6.4: Discussion

6.4.1: Preface

In this discussion, I will analyse the results I obtained before it was discovered that the morpholinos no longer produced the *myoD*, *myogenin*, or *prdm1* phenotype. I will then attempt to address why the morpholinos no longer give the same results.

6.4.2: The expression and regulation of *c125* during zebrafish development

The expression pattern of zebrafish *c125* is conserved with the chick and mouse, with *c125* transcripts detected in the anterior CNS, eye, somites, branchial arches, and the pectoral fins. However, unlike the chick and mouse, the zebrafish does not appear to express *c125* in the ventral neural tube. This indicates a possible divergence in the function of *c125* in the ventral neural tube between the zebrafish and the chick and mouse (discussed in section 6.4.3). Overall, the conserved expression pattern suggests that common regulatory mechanisms control the expression of *c125*.

In the mouse, *C125* expression is dependent upon Shh signalling as *Shh* null and *Gli2*/*Gli3* null mice lack all expression of *C125* (thesis, Antonio Milano 2005). Similarly, *yot*, *smu*, and cyclopamine-treated zebrafish embryos, which lack Hh signalling, display a down-regulation of *c125* expression in the telencephalon and anterior CNS, eyes, somites, and the tail region at 26-28hpf, indicating some level of conservation of the *c125* regulatory mechanisms. However, it is worth noting that in contrast to the mouse, *c125* expression is only partially down-regulated in the absence of Hh signalling. This suggests that additional signalling pathways are involved in *c125* expression. Despite the overlapping expression of *FGF8* and *c125* in the midbrain-hindbrain boundary and the fast muscle domain of the somite (Reifers et al. 1998), FGF8 (and all other FGFs) does not have a role in *c125* regulation. As Retinoic acid (RA) is required for formation of fast muscles (Hamade et al. 2006), RA is a good candidate for a role in the regulation of *c125*.

One might expect that as loss of Hh signalling causes a reduction in *c125* expression, elevated levels of Hh signalling may cause up-regulation of *c125* expression throughout the embryo, although in the chick, Shh is necessary for *C125* expression, but it is not sufficient (thesis, Antonio Milano 2005). Unexpectedly, gain-of-function of Hh signalling actually causes a down-regulation of *c125* expression, which interestingly is greater than in *smu* zebrafish embryos. This suggests that *c125* expression is controlled by a precise ratio of Gli activator to Gli repressor, and a change in this balance caused by increased or decreased levels of Hh signalling results in the repression of *c125* transcription. Neuronal progenitor domains in the neural tube also require a precise Hh concentration threshold for the correct spatial expression of homeodomain transcription factors (Jessell 2000). Despite the overall reduction in *c125* expression, a slight increase of *c125* is detected in the anterior CNS of *ptc1/2* zebrafish.

Results raise the possibility that *c125* is directly regulated by Hh signalling. For instance, Hh signalling is required for normal forebrain development in zebrafish, mouse, and human. Lack of Hh signalling can result in cyclopia, caused by defective floor plate, ventral forebrain, and motor neuron formation (Chiang et al. 1996; Varga et al. 2001). In the forebrain, Hh signalling is required for the induction of genes associated with brain and motor neuron development, and in its absence, like *c125* there is a reduction of Hh-target genes such as *nkx2.2*, *dlx2* and *pax6a* in the ventral forebrain (Varga et al. 2001). Despite the role of Hh signalling in forebrain development, the lens and retina are still formed in the absence of Hh signalling (Macdonald et al. 1995; Varga et al. 2001). Thus, normal *c125* expression in the lens and retina is somewhat expected. However, I observed a reduced expression of *c125* in these tissues. The reduction of *c125* in the brain and eye therefore supports the hypothesis that *shh* directly regulates *c125* expression in the brain and eye. Similarly, over-expression of *shh* is sufficient to alter gene expression and morphology within the brain and anterior CNS (Barth and Wilson 1995; Macdonald et al. 1995). Up-regulation of the Hh signalling pathway is capable of expanding the floor plate, motor neuron and V3 interneuron progenitor domains into dorsal regions (Motoyama et al. 2003). *c125* expressed in the anterior CNS of zebrafish embryos could therefore also be up-regulated in the anterior CNS in response to elevated levels of Hh signalling.

However, it is also possible that the effects of increased Hh signalling on *c125* expression are not direct, and are the consequence of altered tissue morphology in Hh-defective zebrafish embryos. Indeed, by 27hpf, *c125* expression is excluded from the slow muscle lineage. As up-regulation of the Hh signalling pathway causes an expansion of the slow muscle lineage at the expense of the *c125*-expressing fast muscle lineage (Currie and Ingham 1996; Hammerschmidt et al. 1996; Barresi et al. 2000; Koudijs et al. 2008), it is likely that loss of *c125* expression in Hh-gain of function embryos is the consequence of the loss of the fast muscle lineage. Similarly, Hh signalling represses lens development (Macdonald et al. 1995) and results in the failure of midbrain-hindbrain constriction (Ekker et al. 1995). Thus, the lens does not develop in *ptc1/2* and dnPKA mRNA-injected embryos (Hammerschmidt et al. 1996; Koudijs et al. 2008), and the optic tectum structure is greatly disturbed (Ekker et al. 1995). This could account for the loss of *c125* expression in the eye and midbrain-hindbrain of embryos with up-regulated Hh signalling.

To test the possibility that Hh has a direct role in the regulation of *c125* in the anterior CNS and eye, the Hh pathway could be up-regulated in embryos in which these tissues have already formed, by dnPKA mRNA injection. In this way, the eye would still be present, and so *c125* expression would not be altered due to a loss of the eye. However, Hh may have an early function in the regulation of *c125* in the eye, and so a change in expression may not be detected at these later stages of development. To address whether *c125* expression in the fast muscle is directly regulated by Hh signalling, the *prdm1* (*ubo*) mutant zebrafish could be used which cannot form slow muscle, even when Hh signalling is up-regulated. Therefore, dnPKA mRNA injection into the *prdm1* mutant at the one-cell stage will increase Hh signalling without the conversion of fast muscle to slow muscle cells. If Hh directly inhibits *c125* expression in the fast muscle compartment, as *ptc1/2* zebrafish suggest, then a reduction of *c125* expression would be expected in these zebrafish.

As in the case of the gain-of-function approach, loss-of-function of Hh signalling could have indirect effects on *c125* expression. In the somites of Hh mutant zebrafish embryos, embryos fail to form slow muscles (Barresi et al. 2000). Shh also acts at a later stage of development in the formation of fast muscle cells from *pax3/7* expressing progenitor cells located in the dermomyotome (Devoto et al. 2006; Feng et al. 2006). In the absence Hh signalling, cells remain in their progenitor state and do not contribute to the fast muscle domain (Feng et al. 2006). This could partly account for the reduced *c125* expression in Hh-deficient somites at 26-30hpf, although a possible direct mode of regulation by Hh signalling cannot be ruled out. Similarly, there are patterning defects associated with the forebrain including the inability to form an optic chiasm, in the absence of Hh signalling (Varga et al. 2001). Abnormal morphogenesis could therefore give an appearance of reduced *c125* expression. Overall, although Hh signalling plays a role in the regulation of *c125* expression, it remains unknown whether this regulation is direct or indirect.

6.4.3: Zebrafish *c125* does not have a role in neurogenesis or the Hh signalling pathway

In the chick neural tube, *C125* is required in early neural tube patterning, and in its absence, there are alterations to some homeodomain protein boundaries and neuronal progenitor domain positions (unpublished data, Mark Watson 2007). In the motor neuron domain, the down-regulation of *C125* in the chick neural tube does not affect the expression of the progenitor marker Olig2, but causes a reduction in the number of Islet1-expressing motor neurons (unpublished data, Mark Watson 2007). This suggests that *C125* is required for motor neuron differentiation. This function is not conserved in the zebrafish, consistent with the lack of *c125* expression in the ventral neural tube. Furthermore, *C125* over-expression causes increased expression of Olig2 and Islet1 in the chick neural tube (unpublished data, Mark Watson 2007), but has no effect upon *olig2.2* or *islet1* expression in the zebrafish. Therefore, it appears that the chick and the mouse have evolved to express *C125* in the neural tube, perhaps through the loss of a repressor element present in the zebrafish locus which prevents neural tube expression of *c125*, or through the acquisition of a novel regulatory element allowing *C125* expression in the amniote neural tube.

Changes in sizes and positioning of neuronal progenitor domains in the ventral neural tube of the chick lead to the hypothesis that *c125* may be involved in modulating the Hh signalling gradient.

Several other genes are already known to be controlled by Hh signalling and function within the Hh pathway, such as *ptc1* and *HIP* (Chuang and McMahon 1999). *gli1* is also believed to be activated following Hh signalling (Dai et al. 1999). However, over-expression or loss of *c125* does not affect *ptc1* expression in the zebrafish, suggesting that *c125* does not function within the Hh signalling pathway.

The fact that *c125* has no effect on *ptc1* transcription in the zebrafish does not rule out however a role for *c125* within the Hh pathway in amniotes. For instance, loss of *Su(Fu)* in *Drosophila* produces no noticeable effect, yet its loss in the mouse causes death by E9.5 with a Hh gain-of-function phenotype (Svard et al. 2006; Varjosalo et al. 2006). Therefore, *c125* could have a more significant role in amniotes as demonstrated by its importance for correct neural tube patterning and motor neuron differentiation.

6.4.4: *c125* is required for fast muscle determination

Knock-down of *c125* throughout the zebrafish embryo revealed an early defect in fast muscle determination from the 7-somite stage, indicated by a loss of *myoD* in progenitor cells for fast muscles but not in the slow adaxial cells. Another MRF that is initially strongly expressed in the lateral mesoderm is *myf5* (Pownall et al. 2002). However, *myf5* expression is down-regulated in the lateral somite of wild-type embryos following somite formation, and does not drive differentiation of the fast muscle domain (Pownall et al. 2002; Groves et al. 2005), indicating that *myoD* is the primary regulator of fast muscle determination. Accordingly, no effect on *myf5* expression was observed in *c125* morphant embryos.

Interestingly, *myoD* expression in the fast muscle is known to be regulated by a different mechanism to that which controls *myoD* expression in the adaxial cells (Coutelle et al. 2001; Groves et al. 2005). In the fast muscle, RA is required for the expression of *FGF8*, which in turn is required for the expression of *myoD* in the lateral somite (Groves et al. 2005; Hamade et al. 2006). Loss of RA or *FGF8* therefore has the same effect as loss of *c125*, a specific loss of *myoD* expression specifically within the fast muscle compartment (Groves et al. 2005; Hamade et al. 2006). Therefore, *c125* appears to be a novel regulator of fast muscle determination. At present, it is unknown whether *c125* acts in a linear pathway with *RA* and *FGF8* or in a parallel pathway in the control of fast muscle formation. I have shown that *FGF8* is not upstream of *c125*, however *c125* could be upstream of *FGF8*. Future experiments testing the expression of *FGF8* and *RA* in *c125* morphant embryos will address whether *c125* acts in the same regulatory network as *FGF8* and *RA*. The fact that *myoD* expression recovers by the 13-somite stage in *c125* morphant embryos, but not in zebrafish which lack *FGF8* (Groves et al. 2005), suggest that *c125* acts in a parallel pathway during early determination of the fast muscle lineage, in conjunction with *FGF8*. It also suggests that *c125* is no longer needed for *myoD* expression after the 13-somite stage.

There is an antagonistic relationship between the fast and slow muscle determination, such that loss of one muscle type is associated with expansion of the other (Barresi et al. 2000; Ingham and Kim 2005; von Hofsten et al. 2008). Loss of *c125* and defective fast muscle determination is therefore expected to cause an expansion of the slow muscle domain. *prdm1* is an important regulator of slow myogenesis. Therefore, one expectation was that loss of *c125* results in the expansion of *prdm1* expression, although this has not been reported in *FGF8* (*ace*) mutant zebrafish. However, despite an increased intensity of *prdm1* expression, there is little ectopic expression of *prdm1* into the fast muscle compartment. It is possible therefore that *c125* also acts upstream of *prdm1* in slow muscle progenitor cells to repress the expression of *prdm1*. This would provide a mechanism whereby Hh signalling would coordinate the formation of fast muscles with that of slow muscles. The data described in this chapter also support a role for *c125* in the control of early fast muscle differentiation. In line with the fact that *myogenin* is induced by *myoD* (Pownall et al. 2002; Maves et al. 2007), there is a specific loss of *myogenin* expression within the fast muscle domain in *c125* morphant embryos. Despite these early defects in fast muscle determination and differentiation, fast muscle fibres do form eventually suggesting that other signalling pathways compensate for the loss of *c125*. However, I noticed that slow muscle migration to the surface of the somite is abnormal, as fibres appear disorganised and wavy. Defective slow muscle fibre migration could be the result of abnormal fast muscle determination or morphology. Although it is commonly thought that fast muscle differentiation occurs in the wake of slow muscle migration (Henry and Amacher 2004), there is evidence to suggest that slow muscle migration is dependent upon the normal determination and differentiation of the fast muscle domain. In embryos lacking FGF8 signalling, slow muscle fibres fail to migrate across the undifferentiated lateral somitic tissue (Groves et al. 2005).

Together, my results suggest that *c125* is required for early fast muscle determination through the regulation of *myoD* expression, which in turn controls expression of *myogenin*. *c125* also operates in slow muscle progenitor cells to restrict *prdm1* expression (see *Figure 6.35*). To test if *c125* directly regulates *prdm1* in a linear pathway, the expression of *prdm1* could be observed in zebrafish embryos that have been injected with *c125* mRNA. It would be expected that *c125* over-expression would cause down-regulation of *prdm1* expression in the adaxial cells, if it functioned to directly repress *prdm1*. Although *c125* appears necessary for normal *myoD* and *myogenin* expression in the fast muscle during the 7-13-somite stage, it is not sufficient. *myoD* or *myogenin* cannot be prematurely activated, or ectopically expressed, following *c125* over-expression. These data indicate a novel mechanism by which Hh signalling could control early fast muscle determination through the activity of *c125*. Previously, fast muscle determination and differentiation was thought to be Hh independent, yet *c125* is partly regulated by Hh signalling and appears to control fast muscle determination. Therefore, the role of Hh in the determination of slow or fast muscle fibre type may be more complex than previously thought.

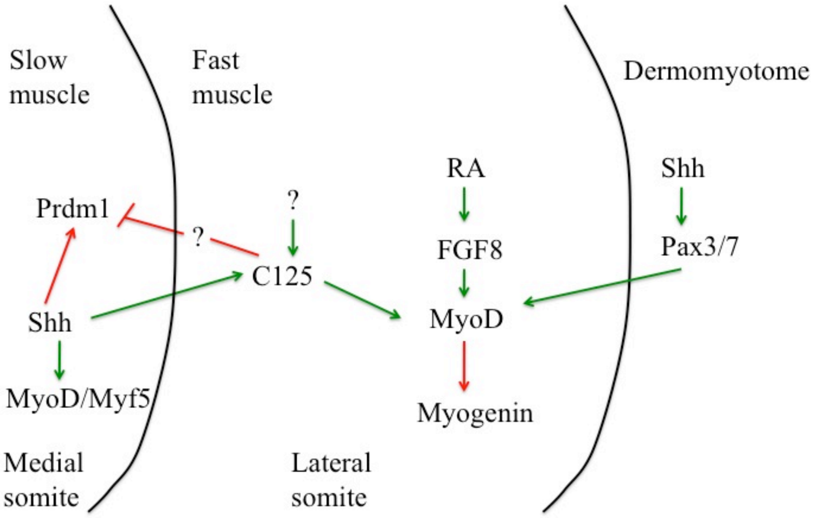

Figure 6.35: *A model to represent the role of c125 within the muscle determination and differentiation network.* By 24hpf, *c125* is excluded from the adaxial cells and is strongly expressed in the fast muscle compartment. It is downstream of Shh, and likely to be regulated by other unknown signalling mechanisms too (indicate with a question mark). *c125* is required for muscle determination through the regulation of *myoD*. It also acts to suppress the expression of *prdm1* in the adaxial cells, although it is unknown is this is through direct regulatory mechanisms. Green arrows represent signalling pathways required for muscle determination, whilst red arrows represent signalling pathways required for muscle differentiation.

6.4.5: *c125* morpholinos no longer have any detectable effects on zebrafish embryonic development

As previously stated, following my initial investigations into how loss of *c125* affects myogenesis, morpholino-mediated knock-down of *c125* stopped giving reproducible defects on *myoD*, *myogenin* and *prdm1* expression.

To try to re-establish the observable defects caused by loss of *c125*, I used a new ATG-translation blocking morpholino (*c125* ATG2), and a new splice blocking morpholino (*c125* splice ex7), and tested them at different concentrations. I used different wild-type zebrafish, and raised them at differing temperatures. None of these approaches reproduced the initial loss of *myoD* phenotype.

It is unknown why *c125* morpholino-mediated knock-down no longer produces any detectable effects. One could suggest that the phenotype was only produced in embryos obtained from a certain wild-type fish within the aquarium. However, non-specific off-target or variable effects are often associated with morpholinos (Bedell et al. 2011). Although the mechanism by which off-target effects is largely unknown, p53-dependent toxicity can consistently account for phenotypes obtained from morpholinos (Bedell et al. 2011). Co-injection of morpholino with p53 morpholino has been shown to mitigate p53 dependent toxicity (Robu et al. 2007), although I did not have the chance to assess the role of p53 in *c125* morphants before the *myoD* phenotype became unreproducible. Similarly, I did not have the chance to perform rescue experiments with *c125* mRNA before the knock-down phenotype stopped occurring.

I suggest that the defects observed with *c125* knock-down were real, as the same phenotype was produced consistently, and with three different morpholinos including both ATG-translation blocking and splice-blocking morpholinos. Both splice-blocking morpholinos effectively reduced the amount of *c125* mRNA transcripts, before (*Figure 6.16*) and after the time (section 6.5.1) at which the loss of *myoD* phenotype was reproducible. The antibody raised against chick c125 protein does not recognise c125 in the zebrafish, and so I could not assess the amount of c125 protein knock-down caused by the ATG-translation blocking morpholino.

A definitive answer as to whether *c125* functions in the determination of the fast muscle domain is now possible. In June 2012, a *c125* mutant zebrafish has been identified by the Zebrafish Mutation Project (ZMP) at the Wellcome Trust Sanger Institute. Analysis of *myoD*, *myogenin*, and *prdm1* expression in these mutant zebrafish will confirm whether the phenotype obtained from *c125* morpholino-mediated knock-down is real or not.

6.5: Appendix

6.5.1: Morpholino-mediated knock-down of *c125*

	Primer pair	WT DNA	Ex1 MO DNA
W	C125 ex1 F, C125 ex4 R	390bp	n/a
M	C125 ex1 F, C125 int1 R	0bp	789bp
	Primer pair	**WT DNA**	**Ex4 MO DNA**
W	C125 ex2 F, C125 ex6 R	481bp	n/a
M	C125 ex2 F, C125 int4 R	0bp	384bp

Primer pairs used in PCR analysis of cDNA from wild-type and *C125* morphant zebrafish embryos (splice exon 1 and splice exon 4). Predicted sizes of bands produced is shown.

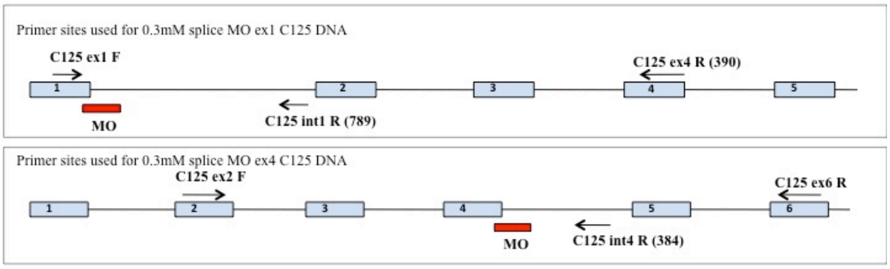

Figure 6.36: *An illustration of morpholino binding sites and the PCR primers used to check for c125 mRNA transcript knock-down*. Splice MO ex1 morpholino targets the splice donor site of intron 1, leading to the retention of this intron in the *c125* transcript, and a premature stop codon at amino acid position 47. Splice MO ex4 morpholino targets the splice donor site of intron 4, leading to the retention of this intron in the *c125* transcript, and a premature stop codon at amino acid position 166.

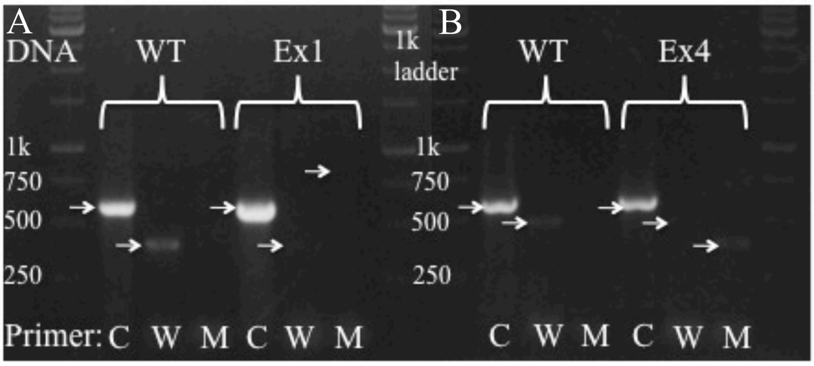

Figure 6.37: *PCR analysis of cDNA obtained from wild-type and c125 morphant embryos.* A: PCR analysis using wild-type (WT) and 0.4mM exon 1 splice morphant (Ex1) cDNA, and the primer pairs used correspond to those listed in *Figure 6.36*. WT cDNA with WT primers (W) produces a 390bp fragment, that is not detected in Ex1 cDNA. This suggests complete knock-down of *c125*. Ex1 cDNA with morphant primers (M) produces a 789bp fragment that should only be detected in Ex1 cDNA. However, this band is not observed, probably due to non-sense mediated decay. B: PCR analysis using wild-type (WT) and 0.4mM exon 4 splice morphant (Ex4) cDNA, and the primer pairs used correspond to those listed in *Figure 6.36*. WT cDNA with WT primers (W) produces a 481bp fragment, that is not detected in Ex4 cDNA. This suggests complete knock-down of *c125*. Ex4 cDNA with morphant primers (M) produces a 384bp fragment. Primer pair C in both A and B represent Elongation factor 1a control primers, which produce a 594bp fragment. This confirms the presence of cDNA in every PCR reaction ran. Expected sizes of bands produced are indicated by white arrows.