migration.

Utilizing the Tg(*shha:gfp*) line, a reporter line which produces GFP expression under the control of *shha* regulatory elements, the expression of GFP during the migratory phase of OPC development, was determined. Expression of GFP was still robust during later stages of development with its expression as expected in the very lower ventral hindbrain. This transgenic line suggested that Shh signalling might still be active at 50hpf (during migration) and at 72 hpf (end of main migratory phase) providing evidence for a potential additional migratory role in OPC development (Figure 3-8). To confirm that GFP was truly marking expression of *shha* during these

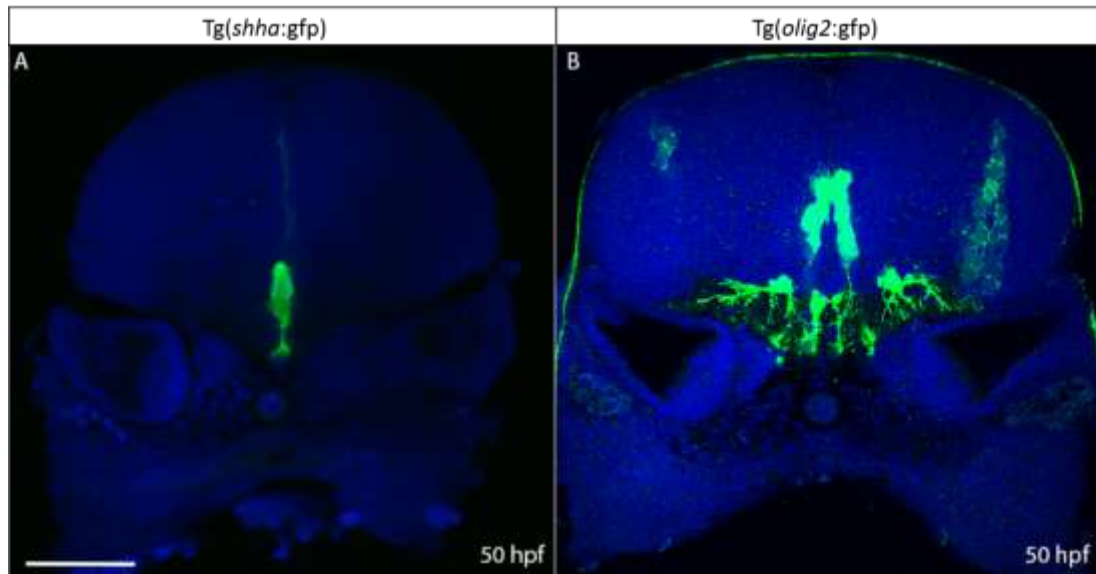
later stages since GFP has a long half-life, expression analysis of *shha* and *shhb* mRNA using *in situ* hybridization studies was investigated in the literature. Expression of both paralogs were seen to be expressed in the midline of the hindbrain at these later stages (Ertzer et al 2007) (Ekker et al 1995) (Thisse et al 2004).



**Figure 3-8: Shh signalling in the *tg(shha:gfp)* zebrafish line**

In the zebrafish hindbrain, expression of GFP is maintained at 50 hpf (A and B), well after the initial specification of OPCs has occurred. Expression of GFP is maintained throughout migration and is still present at 72 hpf (C and D). Panels A and C show dorsal sections and panels B and D show transverse sections. Scale bar 100  $\mu$ M

When expression of GFP in *tg(olig2:gfp)* embryos and GFP in *tg(shha:gfp)* embryos was compared side by side in transverse sections, it revealed that a strip of *shha* promoter activity sat directly below the GFP expressing OPCs. Expression did not seem to overlap in the midline and birthplace of OPCs but there was apparent overlap of expression in the migratory zone which suggested that Shh could be an active chemoattractant and did not act to repel OPCs (Figure 3-9).

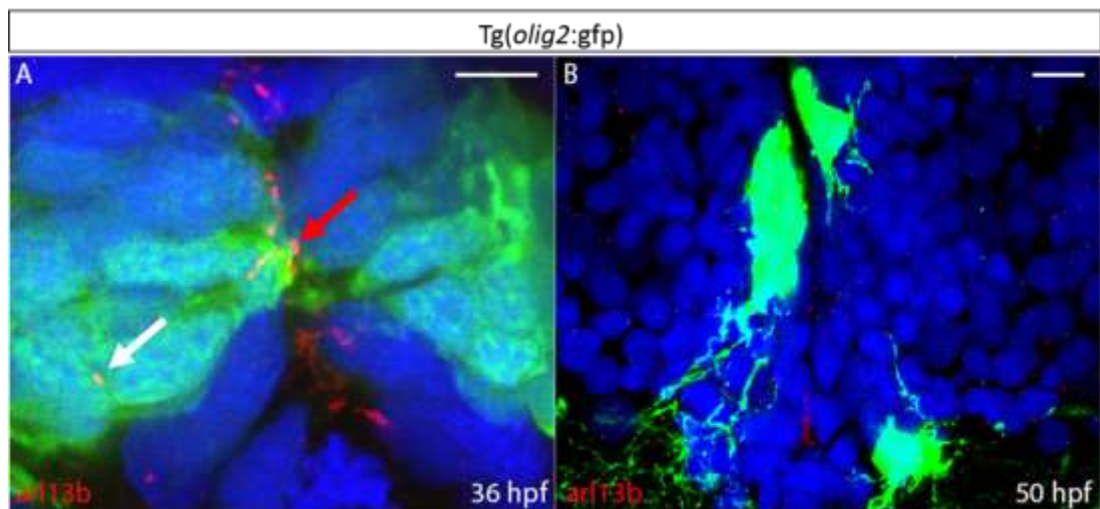


**Figure 3-9: Comparison of *Tg(shha:gfp)* and *Tg(olig2:gfp)* expression in transverse sections through the zebrafish hindbrain at 50hpf**  
 GFP expressing OPCs extended processes ventrally (B) towards GFP-positive Shh expressing cells (A) which appear to be located just below the OPC birthplace in the midline. The nuclei of cells is stained with DAPI (blue). Scale bar 50  $\mu$ M

### 3.3.2 Responding to Shh through primary cilia

If Shh signals to OPCs to direct their migration then these OPCs need to have the ability to respond to this signalling pathway. It is well known that primary cilia play a crucial role in transducing the Hh signal in many cell types, which allows cells to respond to their external environment. If Hh is important for initiating ventral migration of OPCs, then it might be expected that they express primary cilia prior to migration and during migration to transduce the signal and respond accordingly. Responding accordingly would involve up-regulating the expression of cytoskeletal proteins required to physically move the cell in the appropriate direction. Primary and motile cilia are conventionally labelled using primary antibodies against acetylated tubulin, which labels the axoneme of cilia, however acetylated tubulin also labels neurons. Therefore another antibody which exclusively labels cilia was required when analysing cilia in the CNS. An alternative antibody against ADP-ribosylation factor-like protein 13B (Arl13b), which is a component of cilia and is required for their formation, was used to address potential OPC primary cilia (Zhang et al 2013). Arl13b staining in transverse sections showed cilia on OPCs in the ventricular zone which could be a combination of motile and primary cilia (Figure

3-10 A red arrow) but also showed primary cilia on OPCs away from the ventricular zone (Figure 3-10 A white arrow). Primary cilia could be labelled at 36hpf prior to onset of the main migratory phase but were not detected on migrating OPCs at 50hpf in both manually taken larger transverse sections and in thin cryostat sections (Figure 3-10 B). This may have been because during migration primary cilia may have to be constantly disassembled and reassembled (Baudoin et al 2012) so it may have been a case that the imaged sections did not capture primary cilia during such a highly dynamic process, but that they are present transiently.



**Figure 3-10: Primary cilia on OPCs in transverse sections through the hindbrain in *tg(olig2:gfp)* embryos.**

At 36hpf OPCs had primary cilia, as shown by Arl13b staining, prior to migration onset (A). At 50hpf during the main phase of migration, although primary cilia were readily observed in the notochord, no primary cilia were detected on migrating OPCs. Staining of primary cilia in red, staining of GFP *olig2*+ cells in green and the nuclei of cells are stained with DAPI (blue). Red arrow labels cilia in the ventricular zone which could be a combination of primary and motile cilia. White arrow indicates a primary cilium on a pre-migratory OPC. Scale bar 20  $\mu$ M

Analysis of Hh signalling provided evidence that it could be a ventral chemoattractant for OPCs. The *tg(shha:gfp)* line suggested that *shha* is expressed ventral to the birth place of OPCs and might be directly involved in the OPC migratory pathway, since there was apparent overlap with the region of GFP-positive OPC expression. Analysis of Arl13b protein expression showed that OPCs have primary cilia prior to migration and these primary cilia could be responsible for transducing the Shh signal thereby allowing cells to respond and migrate in the

required direction. No primary cilia were detected at 50 hpf during the main period of migration although this could have been because primary cilia go through assembly rounds which meant that they were harder to capture in fixed specimens. It could also be that during these stages, Shh signalling occurs in a primary cilia independent process.

### 3.4 Analysis of OPC development defects in stable Hh pathway mutant zebrafish lines.

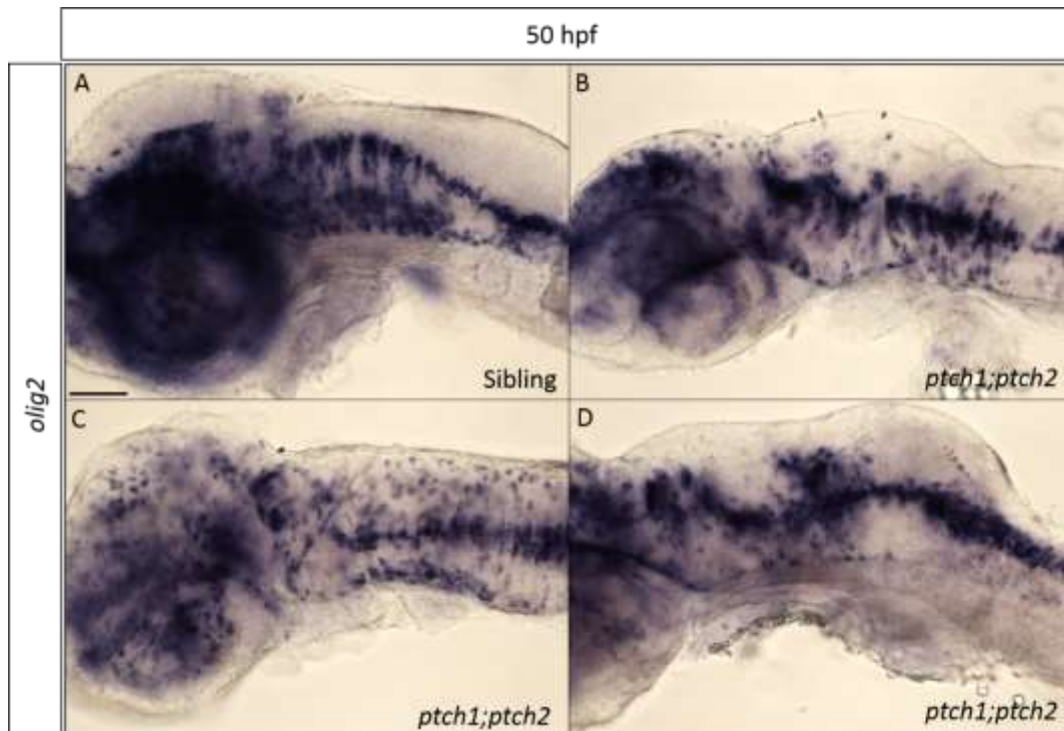
The next question to answer was how OPC development was affected in Hh pathway stable mutant zebrafish lines, in particular focusing on deficiencies in migration direction and capacity. There were several mutant lines available in both zebrafish facilities in which Hh signalling is affected. Mutants either had a loss of function mutation which resulted in a gain of Hh signalling or loss of function mutation which resulted in a loss of Hh signalling through the embryo (Table 15). OPCs were labelled using *in situ* hybridisation with a DIG-labelled anti-sense *olig2* riboprobe in Hh signalling mutants at 50 hpf or 72 hpf and these experiments indicated various OPC development and migration defects.

Zebrafish mutant line	Mutation/phenotype	Reference
<i>ptch1;ptch2</i>	Loss of function Mutation in the <i>ptch1</i> and <i>ptch2</i> receptors leads to constitutively active Hh signalling in cells	(Koudijs et al 2008)
<i>smo</i> <sup>b641</sup>	Loss of function Mutation in the smoothed receptor leads to a loss of Hh signal transduction	(Varga et al 2001)
<i>iguana</i> <sup>ts294e</sup>	Mutation in a ciliary protein resulting in a secondary loss of function in Hh signalling	(Brand et al 1996)

**Table 15: Stable Hh signalling mutant lines used in *olig2* expression analysis**

### 3.4.1 Gain of function mutant analysis

The *ptch1*<sup>(+/-)</sup>/*ptch2*<sup>(+/-)</sup> double mutant line was in-crossed to produce *ptch1*<sup>(-/-)</sup>/*ptch2*<sup>(-/-)</sup> homozygous mutants (which will be referred to as *ptch1;ptch2* mutants from now on) and siblings which were fixed and analysed for OPC migration defects at 50 hpf and 72 hpf. These *ptch1;ptch2* mutant embryos have constitutively active Hh signalling and therefore leading to increased Hh signalling throughout the embryo. When *olig2* expression was scrutinised in these mutants and compared against siblings there were clear changes in *olig2* expression. Positive cells for *olig2* which had extended above the midline into dorsal domains were counted in lateral sections. In wildtype siblings no cells were observed above the midline at 50 hpf (0, n=5), however in the *ptch1;ptch2* mutants several cells were observed above the midline with large variations between individual embryos (18±10, n=5). As discussed earlier, OPC migration is highly regulated and OPCs always formed a uniform distribution pattern which is consistent between wild type embryos. However it was observed in the *ptch1;ptch2* mutants that the expression of *olig2* differed substantially between individual mutants (Figure 3-11B-F). This suggested that a defined gradient of Shh is required to create the ideal chemoattractant environment and excessive transduction of the signal can skew the migratory map that caused it to be deregulated and aberrant cellular migration to occur in these mutants.

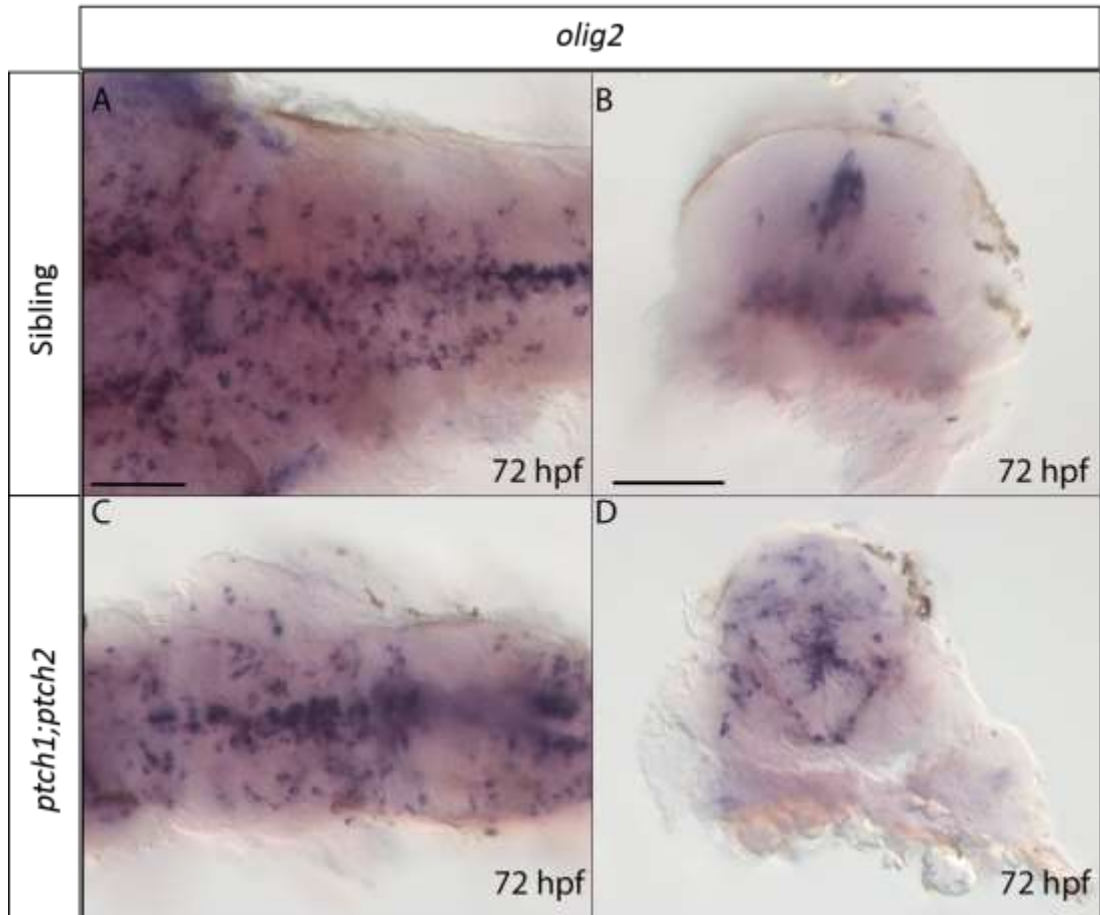


**Figure 3-11: *olig2* positive cell expression pattern in *ptch1;ptch2* mutants at 50 hpf**

The *olig2* positive cell expression pattern in wild type embryos is a midline strip of cells with expression extending ventrally (A) and this is consistent between individuals. No expression of *olig2* is seen in the dorsal hindbrain. In *ptch1;ptch2* mutants however the expression pattern of these cells is altered, the defined midline strip of cells is lost with cells entering the dorsal part of the hindbrain in some cases (C). The expression pattern is also highly varied between individual mutant embryos. Scale bar 100  $\mu$ M

Furthermore the normal ventral pattern of *olig2* was disordered with expression in both ventral and dorsal parts of the hindbrain (compare Figure 3-12 B to D). This demonstrates that constitutive activation of Hh signalling results in abnormal OPC migration. One interpretation of this result is that this type of mutation meant that cells could not determine the location of the signal, although the actual Hh protein remains unaffected and the distribution is unaltered, cells do not interpret this signal properly. Therefore cells continually respond to the signal and do not interpret the signal as ventral leading to a loss of positional information.





**Figure 3-12: Expression of *olig2* positive cells in *ptch1;ptch2* mutant and sibling embryos hindbrains at 72 hpf**

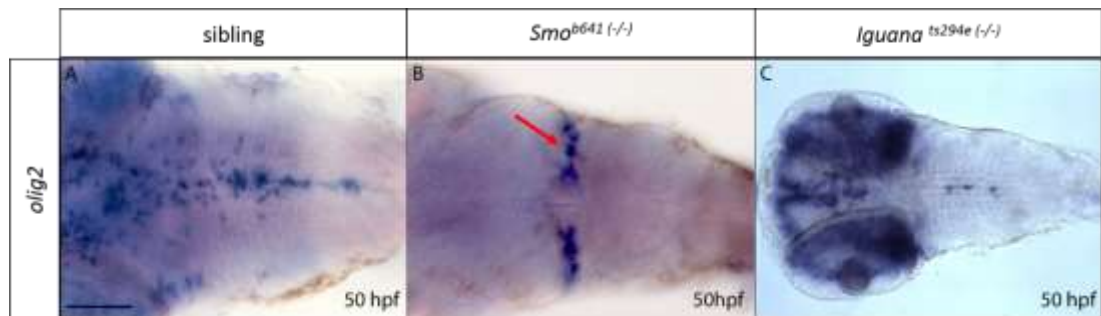
At 72hpf the uniform expression pattern of *olig2* in siblings (A) is lost in *ptch1;ptch2* mutants (C). In transverse sections of the hindbrain, *olig2* expression is mainly restricted to the ventral portion of the hindbrain in siblings (B), whereas in *ptch1;ptch2* mutants extensive expression of *olig2* is seen in the dorsal hindbrain (D). Images in A and C are dorsal views, B and D are transverse sections. Scale bar 100  $\mu$ M

### 3.4.2 Loss of function mutant analysis

An incross of *Smo*<sup>b641 (+/-)</sup> carriers gave homozygous mutants which were analysed for the expression of *olig2*-positive cells and compared with siblings at 50 hpf. No *olig2* positive cells were identified in *smo*<sup>b641</sup> homozygous mutants which was expected since Shh is critical in the initial specification of OPCs and is required to turn on *olig2* expression (Figure 3-13 B). Similarly in *iguana*<sup>ts294e</sup> mutants, in the majority of embryos there was no *olig2* expression, because of the secondary Shh signalling defect, however in some embryos only a small number of OPCs were specified (Figure 3-13 C). These loss of function mutants were therefore unsuitable



to analyse Shh function during later stages of OPC development due to the early specification defect.



**Figure 3-13: Expression of *olig2* positive cells in *smo<sup>b641</sup>* and *iguana<sup>ts294e</sup>* hindbrain embryos at 50 hpf.**

At 50 hpf OPCs have been specified and are actively migrating (A), in *smo<sup>b641</sup>* mutants although cerebellar neurons are specified (red arrow), hindbrain OPCs are not specified due to complete loss of Shh signalling (B). In *iguana<sup>ts294e</sup>* mutants a small number of OPCs are specified but fail to migrate from the midline (C). Scale bar 100  $\mu$ M

Analysis of *ptch1;ptch2* mutants, which possess constitutively active Hh signalling, showed evidence for disordered OPC migration where cells failed to form a uniform expression pattern. OPCs also migrated out of the ventral region where they are normally restricted to into the dorsal hindbrain, suggesting that Hh was unable to be sensed as a ventral source which led to misdirected migration.

Loss of function mutants failed to specify OPCs since Hh signalling is critical for their early development and therefore these were an unsuitable model to analyse how migration is affected upon removal of Hh signalling. An assay which caused a temporal loss of Hh prior to migration and after specification was therefore required to decipher how loss of Hh affects OPC migration.

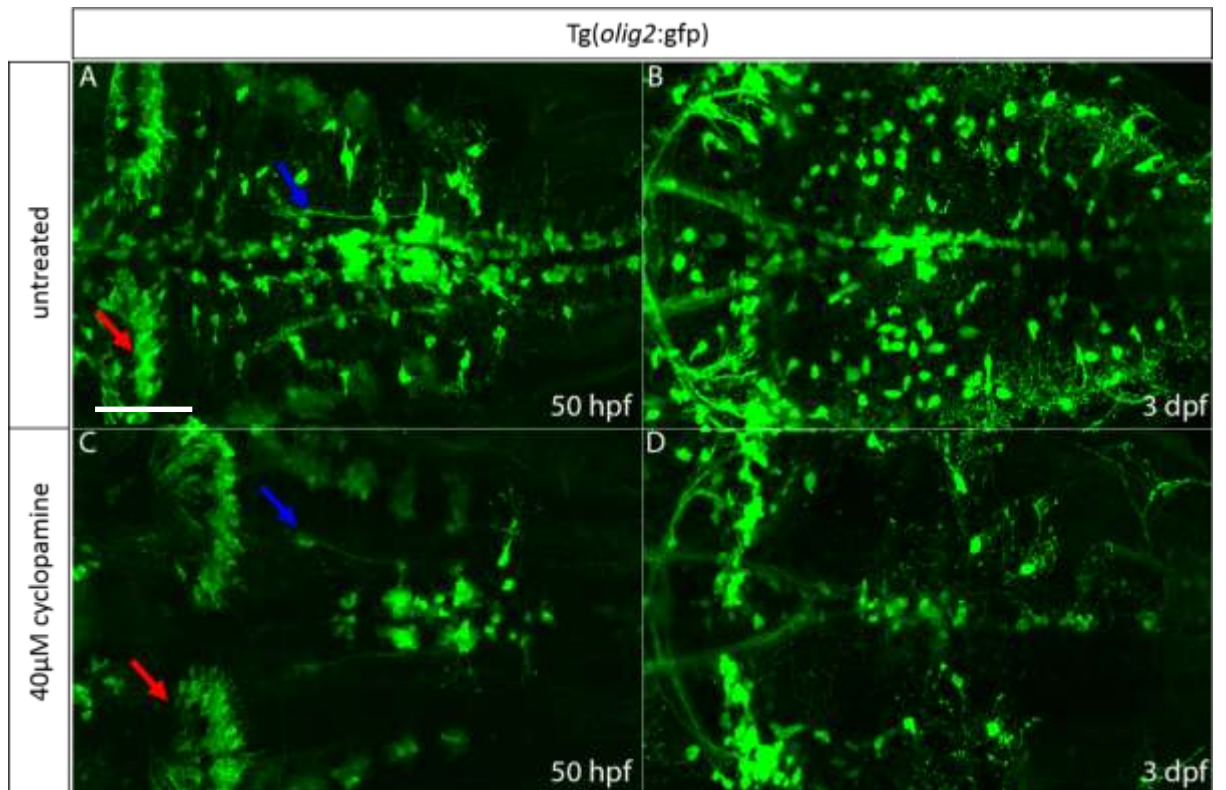
### 3.5 An assay to analyse temporal loss of Hh signalling on OPC migration post-specification

To determine whether Hh could be contributing to OPC development after their initial specification, it was necessary to disrupt Hh signaling in a temporal manner. This was achieved using cyclopamine (cyc), a widely utilized and well characterised chemical inhibitor of Shh signalling, through blocking the receptor pathway (Chen et al 2002). The data obtained thus far has led to the hypothesis that Shh acts as a

ventral external cue providing positional information to OPCs and, upon removal of this signal or the ability to respond to it, OPCs would either fail to migrate away from their birthplace or show an abnormal migratory pattern.

### 3.5.1 Cyclopamine treatment affects OPC migration in the hindbrain

When 40  $\mu$ M cyclopamine was applied to *tg(olig2:gfp)* embryos (n=20) at 34 hpf and fixed specimens analysed at either 50 hpf (n=10) or 3 dpf (n=10) there appeared to be clear defects in proliferation and migration of OPCs in cyclopamine treated embryos. The overall morphology of the embryos was unaffected with no overt signs of toxicity. OPCs failed to migrate fully both along the anterior-posterior axis and laterally to uniformly populate the hindbrain (Figure 3-14). In contrast, the development of GFP-positive cerebellar neurons and the abducens motor neurons was normal which suggests that the development of these cell types are Hh-independent or other pathways could compensate for the loss of Hh signalling (Figure 3-14 B arrows). This suggested that Shh may function as a chemoattractant actively involved in the initial migration of OPCs to their axonal destinations.



**Figure 3-14: *tg(olig2:gfp)* embryos treated with cyclopamine from 34 hpf then fixed at 50 hpf and 3 dpf**

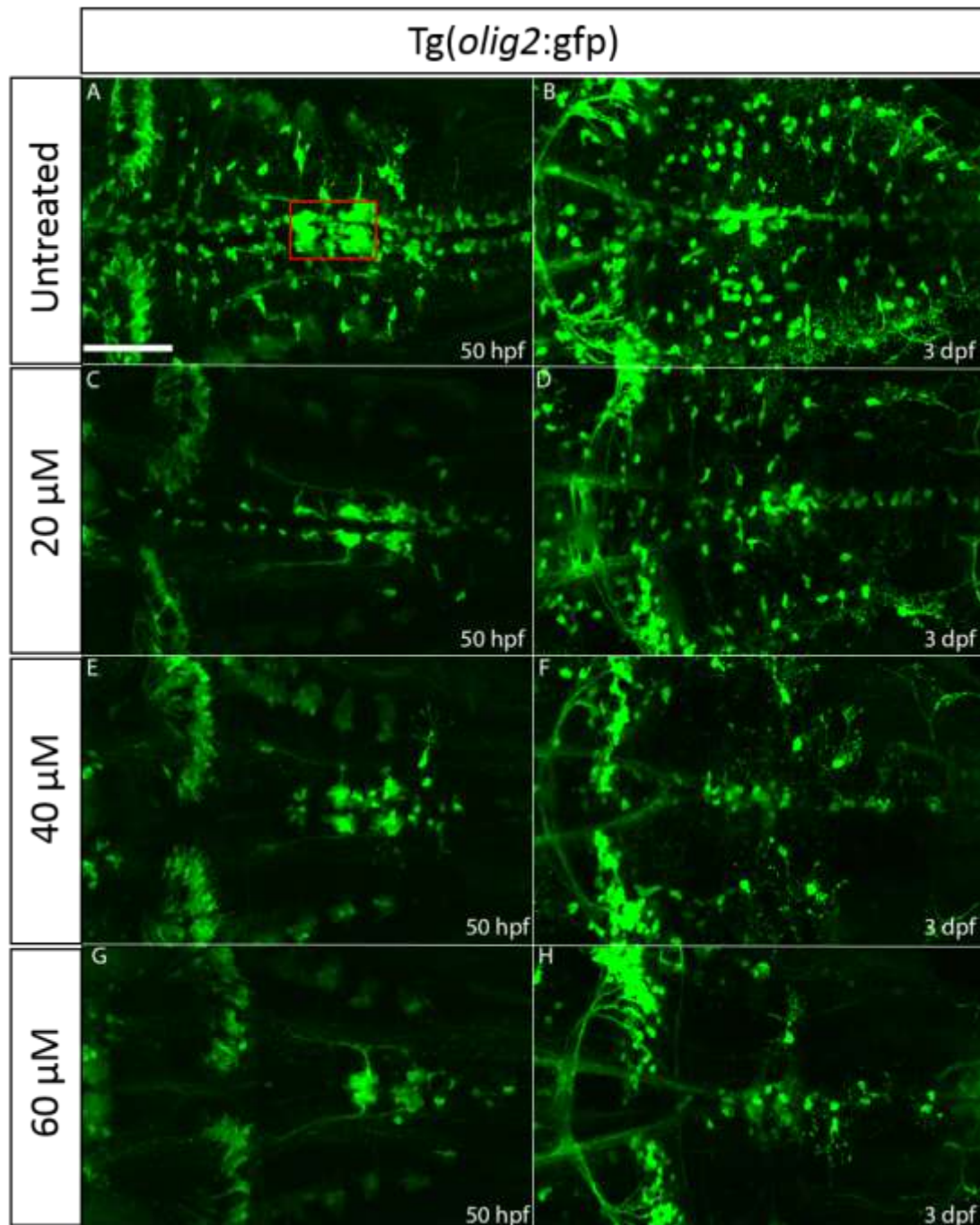
OPCs failed to migrate fully to uniformly populate the hindbrain in cyclopamine treated embryos (C and D) compared with controls embryos (A and B). The development of *olig2*-positive cerebellar neurons (red arrows) and the abducens motor neurons (blue arrows) was normal. At 3 dpf OPCs that had migrated were disordered and lacked even dispersal in the hindbrain (compare D with B). 10 embryos per treatment group were used. Scale bar 100  $\mu$ M

### 3.5.2 Cyclopamine treatment affects migration and proliferation of OPCs in a dose-dependent manner

Varying concentrations of cyclopamine (20, 40 and 60  $\mu$ M) were applied to embryos which resulted in migration defects varying in severity (Figure 3-15) with the least amount of OPCs migrating away from r5/6 in the 60  $\mu$ M treatment group (Figure 3-15 G-H). Quantification of the number of cells that had migrated away from r5/r6 in treated zebrafish (n=5 per treatment group) showed that the number of OPCs that had migrated away from their birthplace reduced dramatically with increasing cyclopamine concentration (Figure 3-16). Individual OPCs that had migrated away from r5/6 were counted in a z-stack projection of the whole zebrafish hindbrain

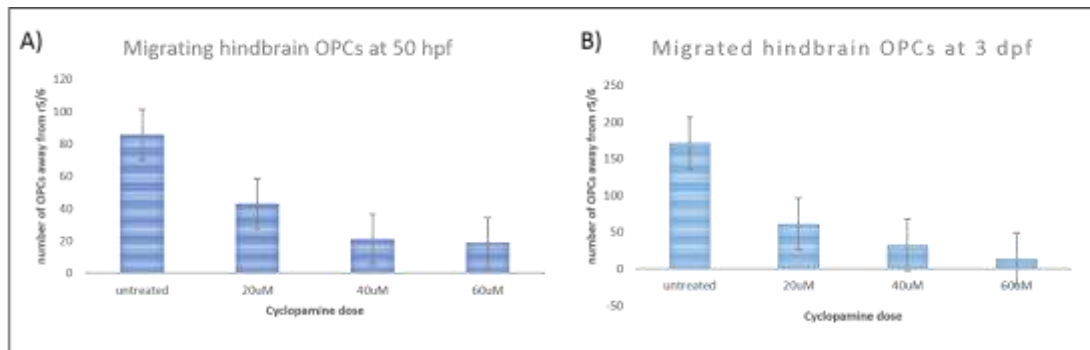
(Figure 3-15 C, depicted by red box). It is important to note that although there is a migration defect there also seemed to be a proliferation defect.

Individual OPCs were readily identified and quantified but the number of OPCs that remained in their birthplace in r5/6 could not be counted since there was a high density of cells in this region. Therefore it was difficult to conclude from these experiments that the observed phenotype was strictly a migration defect, it could be the case that there was deficiency in proliferation which resulted in secondary decrease in migration. Applying cyclopamine at 40hpf when OPCs have started the main period of migration did also affect the number of OPCs migrating away from r5/6 but to a lesser extent. Applying cyclopamine at this late stage however will most likely have resulted in residual Shh signal still being maintained during the early migration phase which provided OPCs.



**Figure 3-15: The effect of different cyclopamine concentrations on OPC migration in the hindbrain**

Treatment of cyclopamine at differing concentrations resulted in differing severities of OPC migration defects. The lowest treatment of 20  $\mu$ M resulted in a less dramatic migration defect than in a higher treatment group of 40  $\mu$ M (compare C and E), increasing the concentration of cyclopamine higher to 60  $\mu$ M reduced the number of OPCs migrating away from the midline even more (compare G to E). 5 embryos were used per treatment group. Scale bar 100  $\mu$ M.



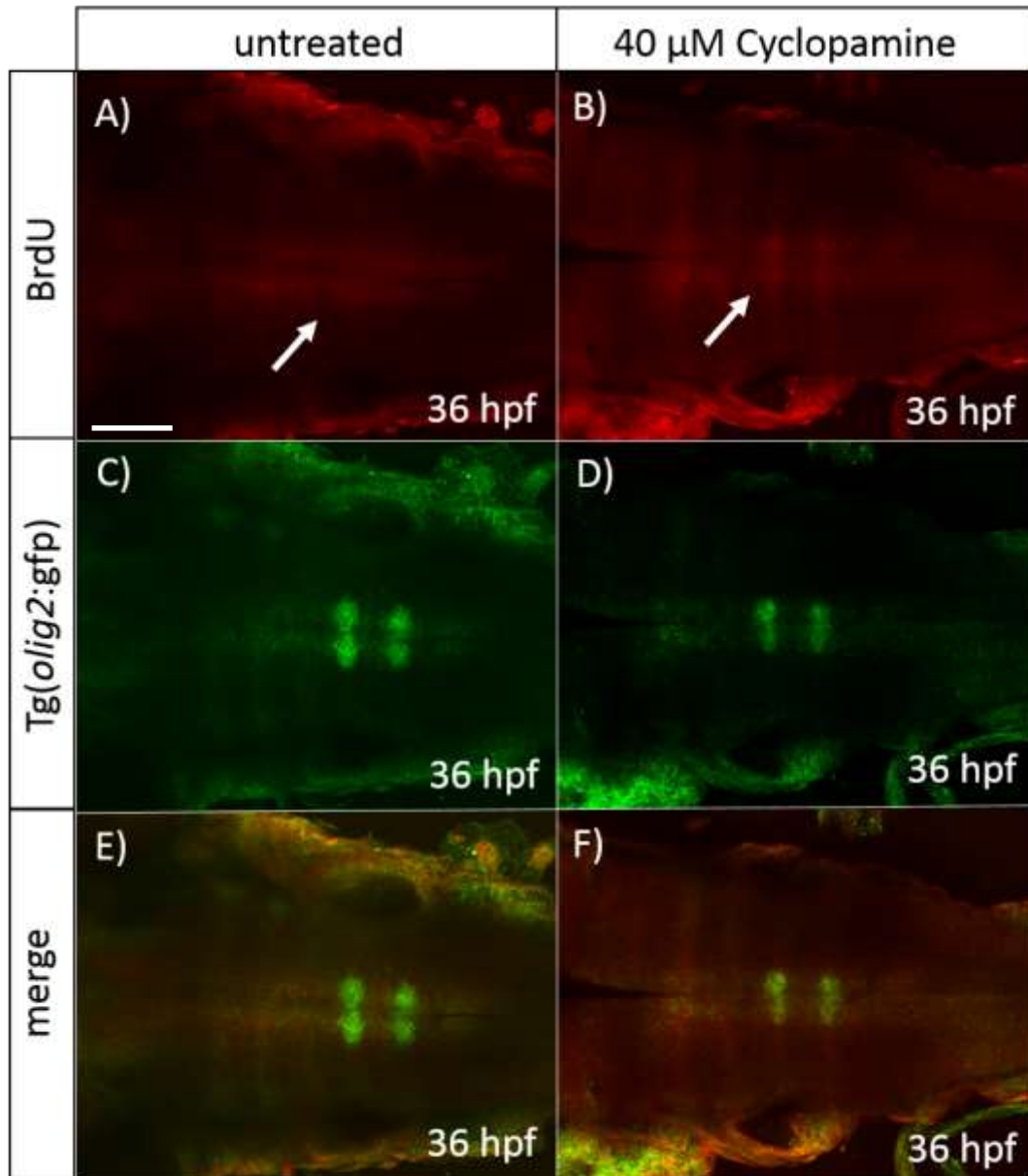
**Figure 3-16: Quantification of OPCs that migrated away from r5/6 in the presence of a range of cyclopamine concentrations**

Quantification of OPC numbers that had migrated away from r5/6 during migration stages shows that in cyclopamine treated fish there was a considerable decrease in cells compared to untreated fish (A). After migration there was a small number of OPCs populating the hindbrain in cyclopamine treated fish compared to controls (B). Each treatment group n=5.

A temporal loss of Shh signalling prior to the main phase of migration, but after the initial specification of the cell type had occurred, resulted in an apparent migration defect but these experiments also suggested that there was a proliferation defect. Although quantification of OPCs that had left their birthplace in r5/6 was determined, OPCs that remained in r5/6 could not be counted individually due to the high density of cells, which were tightly packed in the birthplace that also contained other cell types (Figure 3-16). This meant that it could not be definitively concluded whether this phenotype is a specific migration defect or a combination of migration and proliferation defects. It is known in other animal systems that Hh is a promoter of proliferation and also cell survival so it is reasonable to hypothesise that removing Hh signalling at later stages of development could affect proliferation and OPC cell numbers. However in the zebrafish, Zannino et al produced time lapse movies of OPCs migrating in the hindbrain and it would seem that little division of cells occurred whilst cells were migrating (Zannino & Appel 2009). To determine whether there was a loss of hindbrain OPC proliferation in cyclopamine treated embryos, embryos were pulse labelled with 5-Bromo-2-deoxyuridine (BrdU) at 50 hpf after overnight exposure to cyclopamine from 36 hpf. BrdU is incorporated into newly synthesised DNA and therefore detects proliferating cells. However although several attempts were made to BrdU label embryos, no specific BrdU labelling could be detected. Detection of BrdU requires a harsh acid treatment which can cause

some experimental difficulties and be inconsistent so that could be one of the reasons for the failure in BrdU staining. Since embryos were treated with BrdU at 50 hpf it could also have be that the embryos were too old, with thick skin which could have made it more difficult for the BrdU to penetrate the embryos. Embryos were then pulse labelled with BrdU at an earlier time point of 36 hpf to investigate whether age was the limiting factor. In this experiment cyclopamine was applied at 32 hpf, however again although some staining could be seen in the ventricular zone of the hindbrain (Figure 3-17 A and B arrow), no single cells could be resolved and accurate counts could therefore not be taken. Furthermore OPCs were treated with cyclopamine for a reduced time compared to the overnight treatment in previous experiments and therefore the compound may have not had a long enough exposure time to exert its effect on OPCs. Future attempts could utilize 5-Ethynyl-2'-deoxyuridine (EdU) which does not require the acid treatment and could provide more reliable results. It was of importance to find evidence for a direct role of Shh in OPC migration since no definitive answer could be concluded from BrdU proliferation analysis.





**Figure 3-17: BrdU analysis of hindbrain OPCs in cyclopamine treated embryos at 36 hpf**  
 Tg(*olig2*:gfp) embryos were treated with cyclopamine at 32 hpf for four hours, then at 36 hpf embryos were pulse labelled with BrdU to analyse any proliferative defects. Staining with mouse anti-BrdU antibody showed some BrdU incorporation in the ventricular zone (arrow A and B) which did overlap with some GFP-positive staining (E and F) however single cell incorporation could not be distinguished. Scale bar 100  $\mu$ M



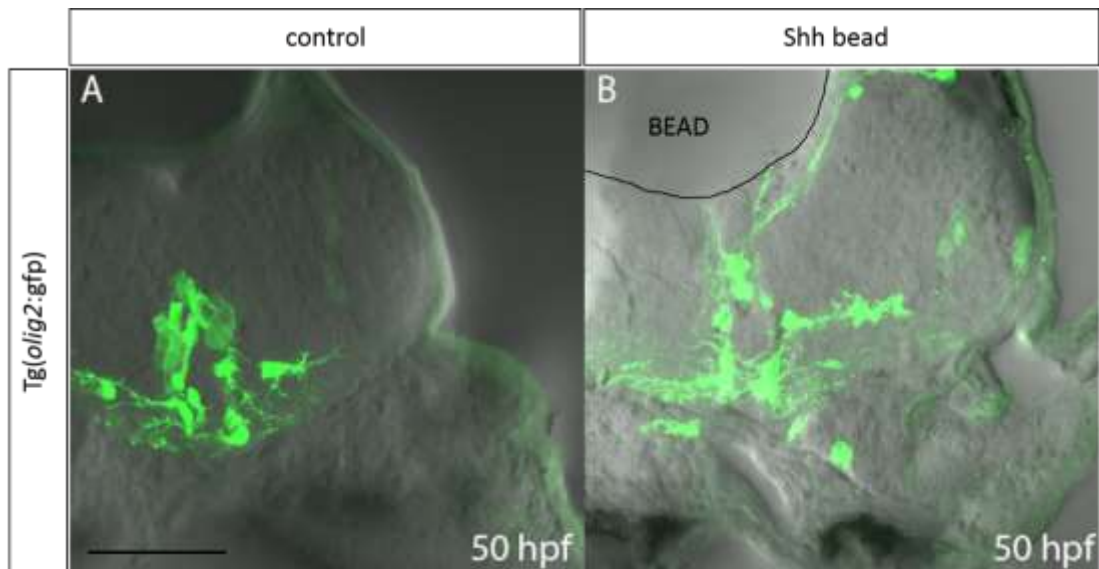
## 3.6 Introducing a dorsal source of Shh into the zebrafish hindbrain

To investigate whether Hh signalling has the potential to direct OPC process extension and migration, an assay was developed whereby a secondary source of Shh was introduced in the dorsal region of the hindbrain, which would compete with the endogenous ventral source. It was reasoned that if OPCs extended their processes up dorsally toward the secondary source of Shh it would suggest that Shh is sufficient to direct OPC migration, and could be responsible for the initial ventral process extension observed in *tg(olig2:gfp)* embryos.

### 3.6.1 Introducing a secondary dorsal source of Shh caused OPCs to alter process extension direction

To provide more direct evidence for an effect of Shh signalling on OPC migration, a dorsal source of mouse N-terminal Shh protein was injected into the dorsal hindbrain at 36 hpf. However this protein was prone to dispersal and it was difficult to ensure that the protein would remain in a fixed dorsal position. To overcome this problem a CM Affi-Gel<sup>®</sup> Blue Gel agarose bead soaked in mouse N-terminal Shh protein was introduced, which resulted in a more reliable fixed dorsal source of protein. A single agarose bead was inserted into the hindbrain of each individual embryo (n=5). Positioning the bead in the exact place was critical. If the bead was too far from midline OPCs they would be unable to detect and transduce the signal, if they were placed too close to the OPCs then too high a concentration may have acted as a stop signal to cells. It was found that beads that were positioned in the correct place (Figure 3-18) were able to induce the OPCs in the immediate vicinity to extend processes dorsally in contact with the beads (Figure 3-18B). This dorsal process extension was not observed in embryos implanted with control beads soaked in buffer alone. Attempts were made to label any primary cilia located on these extension processes through labelling with anti-Arl13b however the antibody failed to detect any primary cilia throughout the entire hindbrain brain which suggested an issue with the protocol or the antibody. Several attempts to increase permeability did not resolve the issue and since the antibody had been kept at 4°C

for two years it could have been denatured over time and lost its binding affinity. The antibody was not commercially available and a fresh aliquot was not available prior to submission of this thesis but will be a focus of future investigations.



**Figure 3-18: The effect of a dorsal Shh-impregnated bead on OPC migration**

To directly assess the effect of Shh on OPC migration, agarose beads soaked in Shh protein were implanted dorsally in the hindbrain at 34 hpf, prior to the main wave of OPC migration. Analysis took place at 50 hpf during the active migration wave when processes were being extended into the surrounding environment. OPCs could be seen extending process dorsally towards the Shh-impregnated beads (B) whereas in control embryos, processes only extended in their normal ventral and lateral directions (A). N=5. Scale bar 50  $\mu$ M

### 3.7 Discussion

This data supports a role for Hh in OPC migration although further analysis is required to separate the migratory phenotype from the proliferation defects observed after temporal loss of Hh. Increased responsiveness of OPCs to Hh signalling in the *ptch1;ptch2* mutants resulted in aberrant migration with cells entering dorsal regions of hindbrain (Figure 3-12). This suggests that a particular level of Hh signalling and responsiveness is required to ensure that cells are provided with and able to respond appropriately to ventral directional information. If OPCs were unable to perceive and transduce the signal as exclusively ventrally then they might be expected to migrate in different directions as they would have lost this positional information. Results to support this were observed in the

*ptch1;ptch2* mutants (see section 3.4.1). Introducing a dorsal source of Shh caused some OPCs in the immediate vicinity of the extraneous dorsal Shh signal, to extend processes in a dorsal upward direction (Figure 3-18). Evidence does need to be provided that this dorsal extension is a primary consequence of the Shh source and not due to the release of other growth factors in response to Shh or cell injury.

As discussed in (section 1.5.6), a study was published implicating FGF signaling in OPC hindbrain development, whereby upon chemical knockdown of FGF signaling, *olig2* cells were not specified. To investigate whether FGF signaling has subsequent roles in OPC migration, *tg(olig2:gfp)* embryos were treated post specification with 20  $\mu$ M SU5402 (following the protocol in original publication (Esain et al 2010)). Embryos were kept overnight and were then fixed at 50 hpf and analysed. This dose was very toxic to embryos, with a high proportion of death and necrosis. It was concluded that overnight exposure with this concentration was too high and the concentration was tested at 10  $\mu$ M and 5  $\mu$ M, however although embryo death was reduced they were still showing signs of toxicity when analysed morphologically. Unlike treatment with cyclopamine where embryos did not display toxicity signs, prolonged exposure to SU5402 showed too much necrosis and analysis for a role in *olig2*-positive cell migration could not be determined. In conclusion, the results present in this chapter suggested that Hh is one of the external signalling cues that promote proliferation and the early ventral migration of newly specified OPCs in the embryonic zebrafish hindbrain.

This project also aimed to address the question: What are the potential downstream target genes of these signalling pathways which lead the physical movement of OPCs in the required direction?

# Chapter 4 *disc1* as a potential downstream target of Hh signalling in OPC development

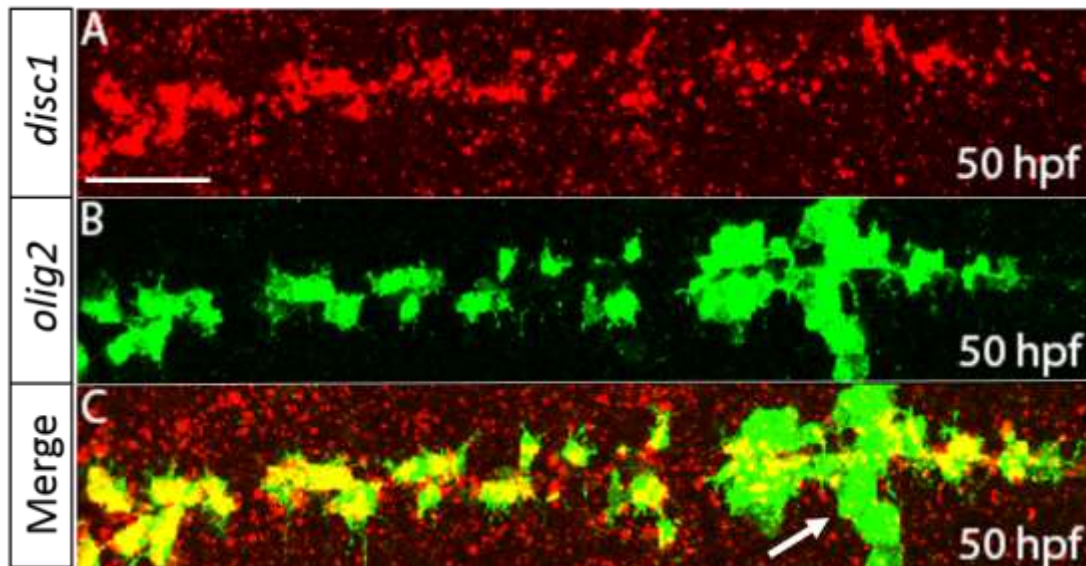
## 4.1 *disc1* is expressed in migrating OPCs in the zebrafish hindbrain

Previous work has shown that morpholino knock down of *disc1* in the zebrafish, a gene implicated in neural crest and neuronal migration (Drerup et al 2009), resulted in defects in proliferation and migration of OPCs in the hindbrain (Wood et al 2009). OPCs remained restricted to their birthplace in rhombomeres 5 and 6 and did not migrate to populate the hindbrain. Subsequently there was a loss of differentiated OPCs in the hindbrain at 5 dpf. Functional analysis in other systems has provided compelling evidence for DISC1 as a cytoskeletal hub protein with a large number of protein binding partners (Camargo et al 2007). The majority of these interacting proteins are components of the cytoskeleton. This led to the hypothesis that in the zebrafish hindbrain, Disc1 could be contributing to OPC migration, through recruitment of cytoskeletal proteins and formation of relevant complexes to cause the physical movement of cells in a ventral direction in response to extracellular signals including Shh.

### 4.1.1 Analysing expression of *disc1* in the zebrafish hindbrain

Previous work suggested that *disc1* was expressed in a similar region to *olig2* positive cells using *in situ* hybridisation comparison during migration however it was not confirmed in same specimen double staining methods (Wood et al 2009). Since there were no antibodies available for Disc1 that were cross-reactive with the

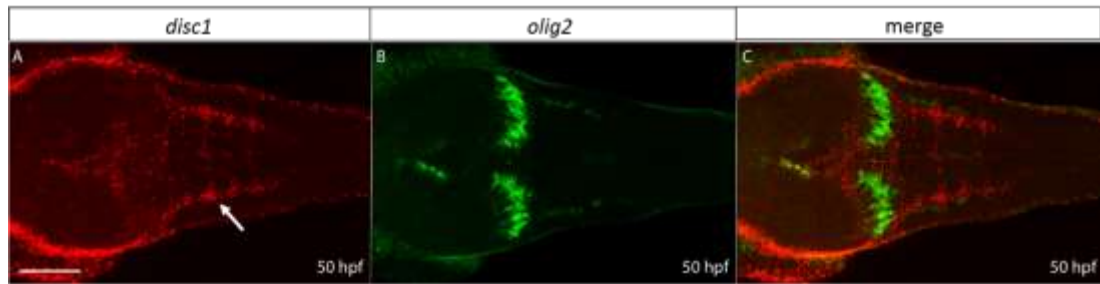
zebrafish protein, a TSA fluorescent *in situ* mRNA stain was performed with immunofluorescence staining for GFP in the *tg(olig2:gfp)* line to look for co-localisation of *disc1* mRNA with GFP. Indeed *disc1* was confirmed to be expressed in GFP positive OPCs at 50 hpf (Figure 4-2 A-C). Midline expression of *disc1* was observed in migrating OPCs but not r5/6 where the OPCs were stationary (Figure 4-1 C). These patches of cells in r5/6 also contained abducens motor neurons which suggested that *disc1* was expressed in migrating OPCs in the midline of the hindbrain (Figure 4-1 C arrow). It should be noted that TSA methods of fluorescent *in situ* hybridisation, although powerful in detecting low abundance mRNA there is also a degree of background staining which can be visualised in some of these images.



**Figure 4-1: Expression of *disc1* in migrating OPCs**

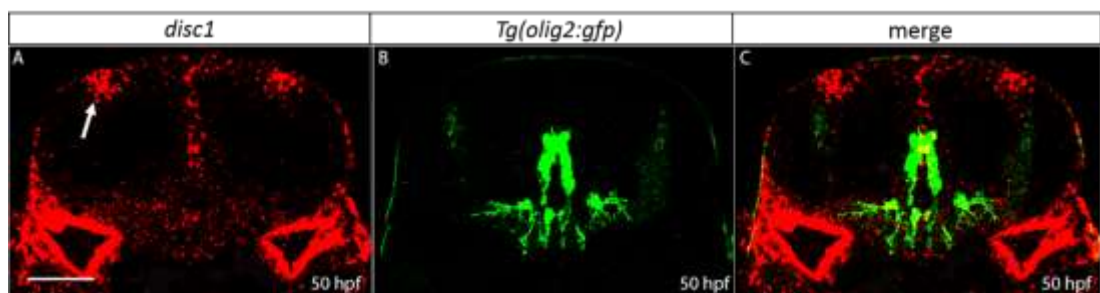
Expression of *disc1* appears higher in migrating OPCs along the anterior-posterior axis than in GFP positive cells in r5/6 which contain stationary OPCs and abducens motor neurons. All images are dorsal with anterior to the left and posterior to the right. Scale bar 20  $\mu$ M

An interesting observation during *disc1* expression of analysis was the discovery of two lateral patches of *disc1* expression in the superficial dorsal regions (Figure 4-2 A arrow) along with expression of *disc1* in migrating OPCs. Transverse sectioning showed that these lateral patches of expression were very superficial in the hindbrain and did not extend down into the hindbrain (Figure 4-3 A arrow).



**Figure 4-2: Expression of *disc1* in superficial dorsal regions of the hindbrain in 50 hpf embryos**

Expression of *disc1* was observed in two lateral strips on either side of the midline in superficial dorsal sections (white arrow in A) which sat more medially to the lateral GFP positive strips which did not overlap suggesting that they are labelling different cell populations (B and C). Scale bar: 100 μM

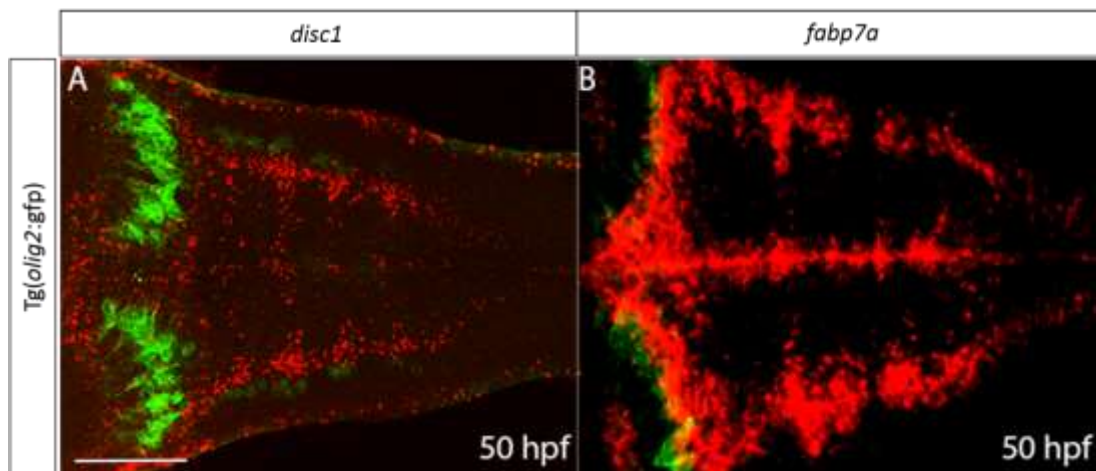


**Figure 4-3: Expression of *disc1* in a transverse section through the hindbrain of 50 hpf embryos**

Expression of *disc1* in transverse sections shows expression in the developing otic vesicle and a strip of expression down the midline (A) which overlaps with GFP expression in *Tg(olig2:gfp)* embryos (C). Lateral patches of expression in the superficial dorsal hindbrain might correspond to radial glia cells (white arrow in A). All images are transverse, dorsal at the top and ventral at the bottom. Scale bar 50 μM

The superficial lateral stripes of *disc1* expression was an interesting observation in light of a paper which showed that another gene, brain fatty acid binding protein (*fabp7a*) had a very similar expression pattern (Esain et al 2010). This gene is known to be expressed in radial glia, neuronal precursors of the brain and when expression of this gene was investigated by fluorescent *in situ* hybridisation then it was apparent that *disc1* shows a very similar pattern of expression to *fabp7a* in the hindbrain (Figure 4-4 compare A to B). Radial glia were originally named due their appearance and function in new born neuronal migration. Radial glia act as a platform for neurons migrating away from their birthplace due to their long radial processes, but more recently they have been identified as key progenitor cells of

the developing CNS which can give rise to neurons, astrocytes and also oligodendrocytes (Sild & Ruthazer 2011).



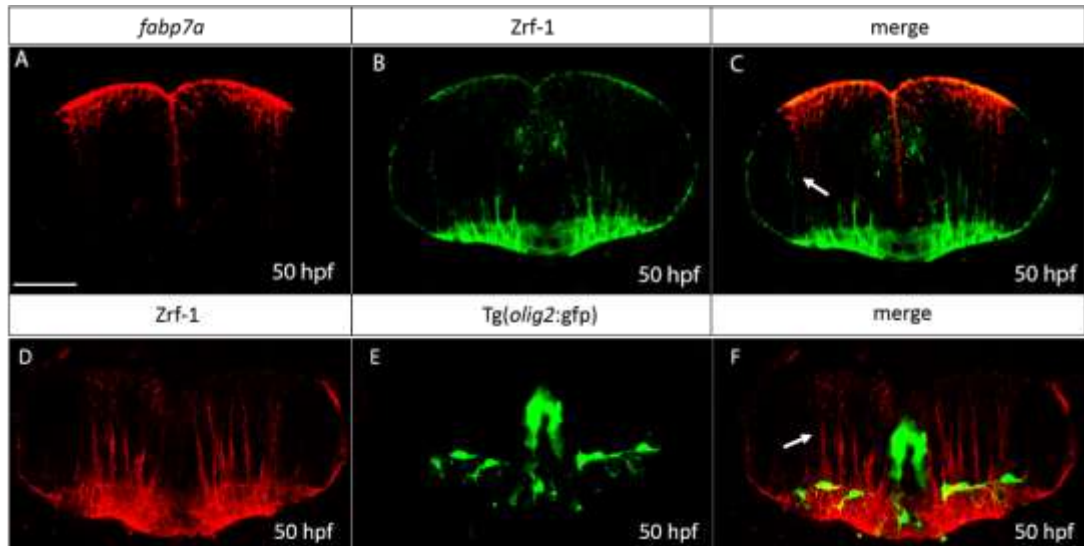
**Figure 4-4: Comparison of *disc1* and *fabp7a* expression in the superficial dorsal hindbrain at 50 hpf**

Expression of *disc1* was observed in lateral strips on either side of midline in the dorsal hindbrain (A) showed a similar expression pattern to *fabp7a*, which is a genetic expression marker for radial glia (B). Scale bar 100  $\mu$ M

This similarity in expression suggests that *disc1* could also have a role in the development of this neuronal cell type which would correlate well with the known roles for *DISC1* in human and mouse neural cell development. Extensive investigation into the role of *disc1* in radial glia development was beyond the scope of this project however some speculations were made as to function of *disc1* in the development of these *fabp7a*-positive cells. Antibody staining of Zrf-1 which labels glial fibrillary acidic protein (GFAP), another marker of radial glia shows expression of long radial glial fibres throughout the hindbrain with extensive expression in the most ventral part of the hindbrain (Figure 4-5). Expression of *fabp7a* mRNA also showed some expression in radial fibres which co-localised with Zrf-1 in superficial lateral patches (Figure 4-5 C arrow). Comparison of Zrf-1 expression in *tg(olig2:gfp)* embryos showed migrating OPCs sitting dorsal to Zrf-1 expression, with radial glia extending fibres on either side of the midline. The data suggests that *disc1* expression and its potential function in radial glia is likely to be independent to its roles in OPC migration. The expression of *disc1* in radial glial cells could identify a function for the cytoskeletal protein in radial fibre extension during the development of radial glia used by migrating new born neurons. Alternatively it



could be expressed in radial glia cells in the context of neuronal or astroglial progenitors. Further experiments and investigations would be required to identify the function of *disc1* in radial glia which could be an interesting avenue for future projects.

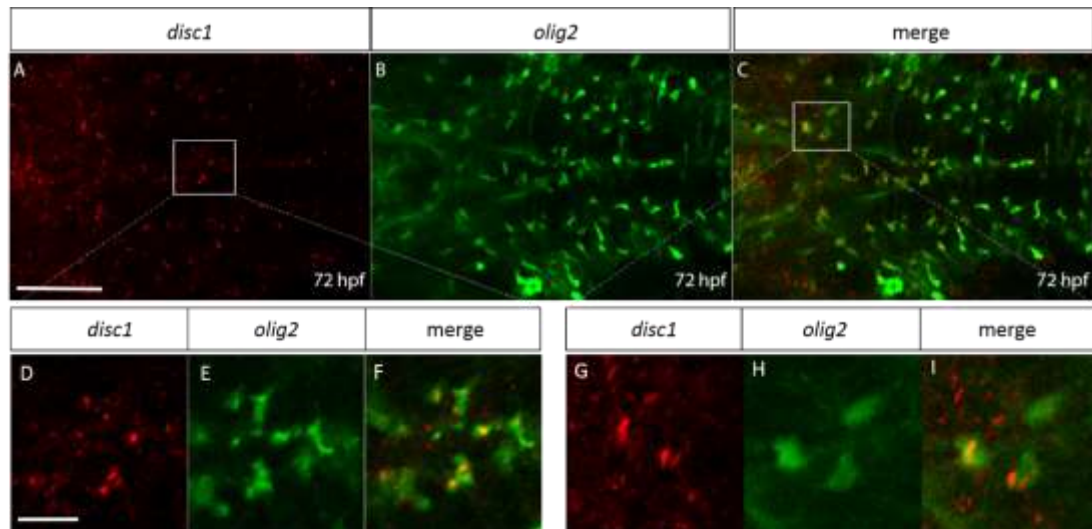


**Figure 4-5: Expression of Zrf-1 shows extension of radial glia processes throughout the hindbrain in transverse sections in 50 hpf embryos.**

Expression of Zrf-1 and *fabp7a* mRNA show radial glia fibres (C arrow). Expression of Zrf-1 sits ventral to *olig2* positive cells and extends process on either side of the midline (F arrow). Scale bar 50  $\mu$ M

Expression of *disc1* was also observed in 72 hpf embryos in OPCs during the later stages of the main migratory period and the onset of differentiation (Figure 4-6). This suggests a possible role for *disc1* at later stages of development as well as during migration, furthermore roles for *disc1* in oligodendrocyte differentiation have recently been postulated (Hattori et al 2014).



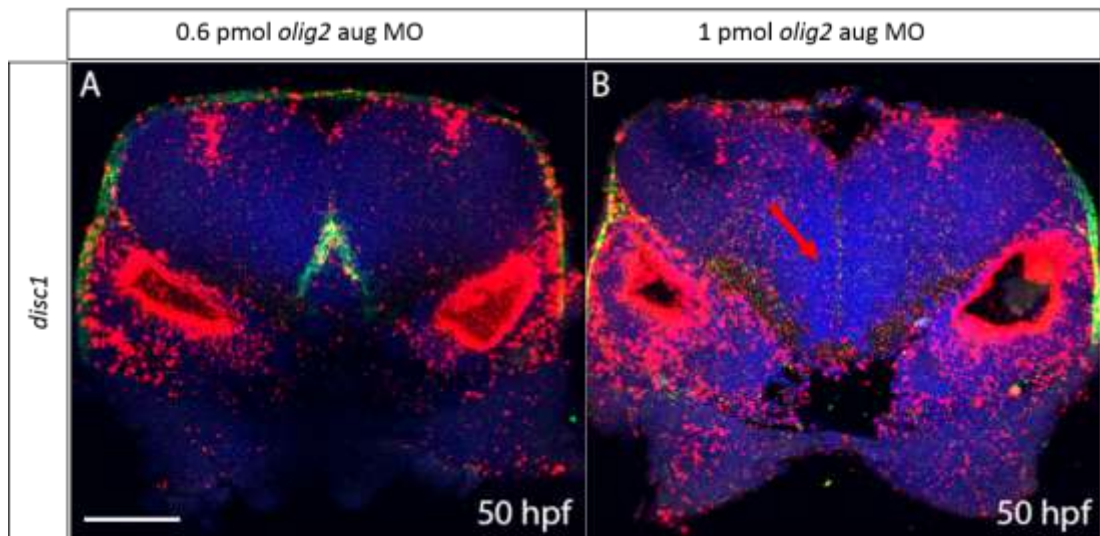


**Figure 4-6: shows *disc1* expression in *olig2* positive cells in 72 hpf embryos**

Expression of *disc1* remained at 72 hpf in *olig2*-positive cells during the final OPC migratory stage and beginnings of differentiation (A-C shows whole zebrafish hindbrain scale bar 75  $\mu$ M). A zoomed in image of OPCs in the midline (D-F) shows co-localization of *disc1* in GFP cells. A zoomed image of migrating OPCs shows *disc1* co-localization in GFP cells (G-I). D-I scale bar 10  $\mu$ M

#### 4.1.2 *olig2* morphants display a loss of *disc1* midline expression

To confirm the fluorescent in situ hybridisation data which suggests that *disc1* is expressed in *olig2*-positive cells, *olig2* was knocked down using a previously described translation blocking morpholino to determine whether midline *disc1* expression is affected upon removal of this cell type (Park et al 2002). If *olig2* expression is lost then OPCs are not specified (Zhou & Anderson 2002). It would therefore be expected that *disc1* OPC expression would be lost in *olig2* morphants, which indeed was the case. Embryos were injected with two doses of morpholino, an intermediate concentration and a higher concentration. Although *disc1* expression was maintained in the developing otic vesicle, the lower jaw and presumptive radial glia, *disc1* was lost in the midline in 1pmol of *olig2* MO injected embryos and reduced in a lower dose of 0.6pmol (Figure 4-7). This result demonstrates that *disc1* is expressed in *olig2*-positive midline cells since upon loss of *olig2* which results in a loss of OPCs, *disc1* is no longer expressed.



**Figure 4-7: shows *disc1* expression in *olig2* morphants**

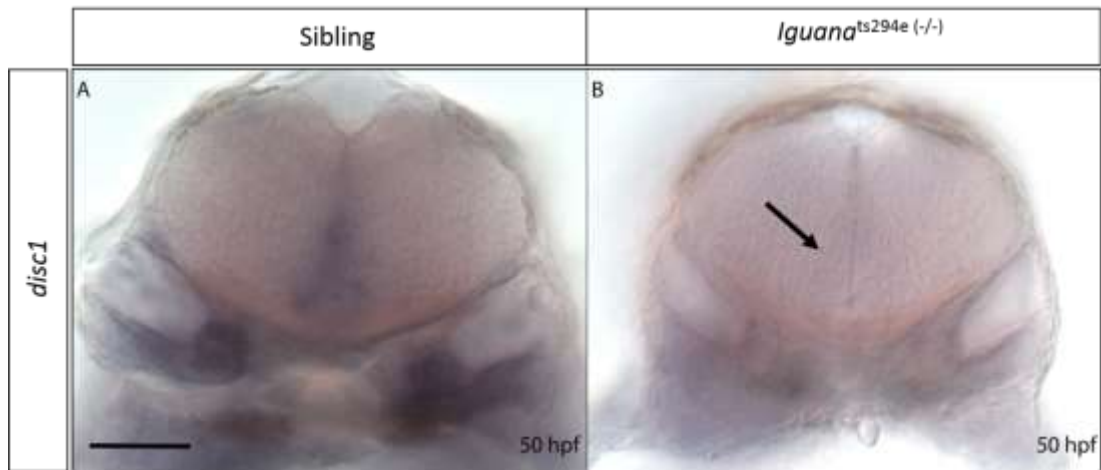
Knock down of *olig2* through a translation blocking morpholino resulted in reduced *disc1* expression (A) or a complete loss of *disc1* expression with a higher dose of morpholino (B) in the midline of the hindbrain. Expression of *disc1* was maintained in the otic vesicle, lower jaw and presumptive radial glia. Red arrow in B shows loss of GFP-*olig2* expression. Scale bar 50  $\mu$ M.

## 4.2 Analysis of *disc1* expression in *shh* pathway mutant lines

To determine whether *disc1* is a downstream target of Shh signalling, *in situ* hybridisation was performed in *smo*<sup>b641 (-/-)</sup>, *iguana*<sup>ts294e (-/-)</sup>, *ptch1;ptch2*<sup>(-/-)</sup> mutants to analyse expression changes of *disc1* in response to defective Shh signalling.

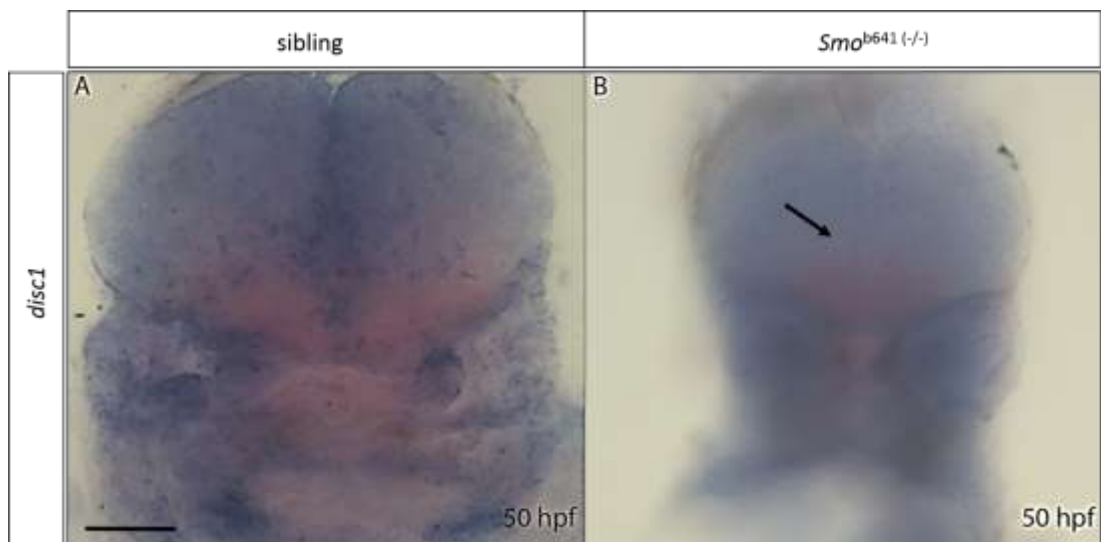
### 4.2.1 Expression of *disc1* around the midline is altered in Shh pathway mutant embryos

As expected *disc1* expression was lost in *iguana*<sup>ts294e (-/-)</sup> (Figure 4-8 B arrow) and *smo*<sup>b641 (-/-)</sup> (Figure 4-9 B arrow) mutants compared to sibling controls at 50 hpf, since the inhibition of Hh signalling in these mutants leads to an OPC specification defect. Mutant embryos were considerably smaller than wildtype siblings which reflect how fundamental Hh signalling is in early embryo development and a loss of global Hh signalling results in decreased survival and proliferation of a wide range of cell types (Chen et al 2001).



**Figure 4-8: Comparison of midline *disc1* expression between sibling and *iguana*<sup>ts294e(-/-)</sup> embryos at 50 hpf.**

Expression of *disc1* was lost in the midline in *iguana*<sup>ts294e (-/-)</sup> mutants (black arrow in B) but expression was maintained in the developing ear but was reduced. Images show transverse sections. 50  $\mu$ M

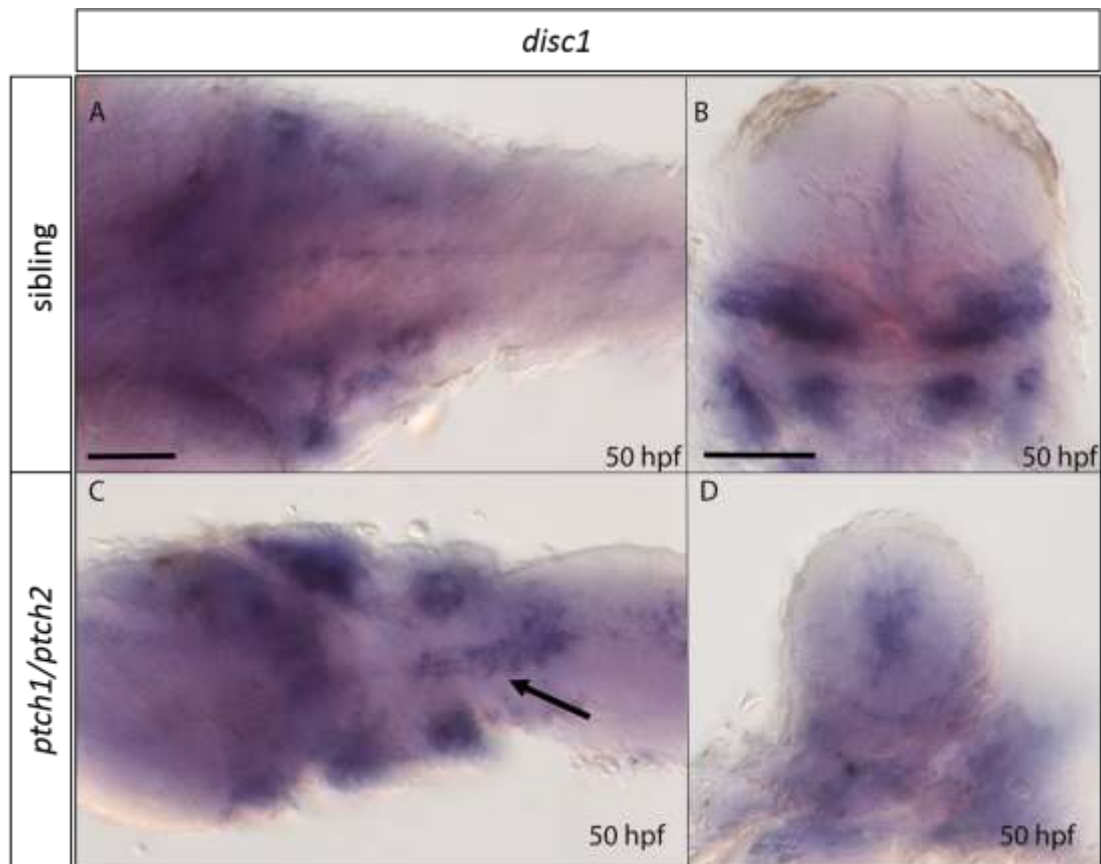


**Figure 4-9: Comparison of midline *disc1* expression between sibling and *smo*<sup>b641(-/-)</sup> embryos at 50 hpf.**

Expression of *disc1* is lost in the midline of *smo*<sup>b641(-/-)</sup> mutants (black arrow in B) compared to sibling *disc1* expression. All images are transverse sections. 50  $\mu$ M

Interestingly in *ptch1;ptch2* mutant embryos *disc1* expression was increased around the midline compared to sibling embryos at 50 hpf (Figure 4-10), which could have been a result of seemingly excessive migration of *olig2*-positive cells in these mutants (Figure 3-12). The expression of *disc1* was altered from a thin strip of expression in the midline which would normally create a diamond shape of expression in the ventral portion of the hindbrain (Figure 4-10B), to expanded

expression on either side of the midline in dorsal views (Figure 4-10 compare A and C).



**Figure 4-10: Comparison of *disc1* expression in *ptch1;ptch2* mutants and sibling controls at 50 hpf**

Expression of *disc1* was increased in *ptch1;ptch2* mutants in the hindbrain midline (arrow in C). A and C are dorsal views, B and D are transverse views. Expression in otic vesicles also show increased expression in *ptch1;ptch2* mutants (C). Scale bar 100  $\mu$ M

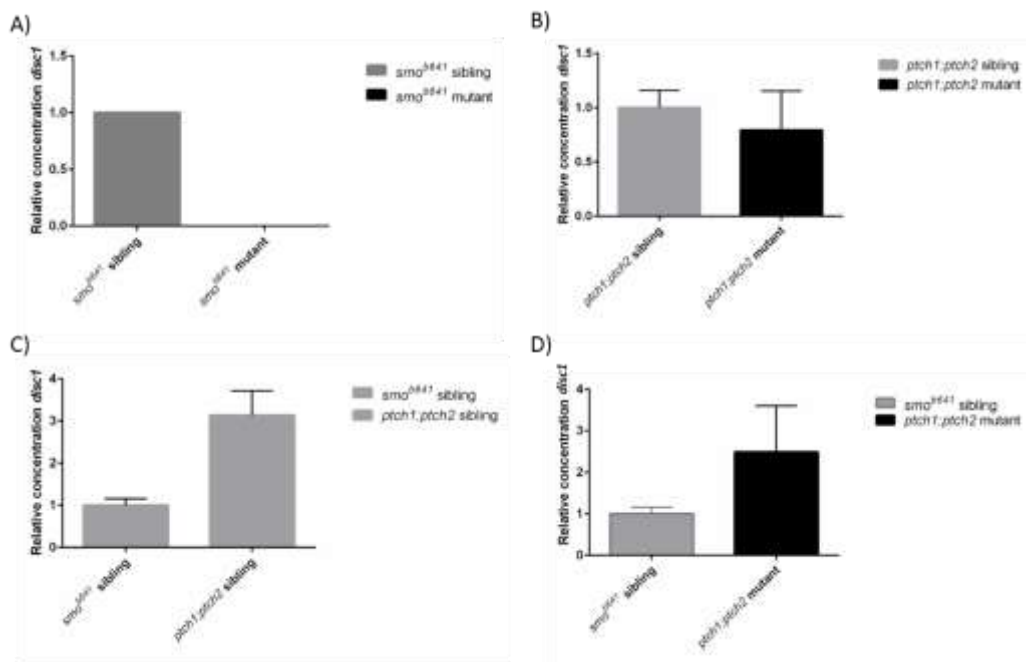
Altered expression of *disc1* mRNA in the different Hh pathway mutant embryos suggests that it may be a downstream target of Hh signalling. In *ptch1;ptch2* mutant embryos, *disc1* expression was considerably increased around the midline, suggesting that increased Hh signalling transduction due to *ptch* loss of function resulted in *disc1* up-regulation. This subsequently could have contributed to the excessive migration witnessed in these mutants (Figure 3-12), with cells migrating out of their normal restricted ventral region into dorsal areas of the hindbrain. Of course it is important to acknowledge here that these mutant embryos have grossly altered morphology due to a severe patterning phenotype. Expression of *disc1* was

completely lost in the midline in the loss of function Shh mutants since OPCs failed to be specified.

### 4.3 Quantification of *disc1* expression in hedgehog signalling mutants

To provide further evidence for regulation of *disc1* expression by Shh we sought to quantify *disc1* expression in the mutant lines using quantitative PCR to verify the *in situ* hybridisation data. The Shh *smo*<sup>b641</sup> and *ptch1;ptch2* mutants and siblings (which were a combination of single homozygote mutants, various combinations of homozygotes and heterozygotes and wild type embryos) were collected at 50 hpf and cDNA was synthesised from RNA extracted from pools of 20 embryos. QPCR expression data was collected and compared against a reference housekeeping gene, *elongation factor 1-alpha (ef1a)* (Livak & Schmittgen 2001) (Tang et al 2007). Expression of *disc1* was compared between *smo*<sup>b641</sup> mutants and their siblings, and as expected there was a loss of *disc1* expression in the homozygous mutants compared to their sibling counterparts. In fact there was complete loss of *disc1* expression in *smo*<sup>b641</sup> mutants compared to siblings i.e. expression didn't pass the threshold suggesting there was no gene product (Figure 4-11 A). Expression of *disc1* was then compared between *ptch1;ptch2* mutants and their siblings. Surprisingly, there was slightly lower expression of *disc1* in *ptch1;ptch2* mutants compared to *ptch1;ptch2* siblings which was inconsistent with the *in situ* hybridisation data which suggested increased expression of *disc1* in the midline (Figure 4-11 B). These sibling pools will have contained a combination of *ptch1*<sup>(-/-)</sup> homozygotes, *ptch2*<sup>(-/-)</sup> homozygotes, *ptch1*<sup>(-/-);ptch2</sup><sup>(+/-)</sup> and *ptch2*<sup>(-/-);ptch1</sup><sup>(+/-)</sup> embryos along with wild type siblings and these combinations of patched mutants could have contained increased *disc1* expression which may have masked any increases in *disc1* expression in the double mutants. The double mutants were very morphologically abnormal and smaller in size and therefore could be lacking cell populations that normally express *disc1* which may also have skewed the results. Comparing the expression levels of *disc1* between the two pools of siblings, revealed that the *ptch1;ptch2* siblings had higher expression than the *smo*<sup>b641</sup> siblings (Figure 4-11 C). When *disc1* expression in *ptch1;ptch2* mutants was compared against *smo*<sup>b641</sup>

siblings there was increased expression in the double mutants as originally expected, thereby suggesting that the *ptch1;ptch2* sibling pools contained an accumulation of increased *disc1* expression from the combination of heterozygotes and single homozygotes (Figure 4-11 D). This could therefore suggest that *disc1* is upregulated in response to increased Shh signalling but this data is a very preliminary finding. One biological set of 20 embryos was used for each group and these were subjected to 3 technical replicates. Further biological replicates are required to confirm these findings and to assess inconsistencies between pools of embryos. Individually pooling *ptch1;ptch2* in-crosses based on genotype would be ideal but difficult, since genotyping of each embryo would be required. Analysing the expression pattern of *disc1* using *in situ* hybridisation in the different single patched homozygous and heterozygous combinations could be used to confirm increased *disc1* expansion in the midline compared to wild type siblings.



**Figure 4-11: Quantification of *disc1* expression in Hh signalling stable mutant lines**

Expression of *disc1* was quantified using qPCR in Shh signaling stable mutants and were compared against *disc1* expression levels in their sibling counterparts. Expression of *disc1* failed to reach the threshold in *smo<sup>b641</sup>* mutants suggesting no gene product compared to sibling control *smo<sup>b641</sup>* embryos (A). Expression of *disc1* in *ptch1;ptch2* double mutants compared to *ptch1;ptch2* siblings showed very similar expression levels (B). When the siblings from both sets were compared it was shown that *disc1* was expressed at three times higher levels in the *ptch1;ptch2* siblings compared to the *smo<sup>b641</sup>* siblings (C). Finally when the expression of *ptch1;ptch2* mutants was compared against expression in the

*smo*<sup>b641</sup> siblings, the mutants displayed 2.4X higher expression of *disc1* (D). Error bars show SEM.

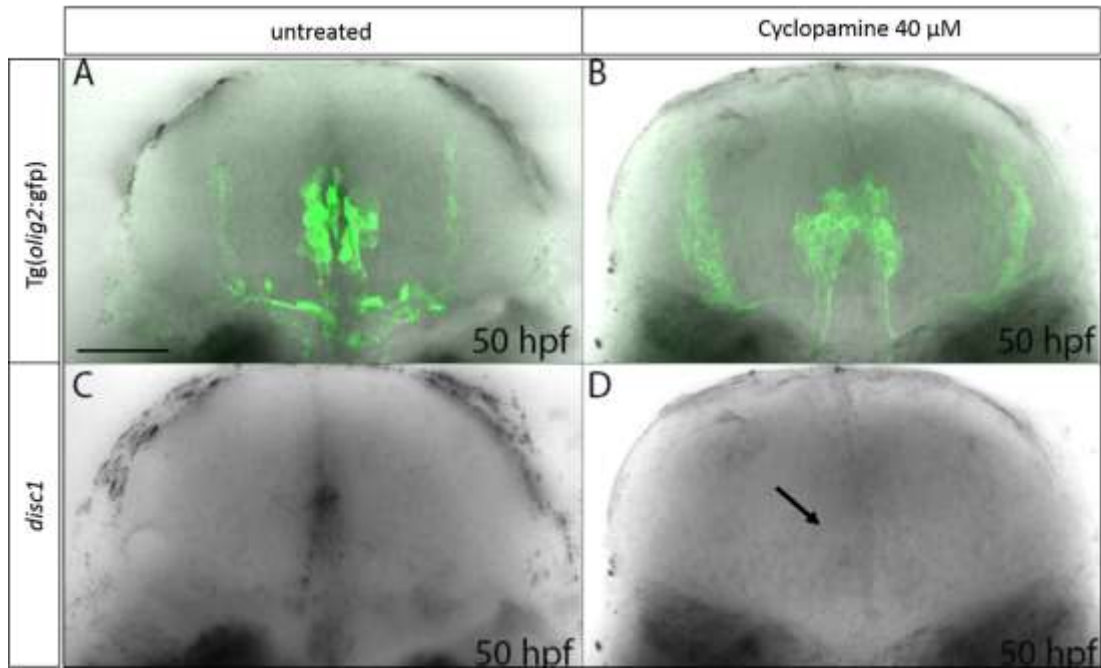
#### 4.4 Loss of *disc1* expression in cyclopamine treated embryos

As discussed before, the Hh loss of function mutant zebrafish lines were not appropriate to investigate the effect of Hh loss on later stages of OPC development since OPCs are not specified in these mutants. Similarly, they were unsuitable to determine the effect of loss of Hh signalling on *disc1* expression in OPCs. However cyclopamine treatment allowed temporal removal of Hh signalling thereby enabling OPCs to be specified as normal in the embryo prior to loss of Hh signalling.

##### 4.4.1 Expression of *disc1* in the midline is lost in cyclopamine treated embryos

Cyclopamine (40 µM) was applied to *tg(olig2:gfp)* embryos (n=10) prior to the main period of OPC migration as with previous experiments and the embryos fixed at 50 hpf. Embryos were then analysed for *disc1* mRNA expression using *in situ* hybridisation. These experiments revealed a clear loss of *disc1* in the midline of cyclopamine treated embryos as well as the previously described apparent reduction in migration of OPCs from the midline (Figure 4-12 Compare D to C). Expression of *disc1* was however maintained around the otic vesicle and developing jaw.





**Figure 4-12: shows *disc1* expression in the midline of cyclopamine treated embryos**

Cyclopamine was applied at 34 hpf prior to migration and fixed at 50 hpf, treated embryos (n=10) had a loss of *disc1* expression (D arrow) compared to untreated embryos (C). Panel B shows a loss of GFP positive cell migration as a result of temporal Hh signalling loss. All images are transverse sections. Scale bar 50  $\mu$ M

These experiments suggest that *disc1* could be a downstream target of Hh signalling, however it whether it is secondary to altered *olig2* expression is not known. These data suggests that Hh signalling ventrally during OPC development could be involved in migration since migration does seem to affect during altered signalling. Whether this is a direct result of proliferation defects is still to be elucidated, and will be of focus for future experiments. It can be hypothesised that if Hh signalling is required for OPC migration it could be through regulation of Disc1 protein and other cytoskeletal proteins, however this is merely speculative.



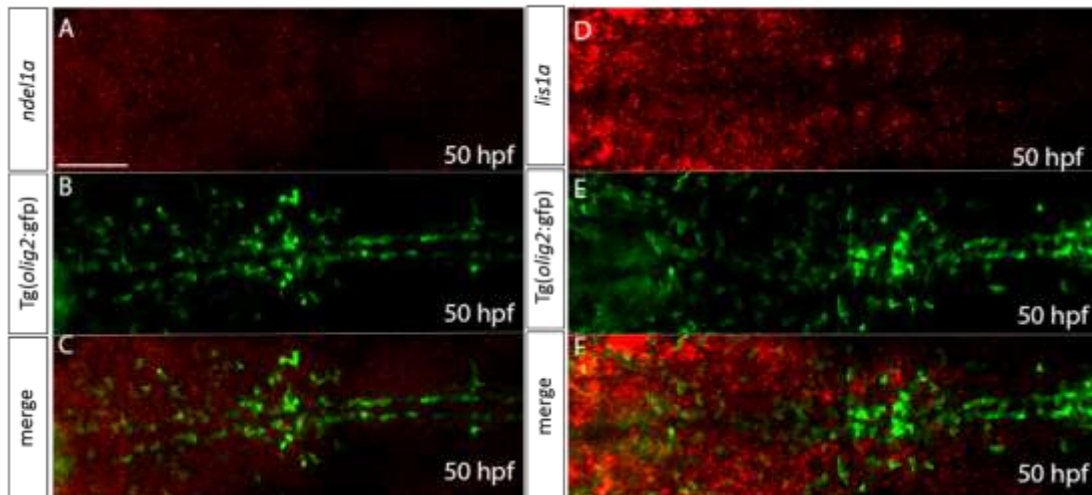
# Chapter 5 Investigating the cellular localisation of Disc1 in OPCs

## 5.1 Introduction

Yeast two hybrid studies have suggested that the DISC1 protein acts as a scaffold with the capacity to bind several proteins (Section 1.3). Many of these multiple binding partners are cytoskeletal proteins and provided the possibility that DISC1 brings proteins together to create cytoskeletal complexes required for processes such as cell migration. As discussed previously, DISC1 has roles in neuronal migration involving such protein complexes so it seemed reasonable to hypothesize that the zebrafish Disc1 protein could contribute to OPC migration through similar protein interactions.

## 5.2 Investigating potential Disc1 OPC protein interactors

To try and identify the relevant protein binding partners of Disc1 which could be involved in OPC migration, fluorescent *in situ* hybridisation of cytoskeletal partners with already established functions with DISC1 in neuronal migration were performed in *tg(olig2:gfp)* embryos. Two proteins known to bind DISC1 and form complexes in cell culture and mouse systems are LIS1 and NDEL1, so expression of their zebrafish orthologues were initially investigated in hindbrain OPCs during the main migration phase (Morris et al 2003) (Kamiya et al 2005).



**Figure 5-1: Expression pattern *lis1a* and *ndel1a* in 50 hpf *tg(olig2:gfp)* embryos**

Fluorescent *in situ* hybridization of *ndel1a*, shows weak expression in the brain that does not overlap with GFP *olig2*-positive cells (A-C). Expression of *lis1a* shows stronger brain expression surrounding GFP *olig2*-positive cells but there is no overlap (D-F). All images are anterior to left and posterior to the right. All images are dorsal views. Scale bar 30  $\mu$ M

No expression of either *ndel1a* or *lis1a* was observed in OPCs (Figure 5-1), suggesting that their function in cell migration with DISC1 may be specific for neuronal migration in other systems and not important in zebrafish OPCs, at least during this stage of development. The expression patterns of *ndel1b* and *lis1b* did not show expression in OPCs either (data not shown). Although this data suggests that these particular proteins are not involved in OPC migration it cannot be completely ruled out. These proteins are ubiquitously expressed, meaning that there may still be some expression but not to a level detectable by *in situ* hybridisation. However such low levels of expression indicate that they are unlikely to have active roles in OPC migration at this time point.

Yeast two hybrid experiments and subsequent validation has identified approximately 600 interacting partners of Disc1 (Camargo et al 2007), and to go through these systematically to look at whether there is expression in OPCs would have been inefficient. Investigating the localisation of the Disc1 protein in OPCs, at all stages of their development would provide a more defined list of candidate proteins to investigate further based on their sub-cellular localisation. Proteins with a similar localisation to Disc1 would be more likely to interact and form complexes and thus contribute to OPC migration. As discussed previously (Section 1.3), the

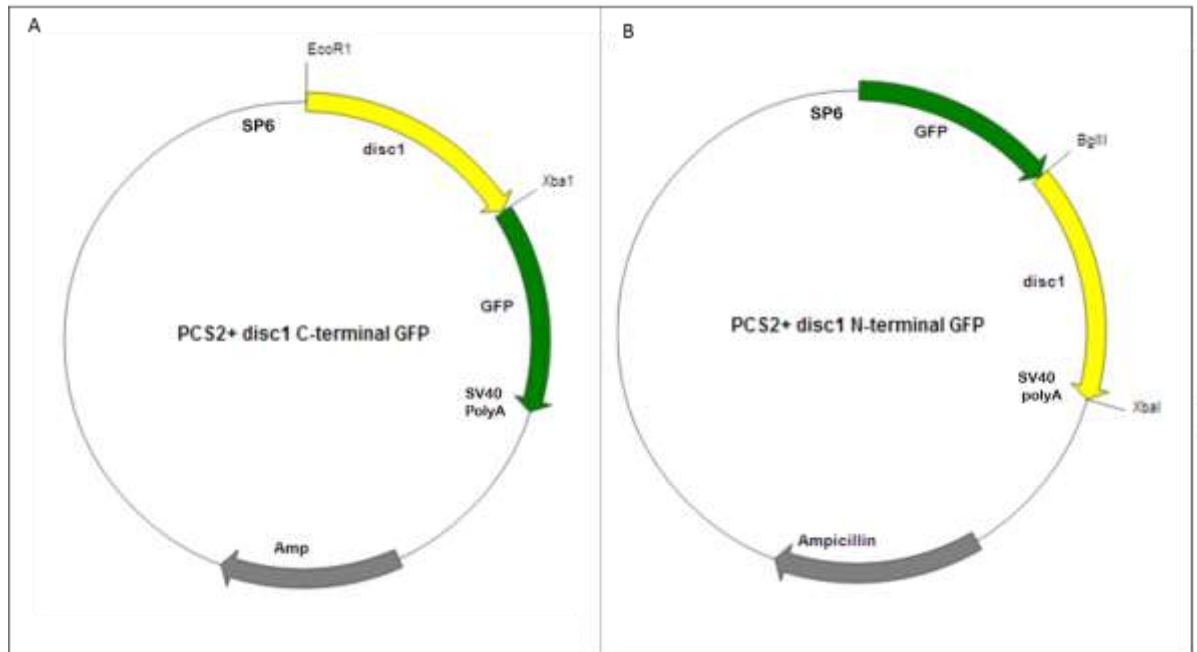
localisation of DISC1 has been debated for several years, with different antibodies showing DISC1 expression in a variety of cellular locations (Figure 1-1) and the majority of this data was obtained with neuronal cells. Localisation information is lacking for DISC1 in OPCs *in vivo* and so investigating the cellular location of Disc1 during OPC development in the zebrafish could provide novel mechanistic information into Disc1 biology.

### 5.3 Overexpression studies of Disc1 to decipher cellular localisation

There are several methods available that can be used in the zebrafish to decipher the subcellular localisation of a protein. Overexpression studies using constructs whereby the gene of interest, in this case *disc1*, is fluorescently tagged and injected either as a linearized DNA construct or as capped mRNA into 1 cell stage embryos is a popular technique that can give a swift indication of protein localisation within cells *in vivo*. Embryos were injected with capped *disc1* mRNA or linearized heat shock-inducible *disc1* expression constructs to analyse the localisation of overexpressed Disc1.

#### 5.3.1 Expression of Disc1-GFP fusion proteins via mRNA injection

To visualise Disc1 mRNA, the protein was fluorescently tagged at the *N*- and *C*-terminal ends with GFP contained in PCS2+ (Figure 5-2). Embryos were injected at the one cell stage and after 6 hours uniform fluorescence was observed throughout the injected embryo. However by 24 hpf fluorescence was no longer visible, indicating that both the injected mRNA and the translated protein had been degraded. Similarly when the protein was tagged at the *N*-terminal end with GFP, loss of fluorescence again occurred before 24 hpf. This made mRNA injections unsuitable for our purposes since the localisation of Disc1 could not be investigated at later stages of development (2 dpf) during OPC migration.

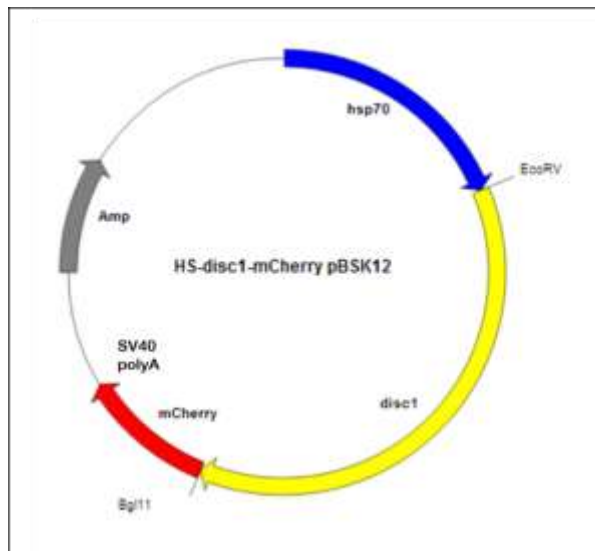


**Figure 5-2: Vector maps of C-terminal and N-terminal GFP tagged *disc1***

The *disc1* sequence was inserted into the PCS2+ GFP vector backbone using the restriction sites EcoR1 and XbaI for the C-terminal GFP vector (A), and BglII and XbaI for the N-terminal GFP vector (B). Both vectors were linearized with Not1 and SP6 polymerase was used to generate mRNA through *in vitro* transcription

### 5.3.2 Expression of Disc1-mCherry fusion proteins using linearized heat-shocked inducible DNA expression constructs

As an alternative to mRNA injection, a *disc1-mCherry* DNA expression construct containing a Heat shock (HSP70) promoter was generated from a heat shock mCherry plasmid in the vector backbone pBSK12 (unpublished Semil Choksi) to allow for temporal control of protein expression (Figure 5-3). The zebrafish Disc1 gene was inserted into the plasmid using restriction sites EcoRV and BglII and the plasmid was linearized for injection using I-SceI sites.

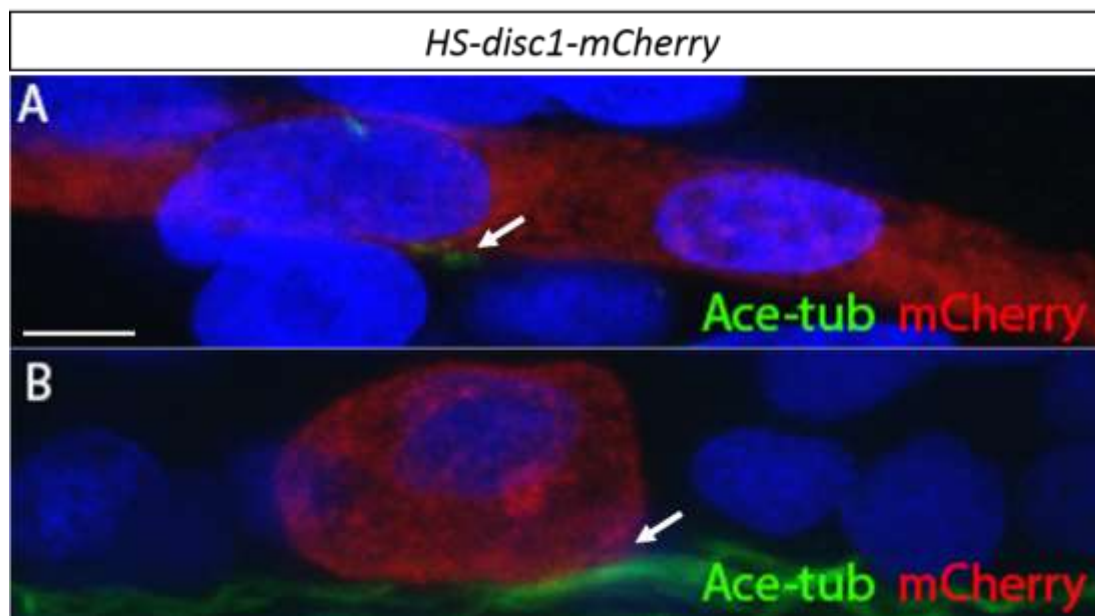


**Figure 5-3: Vector map of HSP70: *disc1*-mCherry**

The *disc1* sequence was inserted into the pBSK12 backbone using the restriction sites EcoRV and BglII. The vector was linearized using IscE-I meganuclease just prior to injection.

One cell stage *tg(olig2:gfp)* embryos were injected with linearised *HSP70-disc1-mCherry* DNA and allowed to develop until 30 hpf. Embryos were then heat shocked in a 37°C water bath which induced expression from the *HSP70* promoter in response to heat stress. After 2-4 hours, cells containing the DNA construct expressed Disc1-mCherry protein. Since DNA is more stable than mRNA, embryos could be injected at fertilisation but then heat shocked at later developmental stages, thereby increasing the chances of observing expression in migrating OPCs. The expression of mCherry however was mosaic, which reduced the chance of the construct being present in OPCs. Unfortunately this was the case; no Disc1-mCherry was detected in OPCs in 20 embryos analysed. However the fluorescence observed was stronger than that during the mRNA expression studies so its localisation was still analysed in other cell types of the embryo. It was reasoned that Shh signalling could be transduced by OPC primary cilia prior to or during migration and there is some evidence to suggest that Disc1 localises to the base of primary cilia in NIH-3T3 fibroblasts (Marley & von Zastrow 2010) (Section 1.3.4). Therefore acetylated tubulin was co-stained with mCherry to investigate whether overexpressed Disc1 localised to primary cilia in the zebrafish. Disc1-mCherry showed diffuse cytoplasmic expression with no clearly defined subcellular localisation, there is also

some expression in the nucleus. No expression was seen on either primary cilia or motile cilia (Figure 5-4).



**Figure 5-4: Cytoplasmic localisation of overexpressed Disc1-mCherry**

Wild type embryos were injected with linearized *HSP70: disc1-mCherry* construct and heat shocked at 37°C for 1 hour at 30 hpf to analyze Disc1 protein expression. Disc1 was not seen to be expressed on primary (A) or motile cilia (B) but showed a general cytoplasmic localization in muscle and pronephric duct cells. Scale bar 5  $\mu$ M

### 5.3.3 Limitations of overexpression

Overexpression via mRNA injection was unsuccessful in identifying the localisation of Disc1 in OPCs. Degradation of both mRNA and protein before 24 hpf was the major limiting factor meaning that the protein localisation in migrating OPCs could not be investigated. This was perhaps not surprising since the Disc1 protein is relatively large and stability could have been compromised. Conversely, the *HSP70-disc1-mCherry* construct could be expressed beyond 24 hpf but posed a different limitation in that injection of this DNA construct created a mosaic pattern of overexpressed Disc1-mCherry, unlike with mRNA injection where the protein was uniformly expressed throughout the embryo. This decreased the likelihood of successfully targeting of the *HSP70-disc1-mCherry* construct into OPCs. Positive embryos for Disc1-mCherry were raised to adulthood in an attempt to establish a stable line to analyse localisation and also to investigate the consequences of

overexpression of *disc1* on migrating OPCs. Unfortunately during screening no mCherry positive embryos were identified after heat shock although when genotyped the *disc1-mCherry* construct could be identified through PCR.

Another limitation to this type of analysis is that it is based on over-expression. The localisation of over-expressed Disc1 may not necessarily truly mimic that of endogenous Disc1. Pumping a cell full of protein could ultimately lead to it being expressed in cellular locations not representative of the normal endogenous localisation. Specifically detecting and labelling only endogenous protein however would eliminate these issues and provide reliable localisation information during all stages of development.

## 5.4 Development of zebrafish antibodies to label endogenous Disc1

The aim shifted to focus on endogenous Disc1 protein through antibody development since overexpression studies were inconclusive and potentially may not be reliable. Commercial antibodies that are cross-reactive with the zebrafish protein are limited compared to those available for use in mammalian systems, making it unlikely that existing commercial anti-DISC1 antibodies would be useful in the zebrafish. Instead it was decided that specifically raising antibodies against zebrafish Disc1 would improve the chances of the antibodies working effectively. Polyclonal antibodies are inexpensive to produce and less time consuming compared to the generation of monoclonal antibodies. Polyclonal antibodies recognise several epitopes on a single antigen which can provide higher affinity since the protein of interest will bind more than one antibody molecule. These antibodies are also more tolerant to antigen changes (for example in the case of polymorphisms) however they vary from batch to batch and can also produce a lot of non-specific antibodies. Previous attempts had been made to make polyclonal antibodies but were unsuccessful in specifically labelling endogenous Disc1 protein and therefore monoclonal antibody production was attempted as an alternative. Monoclonal antibodies are different in that they have high specificity as they only detect one epitope on the antigen and therefore have less background during the

staining process, however they are more expensive and time consuming to produce. Six monoclonal antibodies were raised against zebrafish Disc1 by Abmart against the synthetic peptide EDNLPVQSRD corresponding to residues 119-128 of Disc1. The antibodies were provided at high titre but with no guarantee regarding binding affinity or specificity. Provided these antibodies were successful in labelling endogenous Disc1 through immunofluorescence staining, then the localisation of Disc1 protein could be investigated in OPCs throughout their development, but also in the lower jaw cartilages, otic vesicle and presumptive radial glia cells.

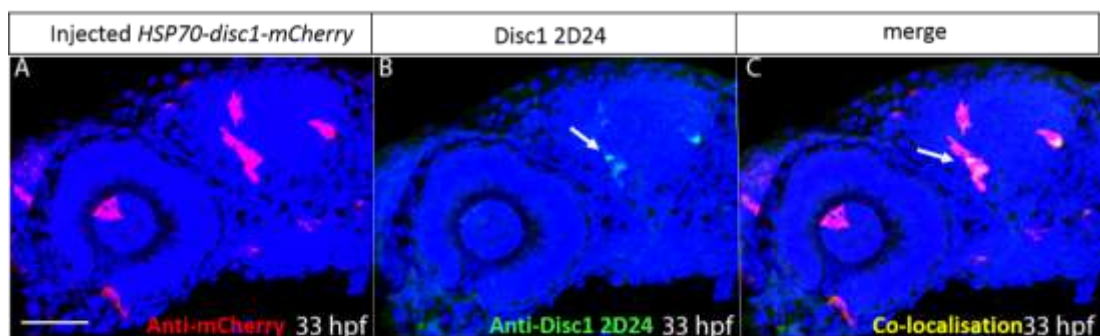
#### 5.4.1 Immunofluorescence staining with Abmart anti-Disc1 monoclonal antibodies

Of the six antibodies raised against zebrafish Disc1 only 2D24 displayed any affinity for detecting Disc1. Western blot analysis detected 3 proteins of approximately 100-160 kDa compared to a predicted  $M_r$  for Disc1 of 112 kDa (NI Alsomali, unpublished). It should be noted that Disc1 contains multiple coiled-coil domains so it might be expected to run with an apparent molecular mass greater than predicted. When used for immunofluorescence staining, the antibody (1:500 dilution) did not detect any endogenous expression in the cell types identified by *in situ* hybridisation analysis of *disc1* mRNA; in fact no staining throughout the entire embryo was detected when a standard immunofluorescence protocol was used. To determine whether the antibody was able to detect non-physiological levels of the Disc1 protein, embryos were injected with the *HSP70-disc1-mCherry* construct then heat shocked for 30 minutes at 24 hpf and then fixed in 4% PFA after mCherry fluorescence was observed. Wholemount embryos were co-stained with anti-mCherry and the 2D24 antibody (1:200 dilution). Co-localisation of 2D24 and anti-mCherry staining would suggest that the 2D24 antibody was capable of detecting zebrafish Disc1 and this was indeed found to be the case (Figure 5-5). Therefore the potential of this antibody to detect endogenous Disc1 was pursued using higher concentrations of the antibody and implementing changes to the protocol and fixation methods that would differ from the standard immunofluorescence protocol.



Antibody name	Concentration (mg/ml)	Specific detection
2D24	36.5	overexpressed <i>HSP70-disc1-mCherry</i> detection
2I13	15.6	Unspecific
6D7	19.8	Unspecific
6G10	26.4	Unspecific
7A10	18.0	Unspecific
7F14	20.8	Unspecific

**Table 16: zebrafish *disc1* antibodies specificity analysis**



**Figure 5-5: Antibody Disc1 2D24 can successfully detect overexpressed Disc1 protein in injected embryos**

Embryos were injected with *HSP70-disc1-mCherry* DNA and heatshocked at 26 hpf for 30 minutes which induced mosaic expression of Disc1-mCherry protein (A). Immunofluorescence staining with the 2D24 anti-Disc1 (B) resulted in detection of overexpressed Disc1-mCherry protein that can be clearly seen in the merged image (C). Images are from whole mount embryos imaged laterally. Scale bar 150μM

Higher concentrations of 2D24 were tested (1:100, 1:50 and 1:10), however no endogenous protein expression was detected even using such high concentrations. This suggested that although 2D24 is able to detect high levels of the Disc1 protein it does not bind with sufficient affinity to detect low levels of the endogenous protein (Table 16). Detection of *disc1* mRNA with *in situ* hybridisation required long incubation times during the staining process compared with other riboprobes applied at the same concentrations, suggesting that Disc1 is expressed at low levels. One interpretation of these results is that Disc1 is enriched in OPCs and other areas detected using *in situ* hybridisation, but the expression of the protein is at a low level that cannot be readily detected in immunofluorescence.

## 5.5 Utilizing homologous recombination to incorporate a fluorescent tag into the endogenous *disc1* gene

### 5.5.1 Precise genome editing

Developing a tool to create fluorescently tagged endogenous Disc1 protein is now a possibility since genes can be precisely edited through new and highly efficient genome editing methods. These reverse genetic strategies allow a gene of interest to be targeted in a chosen region and edited through the induction of double strand breaks (DSBs). These DSBs induce the DNA repair machinery which results in non-homologous end joining (NHEJ) to repair the break in the DNA. This NHEJ creates small deletions and insertions (INDELS), which dependent on the number of base pairs lost or gained can result in frame shift mutations. As well as using these tools to create stable mutant lines, the addition of donor DNA into the system can also lead to insertion of a large fragment of DNA into the desired region via homologous recombination. The insertion of donor plasmid DNA encoding a fluorescent protein can tag the gene of interest leading to an endogenously expressed fluorescently-tagged fusion protein allowing for visualisation of the subcellular localisations of a protein at physiological levels during development. For homologous recombination to occur, the donor DNA plasmid is designed in such a way that homology arms matching the region of the targeting gene are incorporated flanking either side of the fluorescent. These homology arms on either side of the fluorescent protein sequence act as a template for the DNA repair machinery so that it can incorporate the fluorescent protein sequence into the endogenous gene, resulting in a perfectly edited genome containing a fusion protein (Grunwald 2013).

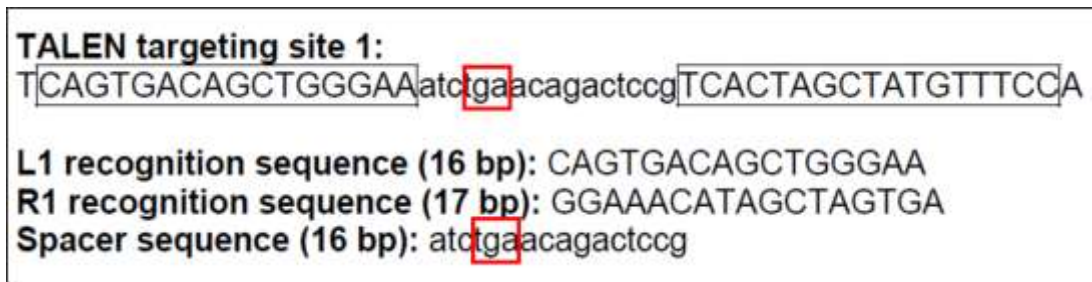
Genome Editing system	Abbreviation	Limitation
Zinc finger nucleases	ZFN	High cost of production Low rate of mutagenesis
Transcription activator-like effector nucleases	TALENs	Highly repetitive which can cause difficulty in the assembly of plasmids

Clustered regularly interspaced short palindromic repeats	CRISPR/Cas9	Requires PAM sequence (NGG)
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**Table 17: Precise genome editing techniques in the zebrafish**

Earlier methods of targeted genome editing in the zebrafish were not widely utilized due to a high cost of production and a relatively low mutation rate; mainly referring to zinc finger nucleases (ZFN) (Chen et al 2013b). However recent developments in genome editing have made the process simpler, more cost effective and highly efficient. Methods such as TALENs (Transcription activator-like effector nucleases) and the CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats) system have revolutionized genome editing in the zebrafish, both in producing stable mutant lines and endogenous fusion proteins through site specific mutagenesis (Bedell et al 2012, Chang et al 2013, Huang et al 2011, Hwang et al 2013). In order to try and address the localisation of endogenous *Disc1*, both the TALEN and CRISPR/Cas9 systems were investigated to determine the possibility of tagging endogenous *disc1* with mCherry at the 3' end of the gene. The CRISPR/Cas9 system was considered first since the plasmids are easier to design. However the guide RNA (gRNA, which is responsible for targeting the gene of interest and guiding the CAS9 to induce DSBs) requires a protospacer associated motif (PAM) sequence (NGG, any nucleotide followed by nucleotides GG) on the 3' end of the target sequence, and no PAM sequence was situated at the end of the *disc1* gene. Therefore the TALEN system which does not have the same sequence restrictions, was selected for *disc1* genome editing (Blackburn et al 2013).

TALENs were designed to create a double strand break at the stop codon of *disc1* (Figure 5-6). Linearized donor DNA was also introduced should be incorporated into DSBs to generate a C-terminal fusion protein termed *disc1-mCherry-pA*. The two TALEN arms and the donor DNA were synthesised by ZGENEBIO, Taiwan.

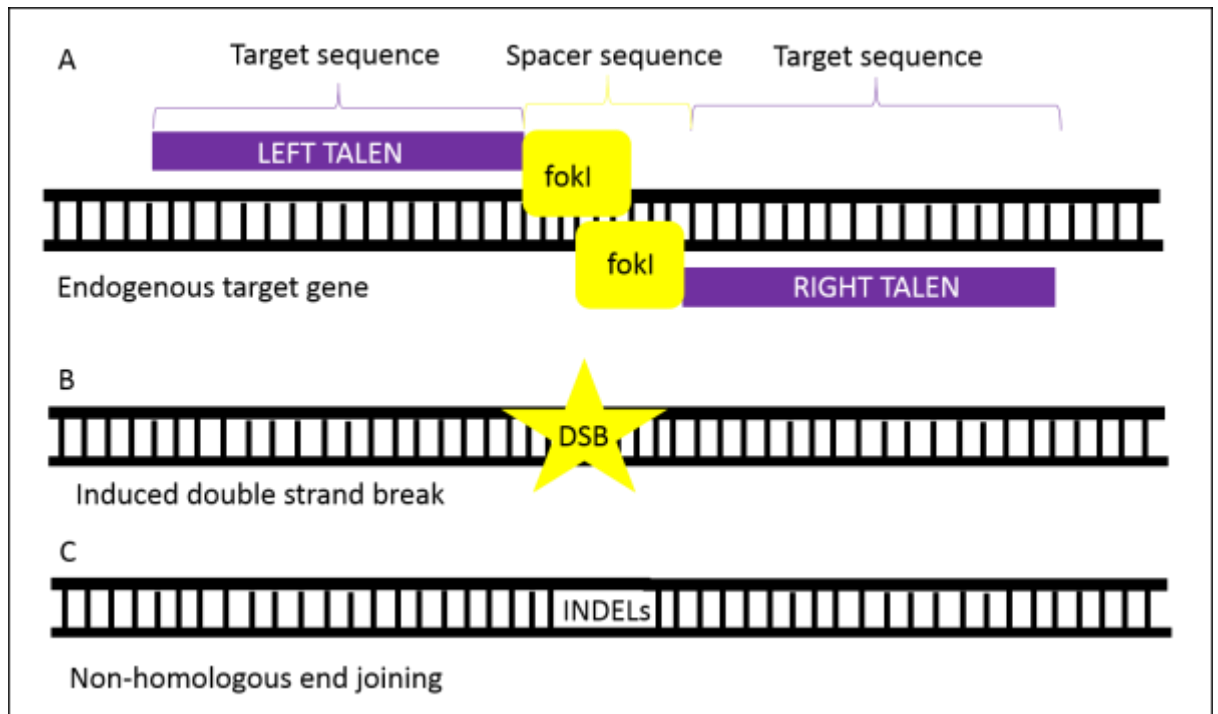


**Figure 5-6: TALEN arms targeting sequence**

The left and right TALEN arms were generated to recognize specific sequences on either side of the target site (spacer region) containing the stop codon of *disc1* (as shown in red box).

### 5.5.2 Function of Transcription activator-like effector nucleases

Each TALEN arm is composed of a TAL effector DNA binding domain fused to a DNA cleavage domain. The TAL effector DNA binding domain acts as the target site to the gene of interest and is comprised of several TALE repeats which are organised into TALE effector arrays, and each repeat recognises a specific nucleotide. Within these highly conserved repeats there are two amino acids referred to as repeat variable di-residues (RVDs) which provide specificity to the target nucleotide sequence (Bogdanove & Voytas 2011). The DNA cleavage domain comprises of a Fok1 endonuclease situated on each TALEN arm and initiates DSBs in the spacer region between the two TALEN targets sites (encoded by the left and right arms) which in this case contained the TGA stop codon of *disc1*. The TALEN arms are incorporated into plasmids which are linearized and transcribed into capped mRNA, then both TALEN arms are co-injected into 1 cell stage zebrafish embryos. Genomic DNA is extracted from injected embryos and screened for double stand breaks in the target region. The function of TALENs is summarised in the schematic below (Figure 5-7).



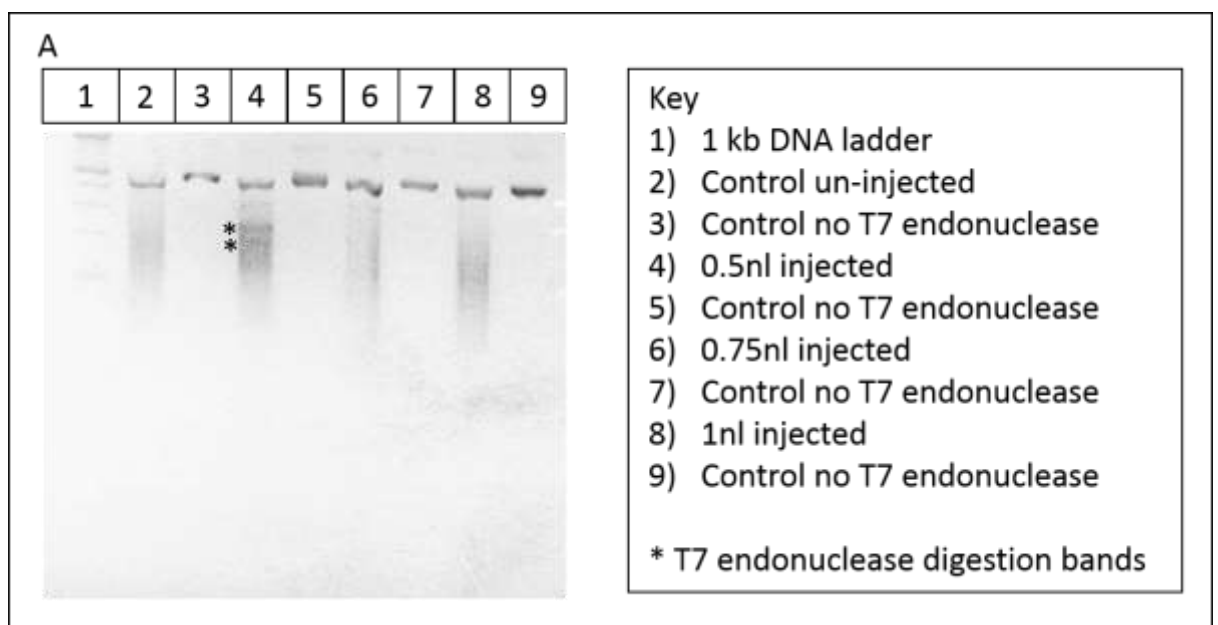
**Figure 5-7: Cartoon illustrating the use of TALENs for gene targeting**

TALENs (Transcription activator-like effector nucleases) are made up of 2 arms targeting specific regions in the gene of interest with a spacer sequence between them. Each arm consists of a DNA binding domain fused to a DNA cleavage domain. TALEN mRNA arms are co-injected to induce double strand breaks in the targeted region which subsequently induce non-homologous end joining (NHEJ) by the DNA repair machinery. NHEJ is error prone and results in small deletions and insertions causing mutations in the gene of interest.

### 5.5.3 Identifying INDELS in zebrafish embryos

Prior to attempting knock-in of the donor DNA via TALEN-induced double strand breaks, it was first important to ensure that the TALENs were capable in inducing double strand breaks in the target region with reasonable efficiency. Wild type AB strain embryos were injected with left and right TALEN-encoding mRNA at varying volumes of 150 ng/ $\mu$ l of each arm to determine toxicity levels; a 50% survival rate was desired for efficient mutagenesis and embryos that survived were genotyped for double strand breaks. After injection, genomic DNA was extracted from injected embryos at 2 dpf in pooled batches of 30 embryos per group. A batch of un-injected embryos was used as a control group. A 450 bp region surrounding the target site was amplified using PCR and purified. The amplified DNA fragments underwent T7 endonuclease I analysis to assay for the presence of DNA mismatches which is indicative of double strand breaks and non-homologous end joining. The T7

endonuclease I functions by recognising imperfectly matched DNA and cleaving that mismatched DNA. The DNA was then electrophoresed in a 3% agarose/TBE gel to screen for any fragments smaller than the 450 bp wild-type product. Embryos injected with 0.5 nl of 150 ng/μl mRNA of each TALEN arm showed two bands below the amplified 450 bp product which suggested that the TALENs had successfully induced DSBs in *disc1* (Figure 5-8). The injections of greater volumes did not result in lower fragments and therefore did not appear to induce DSBs and it could be hypothesised that this level of mRNA caused too much toxicity so embryos that had DSBs would not survive.

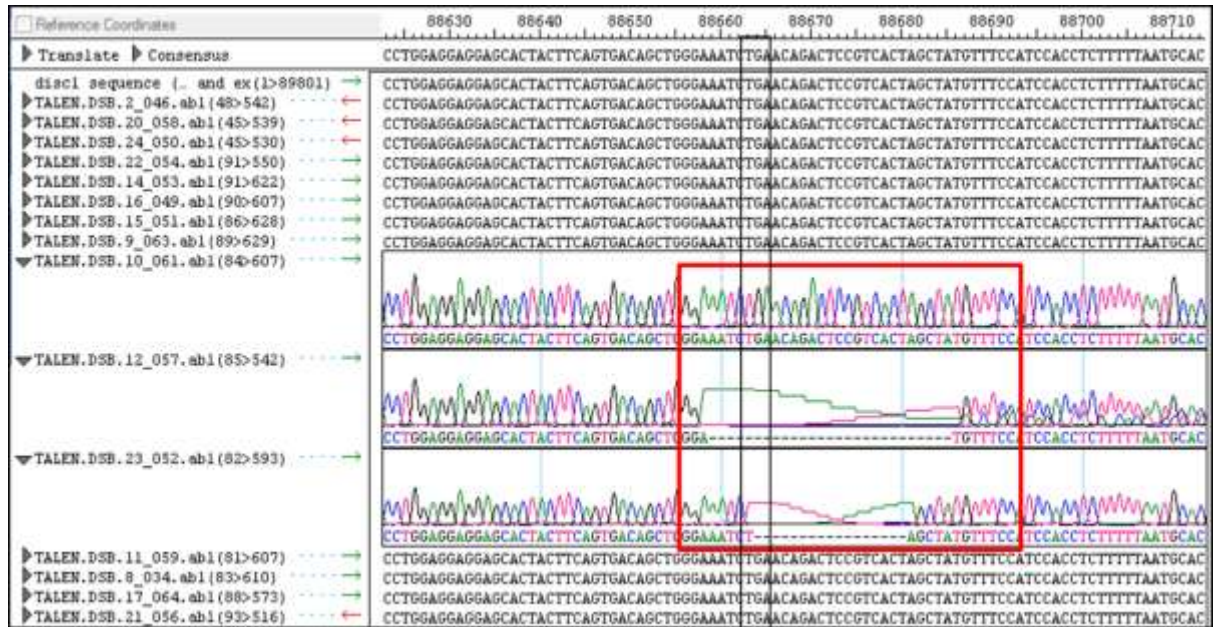


**Figure 5-8: T7 endonuclease I analysis in TALEN injected embryos identified DNA mismatches**

Embryos injected with different volumes of TALENs were analyzed for INDELS using the T7 endonuclease I which recognizes and cleaves mismatched DNA indicative of NHEJ. Each group had a negative control performed with no T7 endonuclease I in the reaction. Embryos injected with 0.5nl of 150ng/μl TALEN mRNA showed digested products below the wild type product (indicated in lane 4 with \*) which indicated that the TALENs had induced DSBs in the region of *disc1* amplified from pooled genomic DNA.

Following T7 endonuclease I analysis, the PCR fragments positive for DNA mismatches were cloned into the pCR™2.1-TOPO® vector in order to confirm the presence for INDELS. Individual DNA fragments had to be cloned since the DNA was amplified from pooled embryos, thereby ensuring that the sequencing was clean and represented single alleles. Sequencing results were aligned with the wild type

zebrafish *disc1* sequence using the DNA analysis software Lasergene (Seqman program), and the results showed that the TALENs induced DSBs in approximately 10% of injected embryos in the spacer target region (Figure 5-9).

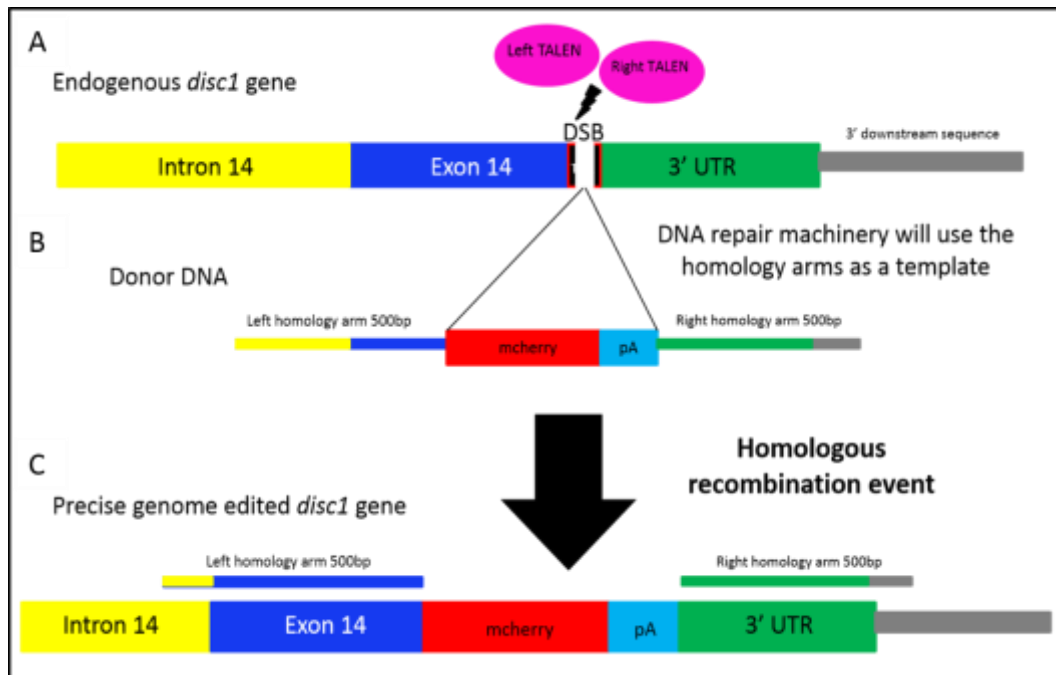


**Figure 5-9: *disc1* sequencing results from individual TALEN injected embryos**  
 A 450 bp region of *disc1* was sequenced in genomic DNA extracted from TALEN injected embryos and aligned against wild type *disc1* to determine the nature of INDELS induced. Ten percent of embryos had DSBs induced by injection of the TALEN mRNAs which resulted in deletions in the target region of *disc1*.

After it was confirmed that the TALENs were efficient in causing specific DSBs, knock-in of the donor DNA was explored.

#### 5.5.4 Homologous recombination

Knock-in of the donor DNA involved co-injecting the TALEN mRNAs with the linearized donor DNA (40 ng/μl). At this point it was observed that co-injection caused a much higher rate of toxicity therefore higher numbers of embryos had to be injected. After induction of DSBs (Figure 5-10A), the homology arms in the donor DNA should act as a template to the DNA repair machinery resulting in a homologous recombination event (Figure 5-10B) and insertion of the mCherry fluorescent tag into the 3' end of *disc1* (Figure 5-10C).



**Figure 5-10: Cartoon of genome editing through homologous recombination using donor DNA as a template in the zebrafish *disc1* gene**

DSBs are induced in the target region of the *disc1* gene by the TALEN pair (A). When donor DNA containing homology arms encoding the *disc1* target region is introduced it acts as a template for the DNA repair machinery (B). Insertion of this template will result in incorporation of a DNA-encoding fluorescent tag into the 3' end of the *disc1* gene (C).

This should theoretically leave a perfectly edited genome containing the *mCherry-pA* sequence (Figure 5-11). The mCherry sequence was selected since the end goal was to cross this line with the *tg(olig2:gfp)* line in the future to study directly the localisation of Disc1-mCherry in GFP positive cells.



Edited endogenous <i>disc1</i> containing a mcherry/pA tag	
<i>disc1</i> exon 14	<p>TCAAATATTATTTACTGTCATCATGACAAAGATAAAAATAAATCAGTTACTAGAAATGAGTCACAACCTATTATGACT  ATTATGATTAGAAAACCTATTATGATTAGAAATGTGTTGAAGAAATCTGCTCTTCGTTAAACAGAAATGGGGAAAA  ACATATTCAGGGTGGTCTAATAATTCTGACTTCAACTGTAATATATCCCTATTGACTTTTTGTTCTCATTTTGAGTT  CTTCCACTGGATTGTTGGTGTGTGAATGAGTAGATGATGTTGTGATCAGATTTTCTTTAACTGAAAAGCCACTCTC  TCTGGAGAGGGAGGTCTGGAGCTGAAGTCTGCTCTTCAGGGGATGCTGTCTCAGCTGAAGGAGGAAGATGAGGAT  GAAGAAGACGAAGAGAAATACTGTGATGTAGAAGAAGAACAGGTTGAAGATGAAGACCTGGAGGAGGAGCACTACT  TCAGTGACAGCTGGGAAATCggtaggttctggt</p>
mcherry	<p>ATCATGACTTAAAGCTGACATGGAGGCTCGTGAAGGGCTAGATTGAGATGAGGGGAGGGGAGGGAGGG  GGCCCTTAAGGGTACCCAAAGCCCAAGCTGAAGGTGACCAAGGGGGTCCCTGGCCCTTGGCCCTGGGAATC  GTCCCTCACTTCATGTAAGCTTCAAGGCTAAGTGAAGACCCCGCCGACATCCCTGACTACTTGAAGCTGT  TTCCCGGAGGCTCAAGTGGAGGGCTGATGAAGTTCAAGGAGGGGGCTGTGTGCTGAGCTGAGGACTTC  TCCTGCAAGACGGGAGTTATTTACAGGTGAAGCTGGGGGACGAAATTCCTCTGAGGGGCGTAATGC  AGAGAAATCTGGGCTGGAGGCTCTCTGAGGGGTTGACCCGAGGAGGGGGCTTGAAGGGCAGATCAA  TAAGGGTGAAGCTGAAGGAGGGGGCAATAGGACGGGAGTTAAGACACTTACAAGGCTCAGAGGCGGG  AGCTGGGGGGCTTAAGCTCAACATCAAGCTGACATCACTTCCAAAGGAGGATACCATTTCTGAAA  AGTAGGGGGGGGAGGGGGTGAATCAAGGGGATGAGGAGCTGTACAAATAGATCCAGACATGATAAG</p>
pA	<p>ATACATTGATGAGTTTGGACAAACCACAACCTAGAAATGCAAGTGAAGAAAAATGCTTTATTTGTGAAATTTGTGATGCT  ATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATGCAATTCATTTTATGTTTCAGG  TTCAGGGGGAGGTTGGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAAATGGGTATGGCTGATTATTCACATAGC</p>
3'UTR	<p>TATGTTTCCATCCACCTCTTTTTAATGCACACTTAAATTTGGAATATTGCATAAAAAATGGGAACGCCCTAGATGTTT  TGATGTGCATGAAAACCTGATGCGGCACAAGTGAAGCTGCTGTACACTAGACTTTTCTCCCATAGACTTCCGTTCA  TACACAAGTGAATGTGGCAGACCAGAAACACAAGCTCATGCGACAAGTTTCTCAGTTCGCTGCGTTTGAAAGTTCA  AGCTTAGTAAACTCTGACCTGCGAAATCGCATCACATGACTGCGTGAGACCAATCAAGGATCAAAACATGACCTCT</p>

**Figure 5-11: The expected edited *disc1-mCherry* fusion after a homologous recombination event.**

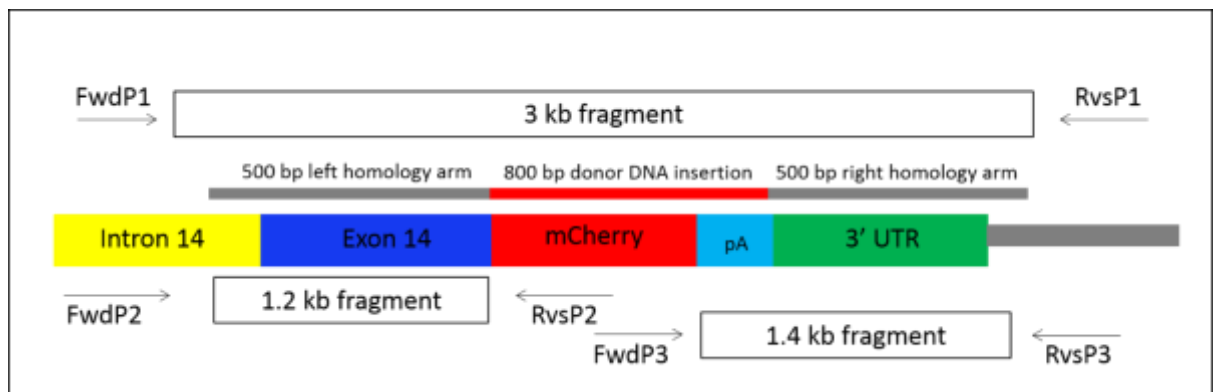
The homologous recombination event is expected to result in a *disc1-mcherry* fusion via a 12bp linker without affecting the reading frame of the endogenous gene.

### 5.5.5 Identifying homologous recombination

To screen for homologous recombination events, primers had to be designed that would not amplify residual donor DNA that had not undergone homologous recombination. Three Primer sets spanning different regions were designed to identify a successful knock-in event (Figure 5-12). Primer set 1 was designed whereby the forward (FWD) primer sat 5' to the left homology arm and the reverse (RVS) primer sat 3' to the right homology arm. If there was a successful homologous recombination event then a 3 kb fragment would be amplified containing the *disc1* gene and the *mcherry/pA* sequence; however if no event occurred then a wild-type product of 2 kb would be amplified instead, which served as a positive control. The wild type alleles were consistently amplified but no 3 kb product was ever amplified in injected embryos. This could have been due to the fact that amplifying a large product from genomic DNA can be inefficient due to the complexity of the template and potential shearing of the DNA during the extraction process. An alternative

method was to design one primer situated in the donor mCherry sequence with the other primer sitting 5' to the left homology arm or 3' to the right homology arm (Figure 5-12 primer sets 2 and 3 respectively). This would amplify a 1.2 -1.4 kb product only in the case of a successful HR event. Although this method has the advantage of amplifying a smaller product, there is no positive control to ensure that the primers were working.

A variety of different primers were used to screen however none have showed consistent and reliable screening of positive homologous recombination events, potential founder fish will continue to be screened for a positive insert although none had been identified before this thesis submission. Although the TALENs cut at 10%, the success rate of a knock in is 0.3% which means that a higher number of potential founders require screening in the future.



**Figure 5-12: Primer pairs used to screen for homologous recombination events in the *disc1* gene**

Three different primers sets were designed to screen for HR events using DNA extracted from injected embryos. P1 set would result in a 3 kb product if there was a positive insertion of the donor DNA or a wild-type 2 kb product if there was no insertion event. P2 and 3 consists of one primer in the *mCherry* donor sequence and the other primer outside of the homology arm sequences so only an HR event would result in an amplified product.

## 5.6 Conclusion

The localisation of zebrafish Disc1 has proved elusive, several attempts to identify the sub-cellular localisation of Disc1 have been unsuccessful. Over-expression assays through the injection of fluorescently tagged Disc1 constructs suggested cytoplasmic expression with no defined localisation. Early degradation and mosaic expression patterns restricted the investigation of Disc1 localisation in migrating

OPCs. Although over-expression studies are popular for localisation studies as discussed previously they do not address the endogenous protein and therefore antibodies were developed to specifically detect endogenous Disc1. Although 2D24 anti-Disc1 was capable at detecting over-expressed Disc1, it did not show any endogenous detection of the protein. Finally as an alternative method, endogenously tagging the gene with mCherry was attempted using precise genome editing techniques to create a useful tool for the Schizophrenia and *DISC1* community.

The TALENs were shown to work efficiently, inducing DSBs in the target sequence of *disc1*. Screening for homologous recombination after co-injection with the donor DNA has proved more problematic than expected. Several primer sets were designed to identify insertion events however none successfully gave the expected fragment size. This could be because the primers were designed to amplify non-coding regions, which are more prone to sequence variations. Sequencing the 3' end of the *disc1* gene of adult wild-type strains in the zebrafish facility in Singapore showed several SNPs in the coding and non-coding regions which resulted in sequence variations between the embryos and the homology arms of the donor DNA. This could have decreased the rate at which the donor DNA was incorporated into the DSB due to homologous recombination requiring the template to match the genomic sequence. However zebrafish were selected that had only highly similar sequences after showing the higher number of SNPs. The low rate of germ line transmission during precise genome editing particularly in knock in studies has made screening for HR directed insertions challenging. A very recent study has attempted to improve the screening and efficiency of germ line transmission of knock ins which could be utilised in the future during the screening process (Dong et al 2014). In the event of the C-terminal tagging of the *disc1* gene is unsuccessful then an attempt to tag the N-terminal end could be considered and tested. The N-terminal end of the protein is less well conserved and so creating the tag at this side of the protein may have less impact on its function. The C-terminal tag was originally attempted due to the *HSP70: disc1-mcherry* showing stability when injected, and the 2D24 anti-Disc1 antibody was able identify the protein suggesting

it did not impact on protein folding and the majority of tagged Disc1 constructs had a C-terminal fluorescent tag.

As an alternative method of screening, instead of waiting for embryos to be raised to adulthood and using a fin clip sample to genotype, embryos could be grown to 2 dpf only and a small larval fin clip taken to genotype. This would mean that only positive embryos are grown to adulthood and analysed which would save time, space and cost and this method will be utilised in the future.

Although currently there is no conclusive evidence as to the localisation of Disc1, this tool is still being developed to endogenously tag the *disc1* gene with mCherry and consequently eliminate the need to rely on overexpression studies or antibodies to detect the protein. To improve the screening assay, embryos at 2 dpf could be fin-clipped directly and screened for homologous recombination events and only those that had an insertion are raised to adulthood. This stable line would not only provide reliable information regarding the true localisation of Disc1 in a variety of cell types but the tool can be used in chemical manipulation to investigate localisation variations to be discussed in further detail in the discussion.

# Chapter 6 A preliminary *in vivo* drug screen to identify compounds modulating OPC development

Conventional drug screens currently use biochemical or *in vitro* cell culture systems, and although these are high throughout, many drugs identified subsequently fail validation or clinical screening when taken into *in vivo* systems. This is most likely because early on, one cannot assess the effects of a drug on an entire tissue or animal. To overcome this major issue, the development of assays in which compounds can be initially screened in an *in vivo* vertebrate system, yet still be high throughput, would potentially improve the hit rate of taking successful drugs to the clinic. The zebrafish, as a small vertebrate and well established research tool, is being increasingly appreciated and utilized in medium to high-throughput *in vivo* drug screens (Baxendale et al 2012, Bruni et al 2014, Buckley et al 2010). Their small size, rapid external development, and genetic similarity to humans make them an ideal candidate in which to screen libraries of compounds and observe drug effects on an entire organism throughout early development. Furthermore, compound libraries can be simply applied to the medium in which the embryos are submerged and removed after the desired treatment time. Finally embryos have a high tolerance for DMSO, a solvent used to dissolve a large majority of compounds. Thus ease of drug administration is therefore another advantage to using zebrafish embryos in medium to high-throughput drug screens to identify new therapeutic targets and treatments for a variety of diseases and developmental disorders. Drug screens can be designed to target a variety of systems and cells in the zebrafish including different cell types of the brain. For example a screen in 2010 utilized the zebrafish to screen for pro-myelinating drugs in attempt to identify new compounds for multiple sclerosis treatment (Buckley et al 2010). Another screen using zebrafish investigated therapeutic targets for epilepsy screen, making the zebrafish an attractive model to investigate drugs that modify a variety of brain diseases (Baxendale et al 2012).

## 6.1 A drug screen to identify novel compounds and signalling pathways involved in OPC migration and proliferation

Another way to understand the signalling pathways governing OPC migration and more specifically which signalling pathways are relevant to *disc1* in OPC development was to design a medium throughput drug screen using the zebrafish. A drug screening assay using whole mount *in situ* hybridisation for *olig2* expression as a read out for OPC development was developed to identify novel compounds involved in OPC migration in the hindbrain of the zebrafish embryo. The screen was designed to focus on OPC migration after the initial specification of the cell type had occurred. Drugs were therefore applied after the initial period of specification and removed during or after the initial migration events but prior to differentiation, after which embryos were fixed and analysed. The reasons for performing this drug screen were two-fold. Firstly, it should allow for the identification of novel signalling pathways involved in OPC migration through analysis of *olig2* expression patterns in compound-treated embryos. In particular, embryos were analysed for restricted migration, excessive migration and migration pattern differences. Although several signalling pathways have been implicated in OPC migration in mouse and human systems, this process is still not fully understood. In the zebrafish hindbrain, how OPCs populate the hindbrain is not completely characterised, although work towards this thesis after the drug screen was performed, has highlighted the importance of Shh signalling in the migration and proliferation of this population of OPCs. The identification of additional signalling pathways would contribute the characterisation of OPC migration in the zebrafish hindbrain, and potentially in other systems. Secondly since *disc1*, is also implicated in hindbrain OPC development in the zebrafish, it would be interesting to investigate whether any of the signalling pathways identified through chemical manipulation, work with or are modulated by *disc1* during development.

## 6.2 Primary drug screening assay development

### 6.2.1 Selection of the compound library

The spectrum library (Microsource delivery systems) was selected to screen for OPC migration deficiencies in the zebrafish. This library contained a collection of 2000 bioactive small molecules. The library included FDA approved molecules (60%), naturally occurring products whose biological functions are not known (25%), and other bioactive compounds (15%). The library was provided in a 25X 96 well plate format so that a high number of compounds can be screened simultaneously. This library gave the opportunity to screen a large variety of different compounds and therefore many potential target signalling pathways in a medium-through put *in vivo* screen.

### 6.2.2 Selection of the appropriate controls

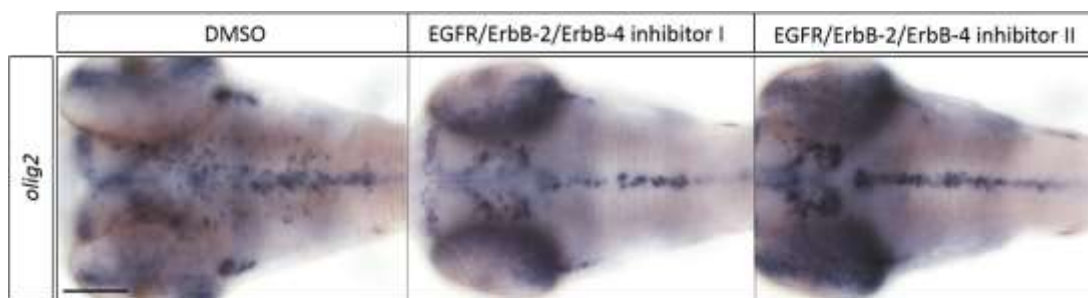
It was important to include different controls with which to compare the *olig2* expression patterns of spectrum library compound-treated embryos. DMSO only-treated embryos served to provide a wild type *olig2* expression pattern reference; these embryos were treated with DMSO at the same time point as the compound-treated embryos and acted as a negative control. The FB148.5 mutant (obtained from an ENU screen in the Ingham group (Wolff et al 2004)) shows a similar phenotype to *disc1* morphant embryos in that OPCs remain restricted to their birth place in r5/6, and therefore acted as positive control for migration deficiency. Chemical inhibition of Nrg-1 signalling via ErbB signaling, a signalling pathway with an established role in OPC migration, was also used as a positive control. ErbB inhibition results in a similar phenotype to that observed in *nrg-1* and *disc1* morphant embryos (Wood et al 2009), again where OPCs remain restricted to the midline of the hindbrain. Two different compounds were used to block Nrg-1-ErbB signalling and these compounds were also used to investigate the most appropriate time to expose embryos to the spectrum library compounds in order to induce readily detected migration defects (Table 18).

Positive control Compound	Mode of action	Test Concentration	Compound supplier and product code
EGFR/ErbB-2/ErbB-4 inhibitor I	Inhibits Nrg-1-ErbB signalling	20 $\mu$ M	324840-1MG Calbiochem
EGFR/ErbB-2/ErbB-4 inhibitor II	Inhibits Nrg-1-ErbB signalling	20 $\mu$ M	324842-5MG Calbiochem

**Table 18: Positive control compound information**

Embryos were exposed to 20  $\mu$ M of each inhibitor drug alongside DMSO-treated embryos as a control group at a range of time points from 26-32 hpf prior to the main phase of OPC migration in order to determine the best time at which to apply the spectrum library compounds to screen for effects on migration. Since it was known that these inhibitors cause an apparent migratory defect they were ideal to investigate at which time point to apply the spectrum compounds. The time point that showed the greatest restriction of OPCs without affecting the overall morphology of the embryos was selected and utilised for the entire drug screen. It was important to establish the optimal time point at which to treat the embryos, since drugs need to be absorbed and then elicit their function on their target signalling pathways and may require a prolonged period of exposure to give an effect. Four time points were tested after the initial specification of *olig2*-positive cells have occurred (26, 28, 30 and 32 hpf). The time of exposure that showed the greatest effect on OPC migration was from 28 hpf so embryos were submerged in drug from this time point throughout the initial screen to give prolonged time for the compounds to take effect (Figure 6-1). Any hit compounds that were identified would then be tested at different time points in future investigations to determine the stage of OPC development it is having its effect on.





**Figure 6-1: Inhibition of Nrg-1 signalling through chemical manipulation at 28 hpf resulted in restricted migration of hindbrain *olig2*-positive cells.**

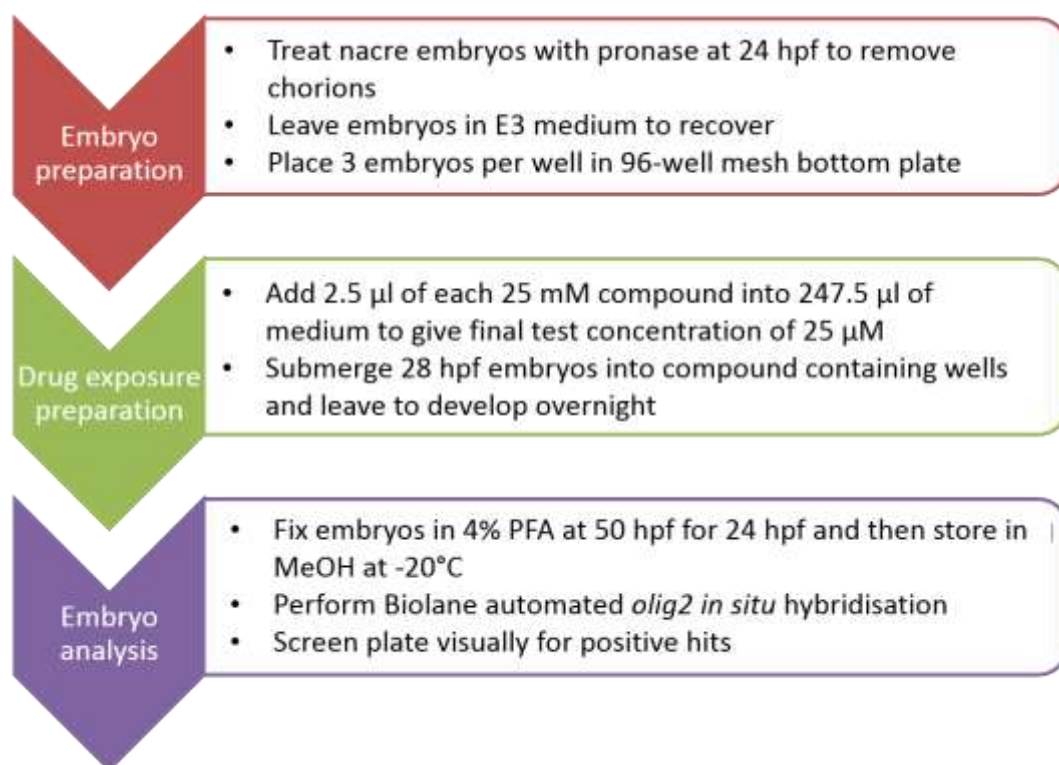
EGFR/ErbB-1/ErbB-4 inhibitor I and II was applied at four time points (26, 28, 30 and 32 hpf) and fixed at 50 hpf during active migration to determine which time point to expose embryos to the spectrum library compound. The time point which showed the strongest effect on OPC migration was at 28 hpf and embryos were exposed at 28 hpf during the drug screen. (20 embryos were exposed to compounds per treatment group) scale bar 100uM.

The objective of this compound screen was not only to screen for compounds that caused a deficiency in migration but also to identify compounds that caused accelerated migration or an altered migration pattern, for example OPCs migrating out of their normal ventral domain into dorsal areas of the hindbrain, but there was not a positive control available at the time of the screen.

### 6.2.3 Compound plate and embryo preparation

Each compound plate had the same set up, with controls in rows 1 and 12 and the spectrum library compounds to be screened in rows 2 through to 11. Spectrum compounds were screened at a test concentration of 25  $\mu$ M. Three Nacre strain embryos were placed in each well and were submerged in medium containing the compounds from 28 hpf. The plates were then incubated at 28°C overnight. Embryos were removed from the compounds at 50 hpf and fixed in 4% PFA overnight. Embryos were fixed at 50 hpf during the active phase of migration to detect early migration defects such as in the initiation of migration. Embryos were then stored in methanol and the following week were processed for *in situ* hybridisation staining. *In situ* hybridisation using an *olig2* anti-sense riboprobe was performed using an automated *in situ* hybridisation Biolane robot and the stained embryos were analysed under a light microscope for expression pattern differences. A flow chart of the preliminary drug screen is summarised below (Figure 6-2). Each plate of compounds was screened once but was visually analysed

twice on two different days and the positive hits were compared. Those hits that had been selected as positive on both days were considered a primary positive hit and were taken forward to validation screening.



**Figure 6-2: Flow chart of preliminary drug screen**

The drug screen was split into 3 parts consisting of embryo preparation (red panel), drug exposure (green panel) and analysis of *olig2* expression (purple panel). Embryos were placed at 3 per well after pronase treatment after which they were exposed to a test concentration of 25  $\mu\text{M}$  at 28 hpf. Embryos were left to develop and fixed at 50 hpf overnight in 4% PFA and were stored in MeOH until OPC analysis using an *olig2 in situ* hybridization. Embryos were screened visually for *olig2* expression abnormalities and positive hits recorded and taken forward for validation screening.

#### 6.2.4 Preliminary screen for positive compound hits

Of the 2000 compounds initially screen, 174 compounds were carried forward for primary validation. These compounds were picked and consolidated into 2 new 96 well plates which were screened and compared for consistency. The validation screen was performed twice (validation screen 1 and validation screen 2). The first validation screen identified 47 hits from the 174 primary compounds. The second validation screen, in which the whole process was repeated, identified 65 hits from

the original 174 primary hits. The validation screening process was carried out using the same conditions as with the primary screen. The results were consolidated Table 19, which shows basic information for the 174 compounds originally identified as positive hits in the primary screen. The 'validation screen' column shows whether these primary hits were also identified as positive in validation screen 1, validation screen 2 or in both screens (1 and 2). Those that were not again identified as positive were labelled as false. The final set of hits has been marked in blue for ease of reference (Table 19).

Plate number	Primary hits	Validation screen	Compound name	Known actions	Formulae	Source
001	A5	1 and 2	Mechlorethamine	Anti-neoplastic, alkylating agent	C5H11Cl2N	Synthetic
	B3	false	Methacholine chloride	Cholinergic, diagnostic acid	C8H18ClNO2	Synthetic
	D4	false	Nitrofurantoin	Antibacterial	C8H6N4O5	Synthetic
	E3	false	Novabiocin Sodium	Anti bacterial	C31H35N2NaO11	Streptomyces niveus and S griseus
	E5	false	Nystatin	Anti-fungal, binds to membrane sterols	C47H75NO17	Streptomyces noursei
	F11	2	Phenylbutazone	Anti-inflammatory	C19H20N2O2	Synthetic
	G6	1 and 2	Fenbendazole	Anthelmintic	C15H13N3O2S	Synthetic
	G8	2	Mefenamic acid	Anti-inflammatory, analgesic	C15H15NO2	Synthetic
	H7	false	Memantine Hydrochloride	Muscle relaxant (skeletal)	C12H22ClN	Synthetic
002	A3	false	Pirenzepine hydrochloride	Antiulcer	C19H23ClN5O2	Synthetic

	A10	1 and 2	Sulconazole nitrate	antifungal	C18H16Cl3N3O3S	Synthetic
	B2	false	'SULPIRIDE	dopamine receptor antagonist, antipsychotic	C15H23N3O4S	Synthetic
	D5	false	'FOLIC ACID	hematopoietic vitamin	C19H19N7O6	liver, kidney, green plants and fungi
	E7	2	'CEFMETAZOLE SODIUM	antibacterial	C15H16N7NaO5S3	Semi-synthetic
	G8	2	VINCRIStINE SULFATE	antineoplastic	C46H58N4O14S	'Vinca rosea; 37231, NSC-67574
	G11	2	DEBRISOQUIN SULFATE	anti-hypertensive	C10H15N3O4S	synthetic; RO-5-3307/1
	H4	1	PENTETIC ACID	chelating agent, diagnostic aid	C14H23N3O10	synthetic; DTPA
	H9	1 and 2	'CEFPROZIL	antibacterial	C18H19N3O5S	'semisynthetic; BMY-28100-03-800
<b>003</b>	A6	false	'SCOPOLAMINE HYDROBROMIDE	anticholinergic, treatment of motion sickness	C17H22BrNO4	'Scopolia, Datura, Atropa spp
	A7	false	THEOPHYLLINE	bronchodilator	C7H8N4O2	Camelia, thea, Paullinia cupana
	B8	false	'PROPAFENONE HYDROCHLORIDE	antiarrhythmic	C21H28ClNO3	Synthetic
	B9	2	FLUCONAZOLE	Anti-fungal	C13H12F2N6O	Synthetic
	B10	1 and 2	LOVASTATIN	antihyperlipidemic, HMGCoA reductase inhibitor	C24H36O5	'Aspergillus spp; mevinolin

	D5	false	'HYDROQUINONE	antioxidant	C6H6O2	synthetic
	E9	1	CANRENONE	aldosterone antagonist; antifibrogenic	C22H28O3	Synthetic
	E11	1	'CLIOQUINOL	antiseptic, antiamebic	C9H5ClNO	Synthetic
	F7	false	'TRAMADOL HYDROCHLORIDE	analgesic	C16H26ClNO2	synthetic; U- 26225A, CG-315E
	G2	false	'LEVALBUTEROL HYDROCHLORIDE	bronchodilator, tocolytic	C13H22ClNO3	synthetic
	G5	2	'TOPOTECAN HYDROCHLORIDE	antineoplastic; topoisomerase I inhibitor	C23H24ClN3O5	Semi-synthetic
	G11	false	PODOFILOX	antineoplastic, inhibits microtubule assembly, and human DNA topoisomerase II; antimitotic agent	C22H22O8	'Podophylum peltatum; podophylotoxin
	H5	false	SPARTEINE SULFATE	oxytoxic	C15H28N2O4S	'Lupinus spp and other Leguminosae
<b>004</b>	B2	1 and 2	IVERMECTIN	antiparasitic	C48H74O14	'semisynthetic
	D4	1 and 2	'AMPHOTERICIN B	Antifungal	C47H73NO17	'Streptomyces nodosus
<b>005</b>	B2	false	'CLEMASTINE	antihistaminic	C25H30ClNO5	synthetic
	B9	1 and 2	CLOXYQUIN	antibacterial, antifungal	C9H6ClNO	Synthetic
	E8	1	DICUMAROL	anticoagulant	C19H12O6	Synthetic
<b>006</b>	C11	false	HALAZONE	antiinfectant	C7H5Cl2NO4S	synthetic
	E9	1	IBUPROFEN	Antiinflammatory	C13H18O2	synthetic
	D8	false	HOMATROPINE METHYLBROMIDE	anticholinergic (ophthalmic)	C17H24BrNO3	Semi-synthetic

	F3	1 and 2	INDOPROFEN	analgesic, antiinflammatory	C17H15NO3	Synthetic
007	A2	2	'OXYQUINOLINE HEMISULFATE	antiinfectant	C9H9NO5S	Synthetic
	B3	1	'PIROXICAM	antiinflammatory	C15H13N3O4S	Synthetic
	B9	2	PREDNISON	glucocorticoid	C21H26O5	Semi-synthetic
	F3	false	'SULFABENZAMID E	antibacterial	C13H12N2O3S	Synthetic
008	A7	false	Trifluoperazine	Anti-psychotic	C21H26Cl2F3N3 S	Synthetic
	A10	false	Trimethoprim	Antibacterial	C14H18N4O3	Synthetic
	B2	false	Tripelennamine citrate	Anti-histaminic	C22H29N3O7	Synthetic
	C6	false	Merbromin	Antibacterial	C20H8Br2HgNa 2O6	Synthetic
	C9	1	Sulfanilamide	Anti-bacterial	C6H8N2O2S	Synthetic
	D2	1	Theobromine	Diuretic, bronchodilator, cardiotonic	C7H8N4O2	Camelia, theobroma, Cola spp
	D4	2	Aconitine	Anesthetic (gastric), anti- pyretic, and cardiotonic	C34H47NO11	Aconitum spp
	F3	false	Iodipamide	Radioopaque agent	C12H11I3N2O4	Synthetic; L- isomer. spectrum
	F9	false	Beknamycin sulphate	Antibacterial	C18H39N5O14S	Semi-Synthetic, Streptomyces kanamyceticus, NK-1006
	F10	false	Budesonide	Anti-inflammatory	C25H34O6	Semi-synthetic
G3	2	Chenodiol	Anti-cholithogenic, anti- lipemic	C24H40O4	Human bile	

	G9	1	Piperine	Analeptic, antibacterial	C17H19NO3	Black pepper
	H2	false	Flumequine	Antibacterial	C14H12FNO3	Synthetic
	H4	1 and 2	Fluphenazine hydrochloride	H1 anti-histamine	C22H28Cl2F3N3 OS	Synthetic
	H8	false	Etodolac	Anti-inflammatory	C17H21NO33	Synthetic
<b>009</b>	A5	false	Nicergoline	Vasodilator	C24H26BrN3O3	Synthetic
	A7	false	Erythromycin estolate	Antibacterial	C52H97NO18S	Synthetic
	B3	1 and 2	Tretinon	Kerolytic, carboxylic acid form of vitamin A	C20H28O2	Semi-synthetic
	B6	false	Cefsulodin sodium	Antibacterial	C22H19N4NaO8 S2	Semi-synthetic
	C6	1 and 2	Cycloheximide	Protein synthesis inhibitor	C15H23NO4	Synthetic
	C11	1	Anisindione	Anti-coagulant	C16H12O3	Synthetic
	D7	1	Abamectin	Anti-parisitic	C48H72O14	Streptomyces avermitilis
	E4	1	Nicotinyl alcohol tartrate	Vasodilator	C10H13NO7	Synthetic
	E5	2	Floxuridine	Anti-neoplastic, anti-metabolite	C9H11FN2O5	Synthetic
	E11	1	Amifostine	Radioprotectant	C5H15N2O3PS	Synthetic
	F9	false	Ceftriaxone sodium trihydrate	Antibacterial	C18H22N8Na2O 10S3	Semi-synthetic
	G7	1 and 2	Chloroxine	Chelating agent	C9H5Cl2NO	Synthetic
	H8	1	Paromomycin sulfate	Antibacterial, antiamebic	C23H47N5O18S	Streptomyces rimosis paramonycinus
<b>010</b>	C4	false	OXETHAZAINE	anesthetic (local)	C28H41N3O3	Synthetic

	H5	false	PAROXETINE HYDROCHLORIDE	antidepressant	C19H21ClFNO3	Synthetic
<b>011</b>	A9	2	TRIMETOZINE	sedative, neurosedative	C14H19NO5	Synthetic
	C2	2	'ORLISTAT	reversible lipase inhibitor, antiobesity	C29H53NO5	Synthetic
	C10	1 and 2	FLUNIXIN MEGLUMINE	analgesic, antiinflammatory	C21H28F3N3O7	'synthetic; SCH- 14714
	E2	1 and 2	'EZETIMIBE	sterol absorption inhibitor	C24H21F2NO3	Synthetic
	H2	false	KETANSERIN TARTRATE	5HT2/5HT1C serotonin antagonist	C22H22FN3O3	Synthetic
<b>012</b>	A7	2	ETHOXZOLAMIDE	carbonic anhydrase inhibitor, antiulcer, antiglaucoma	C9H10N2O3S2	Sythetic
	B3	false	LEVOCARNITINE	cofactor for fatty acid metabolism, replenisher in arterial disease	C7H15NO3	Semi-synthetic
	C5	false	TRICLOSAN	Anti-infective	C12H7Cl3O2	synthetic; CH- 3565
	D8	false	DEXCHLORPHENI RAMINE MALEATE	antihistamine	C20H23ClN2O4	synthetic
	E2	1	NORGESTIMATE	progestin	C23H31NO3	Semi-synthetic
	H7	2	'PREGNENOLONE SUCCINATE	glucocortcoid, antiinflammatory	C25H36O5	Semi-synthetic
<b>013</b>	B2	1	Glycopyrolate	Anti-cholinergic	C19H28BrNO3	Synthetic
	D7	2	Ethinodiol diacetate	Progestin	C24H32O4	Semi-synthetic
	F11	2	Nizatidine	Anti-ulcer, H2 antagonist	C12H21N5O2S2	Synthetic
	H6	false	Aniracetam	Cognitive enhancer	C12N13NO3	Synthetic



<b>016</b>	A2	false	Chiniofon	Anti-protozoal, amebicide	C9H5INNaO4S	Synthetic
	A3	false	Piribedil hydrochloride	Dopamine agonist	C16H18N4O2	Synthetic
	B5	false	Barbital	Sedative	C8H12N2O3	Synthetic
	B6	false	Oxedrine	Anti-obesity	C9H13NO2	Citrus aurantium
	C4	false	Xylocarpus A	N/A	C31H38O11	Meliaceae spp
	D4	1	Euphol acetate	N/A	C32H52O2	Euphorbia spp
	D6	false	Tartaric acid	Pharmaceutical aid	C4H6O6	Common plant constituent
	E2	2	Piromidic acid	Antibacterial	C14H16N4O3	Synthetic
	E7	false	Chloropyramine hydrochloride	Anti-histamine	C16H21Cl2N3	Synthetic
	E11	false	Strophanthidinic acid lactone acetate	N/A	C25H32O7	Semi-synthetic
	G6	1 and 2	Avocadyne acetate	Anti-fungal	C19H34O4	Persea spp
	G8	false	Diacerin	Anti-inflammatory	C19H12O8	Synthetic
	G11	false	Mepiroxol	Anti-hyperlipemic	C6H7NO2	Synthetic
	H6	false	Carzenide	Diuretic, carbonic anhydrase inhibitor	C7H7NO4S	Synthetic
	H9	1 and 2	Chaulmoogric acid	Antibacterial	C18H32O2	Hydnocarpus and oncola spp
H11	false	2,6- Dimethoxyquinon e	Anti-bacterial, induces dermatitis, mutagen	C8H8O4	picrasmus	
<b>017</b>	A2	false	'DECAHYDROGA MBOGIC ACID	N/A	C38H54O8	derivative

A4	false	BISANHYDRORUTILANTINONE	'antibacterial	C22H16O7	Streptomyces spp; also rutilantinone
A6	false	LANOSTEROL ACETATE	N/A	C32H52O2	derivative of lanosterol
A9	false	'GALLIC ACID	antineoplastic, astringent, antibacterial	C7H6O5	'insect galls
A11	false	GLUTATHIONE	'antioxidant	C10H17N3O6S	'plant and animal tissue
B4	false	CARAPIN	N/A	C27H32O7	'Carapa and Cedrela species; mp 180-185
B10	false	ALLOPREGNANOLONE	N/A	C21H34O2	semisynthetic
C4	false	'1,7-DIDEACETOXY-1,7-DIOXO-3-DEACETYLKHIVORIN	N/A	C26H32O7	'Meliaceae spp
C9	false	MEXICANOLIDE	N/A	C27H32O7	'Meliaceae spp
D3	1	'8beta-HYDROXYCARAPIN, 3,8-HEMIACETAL	N/A	C27H32O8	derivative
E2	false	'3-DEOXO-3beta-ACETOXYDEOXYDIHYDROGEDUNIN	N/A	C30H40O7	Meliaceae spp
E7	2	3beta-HYDROXYISOALLOSPIROST-9(11)-ENE	N/A	C27H42O3	semisynthetic
F6	2	'DEOXYKHIVORIN	N/A	C32H42O9	'Meliaceae spp

	F8	1 and 2	DEACETOXY-7-OXOGEDUNIN	Anti-HIV activity	C26H30O6	'Meliaceae spp
	G9	false	BOVINOCIDIN (3-nitropropionic acid)	'antineoplastic	C3H5NO4	'Aspergillus, Streptomyces spp & other microorganisms
	G10	1 and 2	CHRYSIN	diuretic	C15H10O4	'Ulnus sieboldiana, Flourensia resinosa, Oroxylum indicum, Pinus and Scutellaria spp.
	H3	2	7,4'-DIHYDROXYFLAVONE	'antioxidant	C15H10O4	'Pterocarpus marsupium
	H11	false	HECOGENIN	antiinflammatory	C27H42O4	Agave and Yucca spp
<b>018</b>	B3	false	CARNOSINE	N/A	C9H14N4O3	Mammalian Skeletal muscle
	C2	2	LAPACHOL	Anti-neoplastic, anti-fungal	C15H14O3	Heartwood of bignoniaceae
	H5	2	GANGALEOIDIN	N/A	C18H14Cl2O7	Lecanora gangaleoides
<b>019</b>	B3	2	IRIGENIN, 7-BENZYL ETHER	N/A	C25H22O8	Derivative of irigenin from iris spp
	B11	false	VIOLASTYRENE	N/A	C17H18O3	Dalbergia miscolobium

	D3	false	Pachyrrhizin	Insecticide	C19H12O6	Pachyrrhizus erosus mp 206- 207
	E4	1	SPHONDIN	N/A	C12H8O4	Heracleum maximum root; mp 191-192 C
	F3	1 and 2	'EPI(13)TORULOS OL	Anti-fungal	C20H34O2	Cryptomeria japonica and Larix sibirica
	F5	1 and 2	LARIXOL	N/A	C20H34O2	Larix sibirica
	F6	1 and 2	LARIXOL ACETATE	N/A	C22H36O3	Larix europaea
	G6	1 and 2	DEHYDROABIETA MIDE	Anti-viral activity	C20H29NO	derivative
	H2	2	HYMECROMONE METHYL ETHER	N/A	C11H10O3	'Dalbergia volubilis, Eupatorium pauciflorum
<b>020</b>	A3	2	ACACETIN	antiinflammatory, spasmolytic agent, antioxidant	C16H12O5	Robinia pseudoacacia
	A11	false	EPICATECHIN MONOGALLATE	N/A	C22H18O10	tea pigment
	B4	false	RHODINYL ACETATE	N/A	C12H22O2	common constituent of plant essential oils
	B11	false	PHYTOL	N/A	C19H38O	nettles
	C5	false	HESPERIDIN	'capillary protectant	C28H34O15	Citrus spp.
	C8	false	QUASSIN	insecticide, antiamebic	C22H28O6	Quassia amara, Picrasma excelsa

						and <i>Ailanthus glandulosa</i>
	C9	2	APIIN	N/A	C26H28O14	parsley seed; flowers of <i>Anthemis nobilis</i> . 80% + other glycosides
	D8	2	MEVASTATIN	antihyperlipidemic, HMGCoA reductase inhibitor	C23H34O5	<i>Penicillium brevicompactum</i> ; <i>Compactin</i>
	E7	2	PIPERONYLIC ACID	N/A	C8H6O4	<i>Piper longum</i> , <i>Cinnamum</i> spp
	E8	false	PHENACYLAMINE HYDROCHLORIDE	N/A	C8H10ClNO	' <i>Castanopsis cuspidata</i> , <i>Vitis</i> spp
	E9	false	CYCLOVERATRYLENE	N/A	C27H30O6	<i>Halopythus</i> spp
	F3	2	DERRUSTONE	N/A	C18H14O6	<i>Derris robusta</i>
	F9	1	LEOIDIN	N/A	C18H14Cl2O7	lichen metabolite
	G11	false	4-METHYLESCULETIN	N/A	C10H8O4	analog of <i>esculetin</i>
	H3	false	4-METHYLDAPHNETIN	N/A	C10H8O4	analog of <i>daphnetin</i>
<b>021</b>	A2	false	BICUCULLINE (+)	GABA <sub>A</sub> antagonist	C20H17NO6	<i>Dicentra cucullaria</i> , <i>Corydalis</i> spp
	C6	false	GITOXIN	cardiotonic	C41H64O14	<i>Digitalis</i> spp.
	G5	false	TRIPTOPHENOLIDE	N/A	C20H24O3	' <i>Tripterygium wilfordii</i>

	G7	false	alpha-MANGOSTIN	N/A	C24H26O6	Garcinia mangostana, Hydnocarpus octandra, H venenata
	G11	2	SALSOLIDINE	'antihypertensive	C12H17NO2	Salsola Richteri
<b>22</b>	C7	2	DEMETHYLNOBIL ETIN	N/A	C20H20O8	Citrus, Sideritis, Heteropappus and Thymus spp; Mentha piperita, Amaranthus paniculatus
	G11	1 and 2	HEXAMETHYLQUERCETIN	N/A	C21H22O8	Citrus spp
	H3	2	RHIZOCARPIC ACID	N/A	C28H23NO6	Rhizocarpon spp, Calcium hyperelium; mp 178 C
<b>23</b>	D10	false	12a-HYDROXY-5-DEOXYDEHYDROMUNDUSERONE	N/A	C19H18O6	derivative
	H6	false	FARNESOL	N/A	C15H26O	major component in oil of Hibiscus abelmoschus
	H11	2	PERILLIC ACID (-)	inhibits posttranslational cys isoprenylation, blocks G-protein	C10H14O2	Salvia dorisiiana
<b>24</b>	A3	2	ISOROTENONE	N/A	C23H22O6	semisynthetic
	B6	false	APIGENIN DIMETHYL ETHER	N/A	C17H14O5	common plant metabolite
	C5	2	3-HYDROXY-4-(SUCCIN-2-YL)-	N/A	C19H28O4	'derivative of caryophyllene

			CARYOLANE delta-LACTONE			
	E2	2	ESTRADIOL METHYL ETHER	estrogen	C19H26O2	semisynthetic
	E6	1 and 2	4- NAPHTHALIMIDO BUTYRIC ACID	aldose reductase inhibitor	C16H13NO4	synthetic
	E8	false	N- (9- FLUORENYLMETH OXYCARBONYL)-L- LEUCINE	antiinflammatory	C21H23NO4	synthetic; NPC- 15199
	E10	1 and 2	5- FLUOROINDOLE- 2-CARBOXYLIC ACID	'NMDA receptor antagonist (gly)	C9H6FNO2	synthetic
	F8	1 and 2	'PHENETHYL CAFFEATE (CAPE)	'antineoplastic, antiinflammatory, immunomodulator, NFkB blocker	C17H16O4	synthetic
	G6	1	EXALAMIDE	antifungal	C13H19NO2	synthetic
<b>25</b>	A3	1 and 2	ARTENIMOL	antimalarial, antiinflammatory	C15H24O5	semisynthetic; dihydroartemisini n
	4C	false	VALINOMYCIN	antibiotic; LD50 (rat, po) 4 mg/kg	C54H90N6O18	Streptomyces spp
	10G	false	FLOPROPIONE	antispasmodic	C9H10O4	synthetic

**Table 19: Details of the 174 positive drug hits identified from the primary drug screen.**

There were 174 compounds observed as exerting an effect on OPC development, those 174 compounds were screened again twice in validation screening. Those compounds that were selected a positive again in both validation screen I and II were marked in blue to give 28 final primary hits.

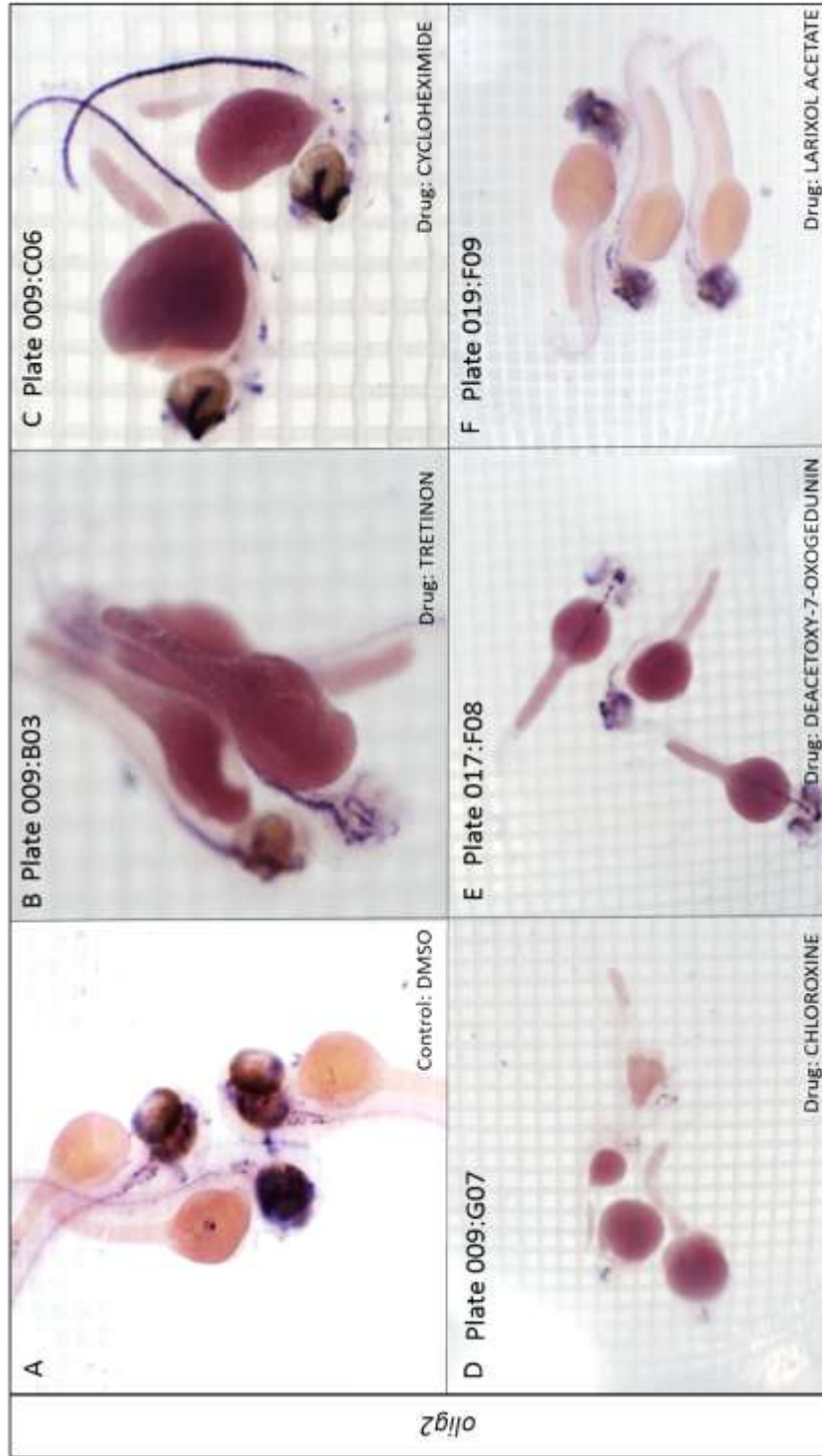
The two sets of hits identified in the validation screens were compared to give a final list of 28 compounds (Table 20) that were positive in all three assays performed (1.4%).

Screen level	Number of compounds screened	Positive hits identified
Primary screen	2000	174 (8.7%)
Validation screen I	174	47
Validation screen II	174	64
Comparison of Validation screens	111	28 (1.4%)

**Table 20: Compounds numbers taken forward through the screening process**

These final 28 hits were consolidated into a table where their effect on *olig2* expression was described (Table 21). The majority of compounds gave a restricted expression phenotype where *olig2*-positive cells remained either along the anterior-posterior axis without any lateral migration (Figure 6-3E and F) or the cells were completely restricted to their initial birthplace in rhombomeres 5/6 (Figure 6-3C). Some compounds, such as Choroquine, resulted in *olig2* expression being completely lost. This could mean that although *olig2* expression was presumably normal before compound treatment *olig2* expression was completely blocked and existing mRNA degraded, alternatively the early OPCs expressing *olig2* were destroyed by this compound leading to the loss of *olig2* expression observed (Figure 6-3D). Interestingly a handful of compounds gave a phenotype in which a long thick strip of *olig2* expression was observed along the midline (Figure 6-3B) where individual OPCs were not clearly visible including compound Tretinon. An explanation for this expression pattern could be that proliferation continues as normal after compound treatment but no lateral migration occurs resulting in an accumulation of *olig2* expressing cells.





**Figure 6-3 Examples of positive hit compounds identified in the validation screening**  
 DMSO control embryos show a normal pattern of *olig2* expression in the hindbrain at 50 hpf (A). TRETINON gives a thick strip of *olig2* expression (B). CYCLOHEXIMIDE shows *olig2* positive cells restricted to r5 and 6 (C). CHLOROXINE gave a complete loss of *olig2* expression except for the eye expression (D). DEACETOXY-7-OXOGEDUNIN and LARIXOL ACETATE gave similar phenotypes with *olig2* positive cells restricted to the midline of hindbrain with a lack of *olig2* cells migrating in lateral directions (E and F).

Plate	Hit well	Compound	Function	<i>olig2</i> expression phenotype
001	A05	MECHLORETHAMINE	antineoplastic, alkylating agent	Restricted to R5/6/7
001	G06	FENBENDAZOLE	anthelmintic	One patch of <i>olig2</i> positive cells in the hindbrain r5?
002	A10	SULCONAZOLE NITRATE	antifungal	Restricted to R5/6, spinal cord expression decreased
002	H09	CEFPROZIL	antibacterial	Pattern of migration is patchy, cerebellar neurons lost
003	B10	LOVASTATIN	antihyperlipidemic, HMGCoA reductase inhibitor	Restricted to r5/6
004	B02	IVERMECTIN	antiparasitic	Patchy midline expression
004	D04	AMPHOTERICIN B	antifungal	Increased <i>olig2</i> expression but pattern normal
005	B09	CLOXYQUIN	antibacterial, antifungal	Decreased lateral migration but migration along A/P axis
006	F03	INDOPROFEN	analgesic, antiinflammatory	Decreased lateral migration
008	H04	FLUPHENAZINE HYDROCHLORIDE	H1 antihistamine	Decreased lateral migration,
009	B03	TRETINON	keratolytic	Continuous thick strip of <i>olig2</i> expression, hard to see individual

				cells, large dip in strip of expression
009	C06	CYCLOHEXIMIDE	protein synthesis inhibitor	Restricted expression to r5 and 6
009	G07	CHLOROXINE	chelating agent	Olig2 expression is lost throughout the entire embryo
011	C10	FLUNIXIN MEGLUMINE	analgesic, antiinflammatory	Restricted expression to r5 and 6
011	E02	EZETIMIBE	sterol absorption inhibitor	
016	G06	AVOCADYNE ACETATE	antifungal	Strip of expression no ventral migration
016	H09	CHAULMOOGRIC ACID	antibacterial (mycobacteria)	Restricted expression to r5 and 6
017	F08	DEACETOXY-7- OXOGEDUNIN	Anti-HIV activity	Lateral migration decreased, very similar to NRg-1 treatments
017	G10	CHRYSIN	diuretic	Restricted expression to r5 and 6
019	F03	EPI(13)TORULOSOL	anti-fungal	Restricted expression to midline or r5 and 6
019	F05	LARIXOL	unknown	Patches of olig2 along midline but no ventral/lateral migration
019	F06	LARIXOL ACETATE	unknown	Patches of olig2 along midline but no ventral/lateral migration

019	G06	DEHYDROABIETAMIDE	Antiviral activity against JC polyomavirus M1/SVEdelta	No ventral expression but A/P midline is okay
022	G11	HEXAMETHYLQUERCETAGETIN	flavonoid	Overall <i>olig2</i> expression decreased
024	E06	4-NAPHTHALIMIDOBUTYRIC ACID	aldose reductase inhibitor	Overall <i>olig2</i> expression decreased
024	E10	5-FLUOROINDOLE-2-CARBOXYLIC ACID	NMDA receptor antagonist (gly)	Thick <i>olig2</i> eye expression, but brain and spinal cord expression lost
024	F08	PHENETHYLCAFFEATE (CAPE)	antineoplastic, antiinflammatory, immunomodulator, NFkB blocker	Restricted to r5 and 6
025	A03	ARTENIMOL	antimalarial, antiinflammatory	Patchy expression in the rhombomeres (4-7) and lost expression in the spinal

**Table 21: Observed *olig2* expression in the hindbrain of embryos treated with final hit compounds**

### 6.2.5 Grouping of Compounds

Compounds were grouped according to their biological activity to identify similarities and compounds targeting similar signalling pathways. Compounds could be grouped into: A) anti-fungal, B) anti-bacterial, C) anti-inflammatory, D) unknown function, E) anti-parasitic, F) Anti-Neoplastic, G) cholesterol reduction compounds and group H) others (single compounds that did not fall into a group). Literature searches that did not bring up any biological function information were grouped into unknown function (Table 22).

<b><u>Group A</u></b> <b><u>Anti-fungal</u></b>	<b><u>Group B</u></b> <b><u>Anti-bacterial</u></b>	<b><u>Group C</u></b> <b><u>Anti-inflammatory</u></b>	<b><u>Group D</u></b> <b><u>Unknown function</u></b>
SULCONAZOLE NITRATE	CEFPROZIL	INDOPROFEN	DEACETOXY-7-OXOGEDUNIN
AMPHOTERICIN B	CHAULMOGRIC ACID	FLUNIXIN MEGLUMINE	LARIXOL
CLOXYQUIN	CLOXYQUIN	PHENETHYL CAFFEATE (CAPE)	LARIXOL ACETATE
AVOCADYNE ACETATE	CHLOROXINE		DEHYDROABIETAMIDE
			HEXAMETHYLQUERCETAGETI N
<b><u>Group E</u></b> <b><u>Anti-parasitic</u></b>	<b><u>Group F</u></b> <b><u>Anti-neoplastic</u></b>	<b><u>Group G</u></b> <b><u>Cholesterol reduction</u></b>	<b><u>Group H</u></b> <b><u>Other</u></b>
FENBENDAZOLE	MECHLORETHAM INE	LOVASTATIN	<b>NMDA receptor antagonist</b> 5-FLUOROINDOLE-2- CARBOXYLIC ACID
IVERMECTIN	PHENETHYL CAFFEATE (CAPE)	EZETIMIBE	<b>H1 antihistamine</b> FLUPHENAZINE HYDROCHLORIDE
			<b>Keratolytic</b> TRETINON
			<b>protein synthesis inhibitor</b> CYCLOHEXIMIDE
			<b>Diuretic</b> CHRYSIN
			<b>aldose reductase inhibitor</b> 4-NAPHTHALIMIDOBUTYRIC ACID

**Table 22: Compounds were grouped according to biological activity to identify compounds potentially working in similar pathways**

### 6.2.6 Secondary validation of 28 initial hits

Several primary hits were identified in the initial drug screen and for these to be taken forward into future screening a number of validation studies would need to be performed to maximise their success rate in later clinical studies. These 28 final hits have not yet been subjected to further validation and it is likely that thorough validation analysing dose responses and toxicity will reduce the final hit number further, thereby leaving a smaller group of compounds that warrant further investigation. Initially one would aim to firstly provide extensive replicates to ensure that each drug consistently gives the same phenotype, these primary hits have undergone 3 replicates prior to secondary validation which does suggest phenotype reliability. Secondly, an important issue to consider is that of toxicity, a common occurrence in drug screening. Screening for toxicity would involve examining embryos for any overall morphological abnormalities such as delayed development, malformed head and brain, curved body axis and cardiac oedema. In some hits there does seem to be a some developmental delay at 50 hpf however this needs more analysis over a longer developmental time period. Since results for only a single test concentration has been achieved thus far dose response curve experiments will help to determine concentrations that cause a migration phenotype without effecting general development. In some cases there will not be a concentration that doesn't cause developmental abnormalities in embryos and these would be removed prior to further validation. Similarly, comparing the behaviour and motility of treated and control embryos will also be an important consideration. Alteration of the behaviour or motility of the developing larvae would also deem the compound as potentially toxic and this can be further analysed with dose response curves. It might be expected that detailed toxicity assays would again reduce the compound hit rate. For those compounds that prove to be consistent in phenotype and also have no overt toxicity, it would next be appropriate for treated embryos to be analysed for cell death. If compounds are shown to be reducing *olig2* expression due to death of the cell type they again would be not be appropriate for further investigation. Dose response curves are also critical for validation screening to determine at which point during OPC

migration are the compounds exerting their effect. With a final set of characterised compounds, a thorough literature investigation to identify potential target signalling pathways and any structural similarity could be performed. The few compounds remaining would then have further structural analysis performed to look for any structural similarity between them or with other drugs that may have a similar function. Screening a small panel of structurally similar compounds would increase the likelihood of understanding mechanistically how they function in disrupting or altering OPC development.

Any compounds that persist through validation screening could be used for *disc1* expression analysis in OPCs in future studies. Investigating whether any of these compounds identified as a positive hit for migratory defects could be further analysed for *disc1* expression changes. Understanding the signalling pathways that regulate *disc1* could potentially improve psychiatric treatment in the future.

## Chapter 7 Discussion

Oligodendrocytes are the myelinating cells of the CNS and primarily function to allow efficient axonal signal transduction through wrapping of neuronal axons with myelin sheaths. Myelination abnormalities have been reported in several brain disorders, including mental illnesses. In schizophrenia, hypothesised to be partly a disorder of neurodevelopment, it is reasonable to suggest that the myelination irregularities reported could be due to early dysfunction in oligodendrocyte development (Weinberger 1987) (Peters & Karlsgodt 2014). Schizophrenia is highly heritable and many genes have been postulated to confer risk to schizophrenia, although no causal gene has been identified. It should be noted that several environmental factors are associated with the disease, so although gene mutations do confer risk, studies based on genetics alone will not paint a complete picture of the disease. However, genetic studies have and will provide further valuable insights into the biological pathways affected, thereby identifying potential targets for future therapeutic development. Several genes reported to confer risk are expressed in the brain and converge on similar biological pathways, implicating neuronal signalling, synapses, neuronal migration and white matter abnormalities in disease pathology. The most recent GWAS did not implicate oligodendrocyte related genes in schizophrenia so it is important to acknowledge that oligodendrocyte dysfunction in the disease could be a secondary result of neuronal defects (Schizophrenia Working Group of the Psychiatric Genomics 2014). Neuronal-glia interactions are important during development so this is a plausible hypothesis. Nonetheless there is wide biological evidence to suggest that white matter abnormalities and oligodendrocyte related genes are involved in schizophrenia pathology. This thesis is based on a previous study which identified a potential role between DISC1, a novel protein with known roles in neurodevelopment and a schizophrenia risk factor gene, and oligodendrocyte migration (Wood et al 2009). At the time of publication, it was one of the first studies to implicate DISC1 in oligodendrocyte development. The majority of studies have concentrated on DISC1 in neuronal development, particularly in migration. In the zebrafish embryo upon knockdown of *disc1*, OPCs in the hindbrain failed to



proliferate and migrate away from their birthplace and the authors suggested that it could have important roles in OPC migration (Wood et al 2009). How *disc1* contributed to migration and what external signalling factors initiate and are responsible for turning on *disc1* expression in zebrafish OPCs was not known and this project aimed to follow up on these unresolved questions. Other groups have since made similar observations and DISC1 function in brain development is now suggested to be more diverse than originally thought, and there is increasing evidence for roles in neuronal, oligodendrocyte and neural crest development (Drerup et al 2009, Hattori et al 2014, Schurov et al 2004, Shimizu et al 2014). In a CNS cell type exon array, *disc1* was found to be most highly expressed in OPCs further encouraging investigation of *DISC1* in OPC development (Cahoy et al 2008). Understanding fully the function of these risk factor genes in all aspects of neurodevelopment, both in neuronal and non-neuronal cell types, is therefore important for understanding brain development and improving knowledge of brain diseases. This thesis provides evidence to suggest that DISC1 acts downstream of the Hh signalling pathway in hindbrain OPC development in the zebrafish embryo.

## 7.1 Hh signaling acts as a ventral external chemoattractant for OPC migration

After observing that OPCs consistently extend their initial processes in a ventral direction and predominantly occupy the ventral portion of the hindbrain, the ventral external cue responsible for this downward movement was investigated. Candidate signaling pathways with known ventral locations were compared and the Sonic Hedgehog signaling pathway was selected for further analysis. The Shh signaling pathway is vital for dorso-ventral patterning of the neural tube and is ventrally located in the notochord and floor plate, including turning on expression of *olig2*. It has also been linked to having roles in migration of optic nerve OPCs and therefore was a suitable and feasible candidate. It was shown that disruption of Hh signaling through cyclopamine treatment resulted in a greatly reduced population of OPCs occupying the hindbrain, indicative of defective OPC migration. However there was also an apparent defect in proliferation of OPCs. This could mean that a proliferation defect is the primary result of Hh signaling abnormalities, which

subsequently results in secondary migration defects. However, introducing a dorsal source of mouse N-terminal Shh protein caused OPCs to extend processes in the opposite direction to their normal ventral path, indicating that Hh could act as a cue for migrating OPCs. In *ptch1; ptch2* double mutants, *olig2* expression was increased and OPCs were observed in dorsal domains of the hindbrain, suggesting abnormal migration out of normally restricted domains. The expression pattern of *olig2* in these mutants could be a result of excessive migration out of the ventral domain, but it was also interpreted that the progenitor domain increased which lead to the subsequent expression in dorsal domains. However when examining these mutants at an earlier time point prior to migration, although there was increased *olig2* expression in r5/6 there was not expression in the dorsal domain. It could also be a result that the increased number of *olig2* positive cells in the progenitor domain meant that cells had to move further apart through contact inhibition leading to movement into dorsal domains. These data demonstrate an essential role for Hh in OPC development after the initial specification of clusters of OPCs in r5/6, both in terms of migration and proliferation, perhaps through primary cilia which are present on OPCs. This data however does not confirm that Shh signaling is the only pathway responsible for causing ventral migration, other recent studies have shown that other Hh signal proteins, including indian hedgehog B which was identified to have roles in OPC development in the zebrafish (Chung et al 2013). There have been other studies which have supported a role for Shh signalling in cellular migration in the brain including *in vitro* and *in vivo* chick studies of the optic nerve (Merchan et al 2007). A more recent *in vitro* study has characterized megalin, a multi-ligand receptor, in relation to Shh OPC migration and proliferation. It was shown to be expressed by astrocytes, to internalize Shh and provide Shh signalling to OPCs of the optic nerve (Ortega et al 2012). There have also been other studies identifying a role for Shh signaling in the migration of other cell types including cancer cell invasion. For example one recent study showed that Shh signaling promoted cell migration in liver cancer (Chen et al 2013a), thus supporting our hypothesis that Shh has additional migratory roles in OPC development. Further work is required to attempt to understand whether Hh signaling has a specific migration roles in OPC development of the hindbrain, and these phenotypes are not a result of altered

specification and proliferation. Cell culturing of hindbrain OPCs in migration assays and altering Hh signaling could help to determine whether there are specific migration cues.

## 7.2 *disc1* is a downstream target of Hh signaling in OPCs

After the identification of a signaling pathway acting as a ventral chemoattractant to initiate process extension and drive ventral migration, the next objective was to determine whether *disc1* was a downstream target of this pathway. Since *disc1* is a cytoskeletal protein and interacts with other cytoskeletal/centrosomal proteins, it is plausible that it contributes to OPC migration by causing the physical movement of cells in response to Hh signaling. Much research has investigated what pathways are regulated by the DISC1 protein, however there is still a lack of research into which signaling pathways sit upstream of DISC1, and control its regulation during development (Chubb et al 2008). There has been one study however which identified FOXP2 targets *DISC1*, but further analysis of *DISC1* regulators is important for understanding further its biological function during development and disease (Walker et al 2012). Experiments performed in this thesis suggested that *disc1* may be a downstream target of Hh signaling that is required for OPC migration. This thesis has provided evidence that *disc1* is expressed in OPCs during their migratory phase as well as in presumptive radial glia, neural crest, otic vesicle and lower jaw cartilage during zebrafish development. Expression of *disc1* in a variety of tissues suggests that it has important roles in overall embryo development, however the function of *disc1* in these tissues was beyond the scope of the project. Hh signalling also has roles in the development of some of these tissues including the presumptive ear and neural crest (Raft & Groves 2014) (Fu et al 2004). It is interesting that *disc1* expression is present in tissues that respond to Hh signalling. It would be interesting in the future to determine whether *disc1* was responsive to Shh in the development of these tissues. Interestingly *disc1* expression was lost in the midline of the hindbrain in *iguana*<sup>ts294e</sup> (-/-) mutants and *smo*<sup>b641</sup> (-/-) mutant embryos, but expression of *disc1* appeared to be maintained in other areas including the lower jaw and otic vesicle. However the qPCR data showed a complete loss of *disc1* in *smo*<sup>b641</sup> compared to siblings which may be because the

expression of the gene did not pass the threshold set by the analysis software but there was still some low level expression. In *ptch1;ptch2* double mutants, *disc1* expression was expanded in the midline along with excessive OPC migration and increased *olig2* expression levels. However this was not confirmed by qPCR when compared to a pool of *ptch1;ptch2* siblings. This likely due to the *ptch1;ptch2* pool of siblings containing a collection of single homozygote and heterozygote mixes which may have increased *disc1* expression and collectively resulted in a higher level of expression. When the expression of *disc1* was compared between *smo<sup>b641</sup>* siblings and *ptch1;ptch2* mutants however there was an increase in expression in the double mutants as expected. The *in situ* hybridisation data suggested that *disc1* is responsive to Hh signalling, and alterations from normal Hh signalling result in aberrations in *disc1* OPC expression and may therefore affect its function in OPC development. When *disc1* expression was analysed in cyclopamine treated embryos, expression was lost in *olig2*-expressing OPCs present in the midline. Hh external signalling transduced by OPCs could be responsible for initiating expression of cytoskeletal proteins, possibly including *disc1*, that are required for process extension towards the ventral signalling source, as well as initiating migration through promoting cytoskeletal gene expression and/or recruitment. How OPCs respond to this Hh signal is yet to be determined; it is expected that for such a quick and dynamic process like migration it could be a non-transcriptional response since transcriptional changes through the Gli family will take time. For example, it has been shown that during fibroblast migration, Shh stimulates small Rho GTPases and this was independent of transcription by Gli proteins (Polizio et al 2011a, Polizio et al 2011b) (Jenkins 2009). Canonical and non-canonical Hh signalling could work in parallel during OPC migration to cause recruitment of cytoskeletal proteins to leading cell edges and over a longer period of time, cause transcriptional changes to increase expression of cytoskeletal proteins to facilitate extensive branching during later stages of migration.

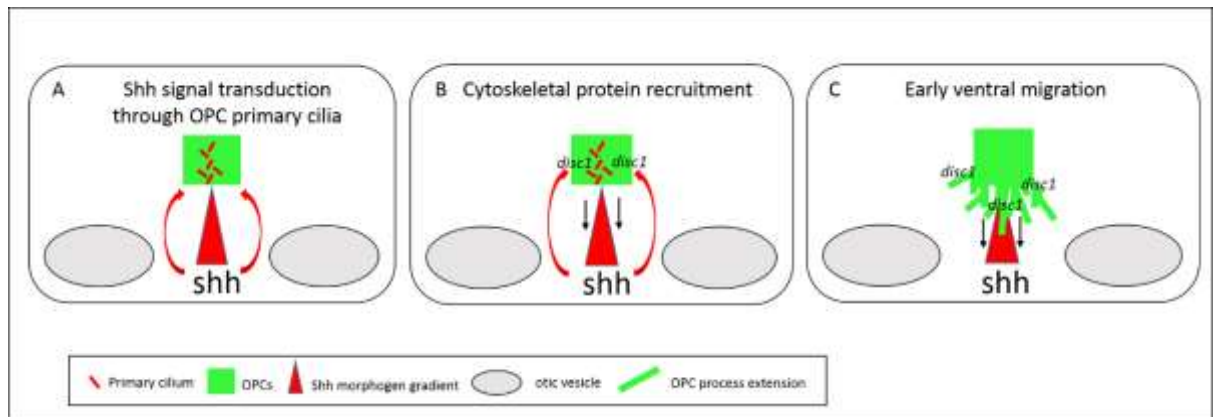
### 7.3 Hedgehog signalling in psychiatric disorders

Since this thesis suggests the *disc1* and Hh signalling are linked during development, it could be feasible that Hh is directly implicated in psychiatric disorders. A

literature search showed that Hh signalling has been implicated in mental disorders. For example craniofacial dysmorphology is a feature of schizophrenia, and is evidence of a developmental origin based disorder (Lane et al 1997). Neural crest gives rise to many craniofacial features, and Hh signalling has known roles in neural crest development (Mishina & Snider 2014). Furthermore, *disc1* has also been implicated in neural crest development in the zebrafish (Drerup et al 2009). Therefore it could be that Hh signalling regulates neural crest development through *DISC1*. Many current antipsychotic medications act on dopamine receptors, and it has been shown that Shh is essential for the development of midbrain dopaminergic neurons (Sillitoe & Vogel 2008). Finally, further evidence for involvement of Hh signaling in psychiatric disease has come from analysis of mouse models. Male *Desert hedgehog (Dhh)* knockout mice, but not females, show increased anxiety-like and depressive behavior (Umehara et al 2006). This data shows the further investigation into Hh signaling regulating *disc1*, in development and in psychiatric diseases could yield some interesting findings.

#### 7.4 Current model for OPC ventral migration in the zebrafish hindbrain

OPCs require directional information in order to migrate to their axonal destination, and this information is provided by external signals to both attract and repel OPCs during migration. These external factors are tightly regulated to create a defined and detailed migratory map which allows OPCs to communicate with their surrounding environment. Perturbation of any of these external factors may cause dysregulation in the entire migratory map leading to aberrant migration which could result in brain disorders. This current model proposes that in the zebrafish hindbrain, OPCs require Hh signalling to act as a ventral chemoattractant to direct cells downwards towards the primary source of the signal located in the notochord and ventral floor plate (Figure 7-1A). Although we have not determined which Hh ligand is responsible, we hypothesize that it is Shh given the already known roles for Shh in OPC development and nervous system development.



**Figure 7-1 Model for OPC migration in the zebrafish hindbrain**

Prior to migration, OPCs transduce a ventral chemoattractant signal proposed to be Shh which provides initiation and positional information through primary cilia (A). OPCs transduce this Shh signal and up-regulate the relevant cytoskeletal proteins required for migration (B). OPCs extend processes in a ventral direction and migrate downwards to eventually populate the ventral hindbrain (C). Cartoon shows a transverse section of the hindbrain. Dorsal top and ventral bottom.

OPCs transduce the Shh signal through primary cilia prior to migration, then signal transduction leads to cytoskeletal protein recruitment and ventral process extension (Figure 7-1B). Such downstream cytoskeletal proteins could include Disc1, a scaffold protein, which is expressed in OPCs during their migratory period (Figure 7-1C). Furthermore, it was suggested from the bead assays (section 3.6) that Shh acts as a directional cue to migrating OPCs, ensuring that cells extend processes towards the ventral signalling source as well initiating migration through promoting cytoskeletal gene expression.

Mechanistic information into how Disc1 might contribute to OPC migration in the zebrafish is an interesting question and has the potential to link schizophrenia oligodendrocyte abnormalities to schizophrenia risk factor genes. However it has to be appreciated that the most recent GWAS study into schizophrenia did not identify DISC1 or any oligodendrocyte genes (Schizophrenia Working Group of the Psychiatric Genomics 2014). To investigate the molecular mechanisms involved, the localisation of the protein in OPCs was explored. Understanding its localisation is important to provide insights into where the protein is recruited during development, for example was it being trafficked to primary cilia, the leading edge of the cell or the centrosome (Marley & von Zastrow 2010, Morris et al 2003)

(Wang & Brandon 2011)? It was expected that Disc1 would be seen to be localised to one or more of these regions as previous studies in mouse and cell culture has shown some evidence for centrosomal and primary cilia localisation, however this has not been convincingly demonstrated in the context of the endogenous protein (Morris et al 2003). Similarly the localisation of Disc1 in the zebrafish has not been addressed before. Since Disc1 has a wide variety of interacting partners, understanding its cellular location in OPCs would present a defined list of potential Disc1 interacting partners based on similarities in cellular localisation, which would provide important mechanistic clues.

## 7.5 Analysis of Disc1 localisation

Extensive localisation analysis was performed *in vivo* using a range of techniques. Over-expression analysis showed cytoplasmic punctate expression with no defined localisation, and this expression pattern was observed in other cells of the embryo and not in OPCs. Furthermore it did not address the endogenous protein. In order to localise the endogenous protein, antibodies were developed against zebrafish Disc1. These antibodies were shown to readily detect over-expressed Disc1. Although several attempts were made to detect the endogenous protein using these antibodies, no specific staining in the regions that have been shown to express detectable levels of *disc1* mRNA were observed. Only anti-peptide antibodies were attempted, other methods for antibody production are available which could be used in the future. As an alternative to antibodies, *in vivo* precise genome editing was attempted to fluorescently label the endogenous Disc1 protein. TALENs were designed to cause a double strand break around the stop codon of *disc1* which initiates the DNA repair machinery. Donor DNA containing a fluorescent *mCherry* sequence flanked by *disc1* homology arms was co-injected with the expectation that it would be incorporated into the genome through homologous recombination. Although the TALENs were shown to work efficiently, at the time of submission no positive founder fish with an incorporated *mCherry* sequence have been identified, although this is an ongoing project. Developing this tool is important, not only will the precise fluorescently-labelled line give valuable localisation information in OPCs but the line could also be used to investigate Disc1

function in other cell types where it is seen to be developmentally expressed. If C-terminal fluorescent tagging of *Disc1* is unsuccessful, the N-terminal end could be tagged.

## 7.6 Project limitations and future directions

At the outset of this project, using morpholino antisense methods to knockdown a gene of interest was the most prominent form of reverse genetics. It provided rapid and full knockdown of a gene which resulted in morphant embryos that could be easily analysed for phenotypic consequences without the need to grow up to adulthood. Although some morpholinos were shown to be highly specific to their target gene, others caused major off-targets effects (Wright et al 2004). This could be alleviated to some extent by co-injection with a p53 morpholino and validation through RNA rescue experiments (Robu et al 2007). Morpholinos have fallen from favour in the zebrafish community since the introduction of precise genome editing methods which have evolved over the last 3-4 years and has become more efficient, accessible and inexpensive for the creation of stable mutant lines (Eisen & Smith 2008) (Kok et al 2015). However, there have been several reported cases where these endonucleases have successfully produce INDELS in a gene without a phenotypic consequence and it seems that in some cases, mutagenized fish have been able to bypass the induced mutation to still produce a functional protein (S.Elworthy, personal communication). Therefore although morpholinos can give indications of biological functions provided the proper controls are in place, it is now expected that through genome editing, stable mutant lines should be generated to confirm morphant phenotypes. There is of course the important discussion point of maternal deposits of transcripts during early development. These zygotic mutants made from genome editing would still have maternal contribution of the transcript of interest, therefore the creation of maternal zygotic mutants would be required to phenocopy start site translation blocking morpholinos, which deplete all transcripts in the embryo including maternal contributions (Vogan 2015).

Previous work based on *disc1* function in OPCs used morpholino antisense techniques and although several parameters were in place to ensure specificity



including using two different morpholinos which gave the same phenotypes and co-injection with a p53 morpholino, one intention has been to confirm such phenotypes using a stable mutant line. The *disc1* mutants obtained from an ENU screen (discussed in section 1.5.5), which harboured mutation points in the poorly conserved N-terminal region, were subsequently acquired by our research group to determine whether these mutants phenocopied the morphant oligodendrocyte abnormalities documented, since these had not been addressed elsewhere (De Rienzo et al 2011). When our group received these stable mutant lines, genotyping of embryos showing reported brain abnormalities demonstrated that they were not exclusively homozygous mutants, but a collection of homozygotes, heterozygotes and wild type embryos. It was concluded that these phenotypes were a result of a secondary mutation. Furthermore when these secondary mutations were removed through outcrossing, *disc1<sup>fh291</sup>* and *disc1<sup>fh292</sup>* homozygote adult mutants were viable and fertile, and lacked the abnormalities described in the original publication (De Rienzo et al 2011). The progeny of maternal zygotic mutants were also viable and did not display the *disc1* morphant phenotypes previously described, there were also no lower jaw cartilage abnormalities described by both groups using *disc1* morpholinos. However subsequent work with these adult mutants has shown some behavioural abnormalities and endocrine changes suggesting that the mutations in *disc1* in these lines are exerting a low penetrance effect, but do not create a full knockout of the gene as originally suggested (Eachus *et al* unpublished). In the future, to validate or disprove the morphant phenotypes the CRISPR/cas9 system is being employed to create stable *disc1* mutant lines harbouring deletions in the conserved coiled-coil domains (Lucas *et al* unpublished).

Another important future direction to focus on is elucidating the localisation of *disc1* in OPCs and other cell types in the zebrafish. Creating a successful endogenous knock in Disc1-mCherry fusion protein line would not only give valuable information regarding Disc1 cellular expression in OPCs but also in protein recruitment and interactions. Furthermore it could prove very useful with regard to the preliminary drug screen which was performed to identify compounds and signalling pathways involved in OPC migration and development (Chapter 6).

Compounds which altered the migration of OPCs either through restriction of migration, excessive migration or an altered migratory pattern of *olig2*-positive cells was considered a positive hit and taken forward for validation screening. In the future, fish containing engineered *disc1* with a fluorescent tag could be crossed with the *tg(olig2:gfp)* line and used to investigate whether any of the 28 compounds identified had any effect on the expression levels or localisation of Disc1. If the signalling pathway that the compound exerts its effect through could be identified this could give an indication of which signalling pathways utilise Disc1 as part of their signalling network. These fish could be then used a readout for subsequent future drug screening.

## 7.7 Final conclusion

Investigating OPC migration in the zebrafish hindbrain has provided valuable information which suggests further roles of Shh signalling after initial specification of OPCs in r5/6 of the hindbrain. Evidence has also been provided which suggests that *disc1* acts downstream of Shh signalling in OPC development. Since migration is critical for subsequent OPC differentiation and myelination of target axons, understanding which signalling pathways are responsible for initiating and directing OPC migration could be used as a therapeutic target in diseases where myelination is defective. For example, in schizophrenia where there is a growing body of evidence to suggest oligodendrocyte abnormalities, understanding which risk factor genes and how they contribute to these abnormalities would improve our knowledge of oligodendrocyte development and potentially schizophrenia pathophysiology. This thesis has linked an important developmental signalling pathway and a schizophrenia risk factor gene in OPC development using the zebrafish as a model system.

# References

- Adams CW, Poston RN, Buk SJ. 1989. Pathology, histochemistry and immunocytochemistry of lesions in acute multiple sclerosis. *Journal of the neurological sciences* 92: 291-306
- Akbarian S, Vinuela A, Kim JJ, Potkin SG, Bunney WE, Jr., Jones EG. 1993. Distorted distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase neurons in temporal lobe of schizophrenics implies anomalous cortical development. *Archives of general psychiatry* 50: 178-87
- Almeida RG, Czopka T, Ffrench-Constant C, Lyons DA. 2011. Individual axons regulate the myelinating potential of single oligodendrocytes in vivo. *Development* 138: 4443-50
- Altshuler LL, Conrad A, Kovelman JA, Scheibel A. 1987. Hippocampal pyramidal cell orientation in schizophrenia. A controlled neurohistologic study of the Yakovlev collection. *Archives of general psychiatry* 44: 1094-8
- Avaron F, Hoffman L, Guay D, Akimenko MA. 2006. Characterization of two new zebrafish members of the hedgehog family: atypical expression of a zebrafish indian hedgehog gene in skeletal elements of both endochondral and dermal origins. *Developmental dynamics : an official publication of the American Association of Anatomists* 235: 478-89
- Barley K, Dracheva S, Byne W. 2009. Subcortical oligodendrocyte- and astrocyte-associated gene expression in subjects with schizophrenia, major depression and bipolar disorder. *Schizophrenia research* 112: 54-64
- Barres BA. 2008. The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron* 60: 430-40
- Baudoin JP, Viou L, Launay PS, Luccardini C, Espeso Gil S, et al. 2012. Tangentially migrating neurons assemble a primary cilium that promotes their reorientation to the cortical plate. *Neuron* 76: 1108-22
- Baxendale S, Holdsworth CJ, Meza Santoscoy PL, Harrison MR, Fox J, et al. 2012. Identification of compounds with anti-convulsant properties in a zebrafish model of epileptic seizures. *Disease models & mechanisms* 5: 773-84
- Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, et al. 2012. In vivo genome editing using a high-efficiency TALEN system. *Nature* 491: 114-8
- Berberi NF, Kin NW, Sharma N, Michaud EJ, Kesterson RA, Yoder BK. 2011. Mutations in *Traf3ip1* reveal defects in ciliogenesis, embryonic development, and altered cell size regulation. *Developmental biology* 360: 66-76
- Blackburn PR, Campbell JM, Clark KJ, Ekker SC. 2013. The CRISPR system--keeping zebrafish gene targeting fresh. *Zebrafish* 10: 116-8
- Blouin JL, Dombroski BA, Nath SK, Lasseter VK, Wolynec PS, et al. 1998. Schizophrenia susceptibility loci on chromosomes 13q32 and 8p21. *Nature genetics* 20: 70-3

- Bogdanove AJ, Voytas DF. 2011. TAL effectors: customizable proteins for DNA targeting. *Science* 333: 1843-6
- Brand M, Heisenberg CP, Warga RM, Pelegri F, Karlstrom RO, et al. 1996. Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* 123: 129-42
- Brandon NJ, Handford EJ, Schurov I, Rain JC, Pelling M, et al. 2004. Disrupted in Schizophrenia 1 and Nudel form a neurodevelopmentally regulated protein complex: implications for schizophrenia and other major neurological disorders. *Molecular and cellular neurosciences* 25: 42-55
- Bribian A, Barallobre MJ, Soussi-Yanicostas N, de Castro F. 2006. Anosmin-1 modulates the FGF-2-dependent migration of oligodendrocyte precursors in the developing optic nerve. *Molecular and cellular neurosciences* 33: 2-14
- Brosamle C, Halpern ME. 2002. Characterization of myelination in the developing zebrafish. *Glia* 39: 47-57
- Bruni G, Lakhani P, Kokel D. 2014. Discovering novel neuroactive drugs through high-throughput behavior-based chemical screening in the zebrafish. *Frontiers in pharmacology* 5: 153
- Buckley CE, Marguerie A, Roach AG, Goldsmith P, Fleming A, et al. 2010. Drug reprofiling using zebrafish identifies novel compounds with potential pro-myelination effects. *Neuropharmacology* 59: 149-59
- Bunge RP. 1968. Glial cells and the central myelin sheath. *Physiological reviews* 48: 197-251
- Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, et al. 2008. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28: 264-78
- Cai J, Qi Y, Hu X, Tan M, Liu Z, et al. 2005. Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. *Neuron* 45: 41-53
- Calver AR, Hall AC, Yu WP, Walsh FS, Heath JK, et al. 1998. Oligodendrocyte population dynamics and the role of PDGF in vivo. *Neuron* 20: 869-82
- Camargo LM, Collura V, Rain JC, Mizuguchi K, Hermjakob H, et al. 2007. Disrupted in Schizophrenia 1 Interactome: evidence for the close connectivity of risk genes and a potential synaptic basis for schizophrenia. *Molecular psychiatry* 12: 74-86
- Campagnoni AT. 1988. Molecular biology of myelin proteins from the central nervous system. *Journal of neurochemistry* 51: 1-14
- Cardno AG, Marshall EJ, Coid B, Macdonald AM, Ribchester TR, et al. 1999. Heritability estimates for psychotic disorders: the Maudsley twin psychosis series. *Archives of general psychiatry* 56: 162-8

- Chang A, Tourtellotte WW, Rudick R, Trapp BD. 2002. Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. *The New England journal of medicine* 346: 165-73
- Chang N, Sun C, Gao L, Zhu D, Xu X, et al. 2013. Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell research* 23: 465-72
- Chen JK, Taipale J, Cooper MK, Beachy PA. 2002. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothed. *Genes & development* 16: 2743-8
- Chen JS, Huang XH, Wang Q, Huang JQ, Zhang LJ, et al. 2013a. Sonic hedgehog signaling pathway induces cell migration and invasion through focal adhesion kinase/AKT signaling-mediated activation of matrix metalloproteinase (MMP)-2 and MMP-9 in liver cancer. *Carcinogenesis* 34: 10-9
- Chen S, Oikonomou G, Chiu CN, Niles BJ, Liu J, et al. 2013b. A large-scale in vivo analysis reveals that TALENs are significantly more mutagenic than ZFNs generated using context-dependent assembly. *Nucleic acids research* 41: 2769-78
- Chen W, Burgess S, Hopkins N. 2001. Analysis of the zebrafish smoothed mutant reveals conserved and divergent functions of hedgehog activity. *Development* 128: 2385-96
- Chen Y, Wu H, Wang S, Koito H, Li J, et al. 2009. The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination. *Nature neuroscience* 12: 1398-406
- Chubb JE, Bradshaw NJ, Soares DC, Porteous DJ, Millar JK. 2008. The DISC locus in psychiatric illness. *Molecular psychiatry* 13: 36-64
- Chung AY, Kim S, Kim E, Kim D, Jeong I, et al. 2013. Indian hedgehog B function is required for the specification of oligodendrocyte progenitor cells in the zebrafish CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 33: 1728-33
- Clapcote SJ, Lipina TV, Millar JK, Mackie S, Christie S, et al. 2007. Behavioral phenotypes of Disc1 missense mutations in mice. *Neuron* 54: 387-402
- Cross-Disorder Group of the Psychiatric Genomics C, Lee SH, Ripke S, Neale BM, Faraone SV, et al. 2013. Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. *Nature genetics* 45: 984-94
- Currie PD, Ingham PW. 1996. Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* 382: 452-5
- Czopka T, Ffrench-Constant C, Lyons DA. 2013. Individual oligodendrocytes have only a few hours in which to generate new myelin sheaths in vivo. *Developmental cell* 25: 599-609
- Davis KL, Stewart DG, Friedman JI, Buchsbaum M, Harvey PD, et al. 2003. White matter changes in schizophrenia: evidence for myelin-related dysfunction. *Archives of general psychiatry* 60: 443-56

- de Castro F, Bribian A. 2005. The molecular orchestra of the migration of oligodendrocyte precursors during development. *Brain research. Brain research reviews* 49: 227-41
- De Rienzo G, Bishop JA, Mao Y, Pan L, Ma TP, et al. 2011. Disc1 regulates both beta-catenin-mediated and noncanonical Wnt signaling during vertebrate embryogenesis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 25: 4184-97
- Dong Z, Dong X, Jia W, Cao S, Zhao Q. 2014. Improving the efficiency for generation of genome-edited zebrafish by labeling primordial germ cells. *The international journal of biochemistry & cell biology*
- Drerup CM, Wiora HM, Topczewski J, Morris JA. 2009. Disc1 regulates foxd3 and sox10 expression, affecting neural crest migration and differentiation. *Development* 136: 2623-32
- Duldulao NA, Lee S, Sun Z. 2009. Cilia localization is essential for in vivo functions of the Joubert syndrome protein Arl13b/Scorpion. *Development* 136: 4033-42
- Dutton KA, Pauliny A, Lopes SS, Elworthy S, Carney TJ, et al. 2001. Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* 128: 4113-25
- Eisen JS, Smith JC. 2008. Controlling morpholino experiments: don't stop making antisense. *Development* 135: 1735-43
- Ekelund J, Hovatta I, Parker A, Paunio T, Varilo T, et al. 2001. Chromosome 1 loci in Finnish schizophrenia families. *Human molecular genetics* 10: 1611-7
- Ekker SC, Ungar AR, Greenstein P, von Kessler DP, Porter JA, et al. 1995. Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Current biology : CB* 5: 944-55
- Ertzer R, Muller F, Hadzhiev Y, Rathnam S, Fischer N, et al. 2007. Cooperation of sonic hedgehog enhancers in midline expression. *Developmental biology* 301: 578-89
- Esain V, Postlethwait JH, Charnay P, Ghislain J. 2010. FGF-receptor signalling controls neural cell diversity in the zebrafish hindbrain by regulating olig2 and sox9. *Development* 137: 33-42
- Flynn SW, Lang DJ, Mackay AL, Goghari V, Vavasour IM, et al. 2003. Abnormalities of myelination in schizophrenia detected in vivo with MRI, and post-mortem with analysis of oligodendrocyte proteins. *Molecular psychiatry* 8: 811-20
- Fogarty M, Richardson WD, Kessar N. 2005. A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. *Development* 132: 1951-9
- Foong J, Maier M, Barker GJ, Brocklehurst S, Miller DH, Ron MA. 2000. In vivo investigation of white matter pathology in schizophrenia with magnetisation transfer imaging. *Journal of neurology, neurosurgery, and psychiatry* 68: 70-4
- Franklin RJ, Ffrench-Constant C. 2008. Remyelination in the CNS: from biology to therapy. *Nature reviews. Neuroscience* 9: 839-55

- Fruttiger M, Karlsson L, Hall AC, Abramsson A, Calver AR, et al. 1999. Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. *Development* 126: 457-67
- Fu M, Lui VC, Sham MH, Pachnis V, Tam PK. 2004. Sonic hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut. *The Journal of cell biology* 166: 673-84
- Funfschilling U, Supplie LM, Mahad D, Boretius S, Saab AS, et al. 2012. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* 485: 517-21
- Georgieva L, Moskvina V, Peirce T, Norton N, Bray NJ, et al. 2006. Convergent evidence that oligodendrocyte lineage transcription factor 2 (OLIG2) and interacting genes influence susceptibility to schizophrenia. *Proceedings of the National Academy of Sciences of the United States of America* 103: 12469-74
- Goetz SC, Anderson KV. 2010. The primary cilium: a signalling centre during vertebrate development. *Nature reviews. Genetics* 11: 331-44
- Grunwald DJ. 2013. A revolution coming to a classic model organism. *Nature methods* 10: 303, 05-6
- Hakak Y, Walker JR, Li C, Wong WH, Davis KL, et al. 2001. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. *Proceedings of the National Academy of Sciences of the United States of America* 98: 4746-51
- Hattori T, Shimizu S, Koyama Y, Emoto H, Matsumoto Y, et al. 2014. DISC1 (disrupted-in-schizophrenia-1) regulates differentiation of oligodendrocytes. *PloS one* 9: e88506
- Higginbotham H, Eom TY, Mariani LE, Bachleda A, Hirt J, et al. 2012. Arl13b in primary cilia regulates the migration and placement of interneurons in the developing cerebral cortex. *Developmental cell* 23: 925-38
- Hikida T, Jaaro-Peled H, Seshadri S, Oishi K, Hookway C, et al. 2007. Dominant-negative DISC1 transgenic mice display schizophrenia-associated phenotypes detected by measures translatable to humans. *Proceedings of the National Academy of Sciences of the United States of America* 104: 14501-6
- Hof PR, Haroutunian V, Friedrich VL, Jr., Byne W, Buitron C, et al. 2003. Loss and altered spatial distribution of oligodendrocytes in the superior frontal gyrus in schizophrenia. *Biological psychiatry* 53: 1075-85
- Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496: 498-503
- Huang P, Xiao A, Zhou M, Zhu Z, Lin S, Zhang B. 2011. Heritable gene targeting in zebrafish using customized TALENs. *Nature biotechnology* 29: 699-700
- Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, et al. 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature biotechnology* 31: 227-9

- Insel TR. 2010. Rethinking schizophrenia. *Nature* 468: 187-93
- Jenkins D. 2009. Hedgehog signalling: emerging evidence for non-canonical pathways. *Cellular signalling* 21: 1023-34
- Kamiya A, Kubo K, Tomoda T, Takaki M, Youn R, et al. 2005. A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. *Nature cell biology* 7: 1167-78
- Kamiya A, Tan PL, Kubo K, Engelhard C, Ishizuka K, et al. 2008. Recruitment of PCM1 to the centrosome by the cooperative action of DISC1 and BBS4: a candidate for psychiatric illnesses. *Archives of general psychiatry* 65: 996-1006
- Katsel P, Davis KL, Haroutunian V. 2005. Variations in myelin and oligodendrocyte-related gene expression across multiple brain regions in schizophrenia: a gene ontology study. *Schizophrenia research* 79: 157-73
- Katsel P, Tan W, Abazyan B, Davis KL, Ross C, et al. 2011. Expression of mutant human DISC1 in mice supports abnormalities in differentiation of oligodendrocytes. *Schizophrenia research* 130: 238-49
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. *Developmental dynamics : an official publication of the American Association of Anatomists* 203: 253-310
- Kirby BB, Takada N, Latimer AJ, Shin J, Carney TJ, et al. 2006. In vivo time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. *Nature neuroscience* 9: 1506-11
- Kok FO, Shin M, Ni CW, Gupta A, Grosse AS, et al. 2015. Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish. *Developmental cell* 32: 97-108
- Kondo T, Raff M. 2000. Basic helix-loop-helix proteins and the timing of oligodendrocyte differentiation. *Development* 127: 2989-98
- Koudijs MJ, den Broeder MJ, Groot E, van Eeden FJ. 2008. Genetic analysis of the two zebrafish patched homologues identifies novel roles for the hedgehog signaling pathway. *BMC developmental biology* 8: 15
- Koudijs MJ, den Broeder MJ, Keijser A, Wienholds E, Houwing S, et al. 2005. The zebrafish mutants dre, uki, and lep encode negative regulators of the hedgehog signaling pathway. *PLoS genetics* 1: e19
- Krauss S, Concordet JP, Ingham PW. 1993. A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* 75: 1431-44
- Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M. 1998. Sox10, a novel transcriptional modulator in glial cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18: 237-50



- Lane A, Kinsella A, Murphy P, Byrne M, Keenan J, et al. 1997. The anthropometric assessment of dysmorphic features in schizophrenia as an index of its developmental origins. *Psychological medicine* 27: 1155-64
- Larson TA, Gordon TN, Lau HE, Parichy DM. 2010. Defective adult oligodendrocyte and Schwann cell development, pigment pattern, and craniofacial morphology in puma mutant zebrafish having an alpha tubulin mutation. *Developmental biology* 346: 296-309
- Lee JE, Gleeson JG. 2011. Cilia in the nervous system: linking cilia function and neurodevelopmental disorders. *Current opinion in neurology* 24: 98-105
- Levavasseur F, Mandemakers W, Visser P, Broos L, Grosveld F, et al. 1998. Comparison of sequence and function of the Oct-6 genes in zebrafish, chicken and mouse. *Mechanisms of development* 74: 89-98
- Li D, Collier DA, He L. 2006. Meta-analysis shows strong positive association of the neuregulin 1 (NRG1) gene with schizophrenia. *Human molecular genetics* 15: 1995-2002
- Liddle PF. 1996. Functional imaging--schizophrenia. *British medical bulletin* 52: 486-94
- Lipska BK, Peters T, Hyde TM, Halim N, Horowitz C, et al. 2006. Expression of DISC1 binding partners is reduced in schizophrenia and associated with DISC1 SNPs. *Human molecular genetics* 15: 1245-58
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>(-Delta Delta C(T))</sup> Method. *Methods* 25: 402-8
- Lu QR, Sun T, Zhu Z, Ma N, Garcia M, et al. 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* 109: 75-86
- Lu QR, Yuk D, Alberta JA, Zhu Z, Pawlitzky I, et al. 2000. Sonic hedgehog--regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. *Neuron* 25: 317-29
- Lyons DA, Naylor SG, Scholze A, Talbot WS. 2009. Kif1b is essential for mRNA localization in oligodendrocytes and development of myelinated axons. *Nature genetics* 41: 854-8
- Lyons DA, Pogoda HM, Voas MG, Woods IG, Diamond B, et al. 2005. erbb3 and erbb2 are essential for schwann cell migration and myelination in zebrafish. *Current biology : CB* 15: 513-24
- Maldonado PP, Angulo MC. 2014. Multiple Modes of Communication between Neurons and Oligodendrocyte Precursor Cells. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry*
- Marley A, von Zastrow M. 2010. DISC1 regulates primary cilia that display specific dopamine receptors. *PloS one* 5: e10902
- Mathews ES, Mawdsley DJ, Walker M, Hines JH, Pozzoli M, Appel B. 2014. Mutation of 3-hydroxy-3-methylglutaryl CoA synthase I reveals requirements for isoprenoid and

cholesterol synthesis in oligodendrocyte migration arrest, axon wrapping, and myelin gene expression. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 34: 3402-12

- McKinnon RD, Matsui T, Aranda M, Dubois-Dalcq M. 1991. A role for fibroblast growth factor in oligodendrocyte development. *Annals of the New York Academy of Sciences* 638: 378-86
- Mehler MF, Mabie PC, Zhang D, Kessler JA. 1997. Bone morphogenetic proteins in the nervous system. *Trends in neurosciences* 20: 309-17
- Merchan P, Bribian A, Sanchez-Camacho C, Lezameta M, Bovolenta P, de Castro F. 2007. Sonic hedgehog promotes the migration and proliferation of optic nerve oligodendrocyte precursors. *Molecular and cellular neurosciences* 36: 355-68
- Millar JK, Wilson-Annan JC, Anderson S, Christie S, Taylor MS, et al. 2000. Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Human molecular genetics* 9: 1415-23
- Mishina Y, Snider TN. 2014. Neural crest cell signaling pathways critical to cranial bone development and pathology. *Experimental cell research* 325: 138-47
- Morris JA, Kandpal G, Ma L, Austin CP. 2003. DISC1 (Disrupted-In-Schizophrenia 1) is a centrosome-associated protein that interacts with MAP1A, MIPT3, ATF4/5 and NUDEL: regulation and loss of interaction with mutation. *Human molecular genetics* 12: 1591-608
- Ng MY, Levinson DF, Faraone SV, Suarez BK, DeLisi LE, et al. 2009. Meta-analysis of 32 genome-wide linkage studies of schizophrenia. *Molecular psychiatry* 14: 774-85
- Niethammer M, Smith DS, Ayala R, Peng J, Ko J, et al. 2000. NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. *Neuron* 28: 697-711
- Nishiyama A, Komitova M, Suzuki R, Zhu X. 2009. Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. *Nature reviews. Neuroscience* 10: 9-22
- Niwa M, Kamiya A, Murai R, Kubo K, Gruber AJ, et al. 2010. Knockdown of DISC1 by in utero gene transfer disturbs postnatal dopaminergic maturation in the frontal cortex and leads to adult behavioral deficits. *Neuron* 65: 480-9
- Nüsslein-Volhard C, Dahm R. 2002. *Zebrafish : a practical approach*. Oxford: Oxford University Press. xviii, 303 p. pp.
- Ono K, Yasui Y, Rutishauser U, Miller RH. 1997. Focal ventricular origin and migration of oligodendrocyte precursors into the chick optic nerve. *Neuron* 19: 283-92
- Orentas DM, Hayes JE, Dyer KL, Miller RH. 1999. Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors. *Development* 126: 2419-29
- Ortega MC, Cases O, Merchan P, Kozyraki R, Clemente D, de Castro F. 2012. Megalin mediates the influence of sonic hedgehog on oligodendrocyte precursor cell migration and proliferation during development. *Glia* 60: 851-66

- Owen MJ, Craddock N, O'Donovan MC. 2005. Schizophrenia: genes at last? *Trends in genetics : TIG* 21: 518-25
- Park HC, Appel B. 2003. Delta-Notch signaling regulates oligodendrocyte specification. *Development* 130: 3747-55
- Park HC, Mehta A, Richardson JS, Appel B. 2002. *olig2* is required for zebrafish primary motor neuron and oligodendrocyte development. *Developmental biology* 248: 356-68
- Paus T, Keshavan M, Giedd JN. 2008. Why do many psychiatric disorders emerge during adolescence? *Nature reviews. Neuroscience* 9: 947-57
- Peters BD, Karlsgodt KH. 2014. White matter development in the early stages of psychosis. *Schizophrenia research*
- Pletnikov MV, Ayhan Y, Nikolskaia O, Xu Y, Ovanesov MV, et al. 2008. Inducible expression of mutant human DISC1 in mice is associated with brain and behavioral abnormalities reminiscent of schizophrenia. *Molecular psychiatry* 13: 173-86, 15
- Pogoda HM, Sternheim N, Lyons DA, Diamond B, Hawkins TA, et al. 2006. A genetic screen identifies genes essential for development of myelinated axons in zebrafish. *Developmental biology* 298: 118-31
- Polizio AH, Chinchilla P, Chen X, Kim S, Manning DR, Riobo NA. 2011a. Heterotrimeric Gi proteins link Hedgehog signaling to activation of Rho small GTPases to promote fibroblast migration. *The Journal of biological chemistry* 286: 19589-96
- Polizio AH, Chinchilla P, Chen X, Manning DR, Riobo NA. 2011b. Sonic Hedgehog activates the GTPases Rac1 and RhoA in a Gli-independent manner through coupling of smoothed to Gi proteins. *Science signaling* 4: pt7
- Porteous DJ, Millar JK, Brandon NJ, Sawa A. 2011. DISC1 at 10: connecting psychiatric genetics and neuroscience. *Trends in molecular medicine* 17: 699-706
- Porteous DJ, Thomson PA, Millar JK, Evans KL, Hennah W, et al. 2014. DISC1 as a genetic risk factor for schizophrenia and related major mental illness: response to Sullivan. *Molecular psychiatry* 19: 141-3
- Prineas JW, Graham JS. 1981. Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. *Annals of neurology* 10: 149-58
- Pringle NP, Yu WP, Guthrie S, Roelink H, Lumsden A, et al. 1996. Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog. *Developmental biology* 177: 30-42
- Qi Y, Cai J, Wu Y, Wu R, Lee J, et al. 2001. Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. *Development* 128: 2723-33
- Raft S, Groves AK. 2014. Segregating neural and mechanosensory fates in the developing ear: patterning, signaling, and transcriptional control. *Cell and tissue research*

- Ripke S, O'Dushlaine C, Chambert K, Moran JL, Kahler AK, et al. 2013. Genome-wide association analysis identifies 13 new risk loci for schizophrenia. *Nature genetics* 45: 1150-9
- Robertson CP, Braun MM, Roelink H. 2004. Sonic hedgehog patterning in chick neural plate is antagonized by a Wnt3-like signal. *Developmental dynamics : an official publication of the American Association of Anatomists* 229: 510-9
- Robinson S, Tani M, Strieter RM, Ransohoff RM, Miller RH. 1998. The chemokine growth-regulated oncogene-alpha promotes spinal cord oligodendrocyte precursor proliferation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18: 10457-63
- Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, et al. 2007. p53 activation by knockdown technologies. *PLoS genetics* 3: e78
- Schizophrenia Working Group of the Psychiatric Genomics C. 2014. Biological insights from 108 schizophrenia-associated genetic loci. *Nature* 511: 421-7
- Schurov IL, Handford EJ, Brandon NJ, Whiting PJ. 2004. Expression of disrupted in schizophrenia 1 (DISC1) protein in the adult and developing mouse brain indicates its role in neurodevelopment. *Molecular psychiatry* 9: 1100-10
- Semple CA, Devon RS, Le Hellard S, Porteous DJ. 2001. Identification of genes from a schizophrenia-linked translocation breakpoint region. *Genomics* 73: 123-6
- Seshadri S, Kamiya A, Yokota Y, Prikulis I, Kano S, et al. 2010. Disrupted-in-Schizophrenia-1 expression is regulated by beta-site amyloid precursor protein cleaving enzyme-1-neuregulin cascade. *Proceedings of the National Academy of Sciences of the United States of America* 107: 5622-7
- Shimizu S, Koyama Y, Hattori T, Tachibana T, Yoshimi T, et al. 2014. DBZ, a CNS-specific DISC1 binding protein, positively regulates oligodendrocyte differentiation. *Glia* 62: 709-24
- Shin J, Park HC, Topczewska JM, Mawdsley DJ, Appel B. 2003. Neural cell fate analysis in zebrafish using olig2 BAC transgenics. *Methods in cell science : an official journal of the Society for In Vitro Biology* 25: 7-14
- Sild M, Ruthazer ES. 2011. Radial glia: progenitor, pathway, and partner. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 17: 288-302
- Sillitoe RV, Vogel MW. 2008. Desire, disease, and the origins of the dopaminergic system. *Schizophrenia bulletin* 34: 212-9
- Small RK, Riddle P, Noble M. 1987. Evidence for migration of oligodendrocyte--type-2 astrocyte progenitor cells into the developing rat optic nerve. *Nature* 328: 155-7
- Sorensen HJ, Mortensen EL, Schiffman J, Reinisch JM, Maeda J, Mednick SA. 2010. Early developmental milestones and risk of schizophrenia: a 45-year follow-up of the Copenhagen Perinatal Cohort. *Schizophrenia research* 118: 41-7

- Spassky N, de Castro F, Le Bras B, Heydon K, Queraud-LeSaux F, et al. 2002. Directional guidance of oligodendroglial migration by class 3 semaphorins and netrin-1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22: 5992-6004
- St Clair D, Blackwood D, Muir W, Carothers A, Walker M, et al. 1990. Association within a family of a balanced autosomal translocation with major mental illness. *Lancet* 336: 13-6
- Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, et al. 2002. Neuregulin 1 and susceptibility to schizophrenia. *American journal of human genetics* 71: 877-92
- Steinecke A, Gampe C, Valkova C, Kaether C, Bolz J. 2012. Disrupted-in-Schizophrenia 1 (DISC1) is necessary for the correct migration of cortical interneurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32: 738-45
- Stolt CC, Rehberg S, Ader M, Lommes P, Riethmacher D, et al. 2002. Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. *Genes & development* 16: 165-70
- Stuermer CA, Bastmeyer M, Bahr M, Strobel G, Paschke K. 1992. Trying to understand axonal regeneration in the CNS of fish. *Journal of neurobiology* 23: 537-50
- Sugimori M, Nagao M, Parras CM, Nakatani H, Lebel M, et al. 2008. Ascl1 is required for oligodendrocyte development in the spinal cord. *Development* 135: 1271-81
- Sullivan PF, Kendler KS, Neale MC. 2003. Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. *Archives of general psychiatry* 60: 1187-92
- Takada N, Kucenas S, Appel B. 2010. Sox10 is necessary for oligodendrocyte survival following axon wrapping. *Glia* 58: 996-1006
- Takahashi N, Sakurai T, Davis KL, Buxbaum JD. 2011. Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. *Progress in neurobiology* 93: 13-24
- Takebayashi H, Yoshida S, Sugimori M, Kosako H, Kominami R, et al. 2000. Dynamic expression of basic helix-loop-helix Olig family members: implication of Olig2 in neuron and oligodendrocyte differentiation and identification of a new member, Olig3. *Mechanisms of development* 99: 143-8
- Tang R, Dodd A, Lai D, McNabb WC, Love DR. 2007. Validation of zebrafish (*Danio rerio*) reference genes for quantitative real-time RT-PCR normalization. *Acta biochimica et biophysica Sinica* 39: 384-90
- Thisse B, Heyer V, Lux A, Alunni V, Degraeve A, et al. 2004. Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. *Methods in cell biology* 77: 505-19
- Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, et al. 2003. Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. *Lancet* 362: 798-805

- Tsai HH, Frost E, To V, Robinson S, Ffrench-Constant C, et al. 2002. The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* 110: 373-83
- Umehara F, Mishima K, Egashira N, Ogata A, Iwasaki K, Fujiwara M. 2006. Elevated anxiety-like and depressive behavior in Desert hedgehog knockout male mice. *Behavioural brain research* 174: 167-73
- Varga ZM, Amores A, Lewis KE, Yan YL, Postlethwait JH, et al. 2001. Zebrafish smoothed functions in ventral neural tube specification and axon tract formation. *Development* 128: 3497-509
- Vartanian T, Fischbach G, Miller R. 1999. Failure of spinal cord oligodendrocyte development in mice lacking neuregulin. *Proceedings of the National Academy of Sciences of the United States of America* 96: 731-5
- Vogan K. 2015. Zebrafish mutants versus morphants. *Nature genetics* 47: 105
- Walker RM, Hill AE, Newman AC, Hamilton G, Torrance HS, et al. 2012. The DISC1 promoter: characterization and regulation by FOXP2. *Human molecular genetics* 21: 2862-72
- Wang Q, Brandon NJ. 2011. Regulation of the cytoskeleton by Disrupted-in-schizophrenia 1 (DISC1). *Molecular and cellular neurosciences* 48: 359-64
- Wang S, Sdrulla AD, diSibio G, Bush G, Nofziger D, et al. 1998. Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* 21: 63-75
- Weinberger DR. 1987. Implications of normal brain development for the pathogenesis of schizophrenia. *Archives of general psychiatry* 44: 660-9
- Wolff C, Roy S, Lewis KE, Schauerte H, Joerg-Rauch G, et al. 2004. iguana encodes a novel zinc-finger protein with coiled-coil domains essential for Hedgehog signal transduction in the zebrafish embryo. *Genes & development* 18: 1565-76
- Wood JD, Bonath F, Kumar S, Ross CA, Cunliffe VT. 2009. Disrupted-in-schizophrenia 1 and neuregulin 1 are required for the specification of oligodendrocytes and neurones in the zebrafish brain. *Human molecular genetics* 18: 391-404
- Wright GJ, Leslie JD, Ariza-McNaughton L, Lewis J. 2004. Delta proteins and MAGI proteins: an interaction of Notch ligands with intracellular scaffolding molecules and its significance for zebrafish development. *Development* 131: 5659-69
- Xin M, Yue T, Ma Z, Wu FF, Gow A, Lu QR. 2005. Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25: 1354-65
- Ye P, Li L, Richards RG, DiAugustine RP, D'Ercole AJ. 2002. Myelination is altered in insulin-like growth factor-I null mutant mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22: 6041-51
- Yin W, Hu B. 2014. Knockdown of Lingo1b protein promotes myelination and oligodendrocyte differentiation in zebrafish. *Experimental neurology* 251: 72-83

- Young-Pearse TL, Suth S, Luth ES, Sawa A, Selkoe DJ. 2010. Biochemical and functional interaction of disrupted-in-schizophrenia 1 and amyloid precursor protein regulates neuronal migration during mammalian cortical development. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30: 10431-40
- Yu Y, Chen Y, Kim B, Wang H, Zhao C, et al. 2013. Olig2 targets chromatin remodelers to enhancers to initiate oligodendrocyte differentiation. *Cell* 152: 248-61
- Zannino DA, Appel B. 2009. Olig2+ precursors produce abducens motor neurons and oligodendrocytes in the zebrafish hindbrain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 2322-33
- Zhang Q, Hu J, Ling K. 2013. Molecular views of Arf-like small GTPases in cilia and ciliopathies. *Experimental cell research* 319: 2316-22
- Zhang X, Tochigi M, Ohashi J, Maeda K, Kato T, et al. 2005. Association study of the DISC1/TRAX locus with schizophrenia in a Japanese population. *Schizophrenia research* 79: 175-80
- Zhou Q, Anderson DJ. 2002. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* 109: 61-73
- Zhou Q, Wang S, Anderson DJ. 2000. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. *Neuron* 25: 331-43