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 \textit{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 \textit{c125} there is a reduction of Hh-target genes such as \textit{nkh2.2}, \textit{dlx2} and \textit{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 \textit{c125} expression in the lens and retina is somewhat expected. However, I observed a reduced expression of \textit{c125} in these tissues. The reduction of \textit{c125} in the brain and eye therefore supports the hypothesis that \textit{shh} directly regulates \textit{c125} expression in the brain and eye. Similarly, over-expression of \textit{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). \textit{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 \textit{c125} expression are not direct, and are the consequence of altered tissue morphology in Hh-defective zebrafish embryos. Indeed, by 27hpf, \textit{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 \textit{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 \textit{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 \textit{ptc1/2} and \textit{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 \textit{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 \textit{c125} in the anterior CNS and eye, the Hh pathway could be up-regulated in embryos in which these tissues have already formed, by \textit{dnPKA} mRNA injection. In this way, the eye would still be present, and so \textit{c125} expression would not be altered due to a loss of the eye. However, Hh may have an early function in the regulation of \textit{c125} in the eye, and so a change in expression may not be detected at these later stages of development. To address whether \textit{c125} expression in the fast muscle is directly regulated by Hh signalling, the \textit{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

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>WT DNA</th>
<th>Ex1 MO DNA</th>
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<tbody>
<tr>
<td>C125 ex1 F, C125 ex4 R</td>
<td>390bp</td>
<td>n/a</td>
</tr>
<tr>
<td>C125 ex1 F, C125 int1 R</td>
<td>0bp</td>
<td>789bp</td>
</tr>
</tbody>
</table>

 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.