Chapter 7

General Discussion

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A vast amount of research has been performed to date towards understanding the complex processes that underlie muscle formation and function in vertebrates. However, further research will be required to fully comprehend how the muscle develops, especially *in vivo*. In this thesis I have described the positional cloning of the zebrafish muscle mutant *sne* to *capzα1* and determined that CapZ, in conjunction with other sarcomeric proteins, is crucial for maintaining the integrity and architecture of striated skeletal muscle.

7.1 The *sne* locus is *capzα1*

SSLP and indel markers were used to map the *sne* locus to a 0.7Mb region on chromosome 8. The primary candidate within this region was *capzα1* and sequencing of this gene identified a G-A mutation at the donor splice site of exon 9. The mutation affected splicing of the 3′ region of *capzα1* and three mis-spliced transcripts were identified in the *sne* mutant (Fig. 3.8). The mutant was phenocopied using a MO that targeted the exon 9 donor splice site (Fig. 5.8) and the phenotype of the capZα1 ATG morphant was also rescued by expression of exogenous *capzα1*-GFP (Fig. 5.22). These experiments provided conclusive evidence that the *sne* mutant phenotype is due to a mutation in *capzα1*.

7.2 The mutation in *capzα1* affects the sarcomere and myofibrillar structure of skeletal muscle

The *sne* mutant has a distinctive muscle phenotype that affects embryonic motility as well as the morphology of skeletal muscle. The motility defect is first apparent at 4 dpf and mutant embryos do not survive to adulthood, presumably as they are unable to swim effectively and therefore cannot independently source food. Immunostaining of *sne* mutant skeletal muscle

revealed that the myofibres were wavy, CapZα1 was not localized to the Z-lines and the Z-lines were misaligned between myofibrils. Moreover, the myofibrils did not appear to attach cleanly to the myoseptum (Fig. 4.16 and 4.19). Closer inspection of striated muscle architecture by TEM also indicated that, although all the sarcomeric components were present, the structure of the sarcomere was disrupted; thin filaments were splayed near the Z-line and the Z-lines were more diffuse compared to the sibling controls (Fig. 4.20).

It is evident from the morphology of the *sne* mutant that the mutation in *capzα1* does not affect the assembly of the sarcomere *per se*, but rather its integrity is lost. It is tempting to speculate that in the absence of CapZ the thin filament is not tethered securely to the Z-line. Once the sarcomere starts to contract, the link between the thin filament and the Z-line is not strong enough to withstand the forces generated and subsequently the thin filament starts to dissociate from the Z-line. Moreover, as the barbed end of the actin filament is not capped by CapZ polymerization is likely to continue, resulting in the extension of thin filament across the Z-line which in turn leads to the splayed thin filaments and diffuse, wider Z-lines that are observed in the TEM images of mutant muscle.

The C-terminus of nebulin has been shown to interact with the actin thin filament, capZ and the Z-line protein α-actinin (Chen et al., 1993; Gonsior et al., 1998; Papa et al., 1999; Witt et al., 2006). It is likely that in the absence of capZ, nebulin may act as a stabilizer for the thin filament and partially tether the thin filament to the Z-line. Therefore it may be possible that more extreme phenotypes such as complete loss or massive elongation of the thin filament are not observed in *sne* mutants due to the compensatory roles of nebulin. If thin filaments were extended due to the lack of capZ then it would be expected that the width of each sarcomere would be longer.

Surprisingly, measurements of the sarcomere width from TEM images indicated that the sarcomeres were shorter in the mutants (Fig. 4.21). This puzzling result may be due to the interaction of capZ with other components that are involved in aligning the myofibrils and linking them to the sarcolemma. One could speculate that in the *sne* mutants the smaller sarcomeres are generated as the Z-lines are unable to return to their original position within the myofibril after contraction. This in turn contributes to the myofibrillar disorganization and misaligned Z-lines which are characteristic of the *capzα1* mutant.

The misalignment of myofibrils and Z-lines has also been observed in the skeletal muscle of the obscurin zebrafish morphant (Raeker et al., 2006). Obscurin is a giant sarcomere associated protein that localizes to the Z-line and M-lines. Knock down of this protein in zebrafish produces disarrayed myofibrils as well as somite segmentation defects. This finding indicates that sarcomeric proteins are important not only in facilitating contraction but are also essential for organization of myofibrils within muscle cells. Indeed, Raeker and colleagues suggest that obscurin links myofibrils to the ECM and disruption of this linkage affects the stability and organization of the myofibril architecture and myosepta.

To demonstrate that the smaller sarcomeres are due to muscle contraction in the *sne* mutants one would need to show that the sarcomere width is normal at earlier developmental stages, prior to muscle contraction. It seems likely that muscle contraction does contribute to the *sne* muscle phenotype as inhibition of muscle use with an anesthetic (tricaine) was able to partially rescue the mutant phenotype. The actin dynamics in *sne* mutants could also be examined to determine whether actin polymerizes from the barbed end in the absence of functional capZ.

This could be performed by photobleaching of Actin-GFP expressed in *sne* mutants, in a similar fashion to the experiments that were performed in cultured muscle cells (Littlefield et al., 2001).

Characterization of the *sne* mutant phenotype has shown that CapZα1 and thus CapZ is important in maintaining the myofibrillar and sarcomeric structure of striated skeletal muscle. To date *sne* is the only vertebrate mutant of *capzα1*, therefore in depth analysis and manipulation of this mutant will no doubt provide further insight into the complex interactions that occur within striated skeletal muscle.

7.3 The *sne* allele is hypomorphic

Of vital importance to an informed analysis of *sne* has been defining whether the mutant is a complete null (i.e. where none of the aberrant transcripts are translated and the function of CapZα1 is completely abolished) or a hypomorph. CapZα1 immunostaining and MO knockdown of the various CapZ subunits has provided substantial evidence to suggest that the *sne* mutant is a hypomorph, and the aberrant CapZα1 protein product that is produced in the mutant is non-functional in its capacity to cap actin filaments at sarcomeric Z-lines.

7.3.1 The *capzα1* mis-spliced transcripts are translated in the *sne* mutant

By using a polyclonal CapZα1 antibody to detect CapZα1 in wholemount embryos I was able to establish that a mis-spliced form of CapZα1 was translated in the mutants (Fig. 4.19). However, the distinctive striated pattern seen in normal muscle tissue was absent and positive staining was instead observed in aggregations adjacent to the myoseptum . This result indicated that the mutant form of CapZα1 is unable to localize to the Z-line and cap the barbed end of the actin filament in skeletal muscle. There are a number of possible explanations for this finding:

Firstly, the mutant isoform may not be able to dimerize with the β subunit, therefore no functional CapZ is produced and no capping takes place. Secondly, CapZα1 is still able to dimerize with the β subunit but cannot bind to actin. Thirdly, CapZ dimerizes with the β subunit and is able to bind to the barbed end of actin, however, it is unable to localize to the Z-line and therefore cannot cap the barbed end of the actin filament in skeletal muscle. In light of the MO analysis discussed below the first possibility appears to be the most feasible.

7.3.2 Knockdown of CapZ subunits suggests that the *sne* mutant protein is functional in non-muscle cells

Complete ablation of CapZα1 expression by injection of the capZα1 ATG and splice 1 MOs into 1-2 cell stage embryos resulted in a more severe defect than was observed in the *sne* mutant (Fig. 5.2-5.5). This finding implies that the mutant form of CapZα1 has some function, particularly in the early stages of development prior to muscle differentiation. It would also suggest that the aberrantly spliced CapZα1 must be able to dimerize with a β subunit and cap the actin filaments in non-muscle cells. If this is indeed the case then why does the mutant form of CapZα1 only affect skeletal muscle tissues? One explanation for the different phenotypes observed between the *capzα1* mutant and morphant is perhaps that CapZα1 is able to bind to more than one β subunit. Therefore, this may result in the formation of CapZ isoforms that localize to unique sub-cellular locations within different tissues. In cardiomyocytes of other vertebrates the β subunits are differentially localized. The β1 subunit is located at Z-lines and the β2 subunit is localized to the intercalated disc and cell to cell junctions (Hart and Cooper, 1999; Schafer et al., 1994). Due to their different locations within the cardiomyocytes the two isoforms of CapZβ are thought to have distinct functions *in vivo*. If more than one β subunit exists in zebrafish and they are differentially localized, one could speculate that the CapZα1 mutant

isoforms are able to bind to the β2 subunit and function early in development. However, are unable to dimerize with the β1 subunit located at the Z-line in striated muscle, resulting in the muscle specific defects observed in the *sne* mutant. This theory is supported by studies that recombinantly generated a capping protein comprised of the mouse α1 and β2 subunit, and showed that this isoform can bind to Twinfilin and CARMIL (Palmgren et al., 2001; Yang et al., 2005). Both these proteins are thought to be important in regulating non-sarcomeric actin filament dynamics. This finding supports the hypothesis the different α and β subunits can dimerize to form capping proteins that have distinct functions within muscle and non-muscle cells.

Different levels of CapZβ knock down also suggest that more than one isoform of CapZ exists in zebrafish. Additionally, they indicate that the *sne* mutant forms of CapZα1 are unable to bind to the β subunit expressed in skeletal muscle. Low doses of both the capZβ splice and ATG MOs produce morphants that have a very similar phenotype to the *sne* mutant (Fig. 5.17 and 5.18). Moreover, wholemount CapZα1 antibody staining revealed that CapZα1 was mis-localized to the myoseptum in these morphants. These results illustrate that CapZβ is required for the correct localization of these capping proteins; in the absence of CapZβ, although functional CapZα1 is produced, it is unable to dimerize with the β subunit to form CapZ, and therefore it cannot localize to the Z-line and cap the barbed end of the actin filaments.

Complete knock down of the CapZβ subunit using high doses of the capZβ ATG MO (which would be able to knock down the CapZβ1 isoform if it exists) produced a severe phenotype similar to the knock down of both the α subunits with the capZα2 ATG MO (Fig. 5.19). This phenotype supports the previous findings that the *sne* mutant must produce some form

of functional CapZ and confirms that severe defects are induced in the complete absence of CapZ, either by knockdown of all α or β subunits. It also verifies the importance of CapZ in the early stages of development, long before sarcomere assembly. Indeed, similar findings were observed in a *Drosophila capzβ* mutant (Hopmann et al., 1996), where the generation of a complete null resulted in lethality in the early larval period, indicating that CapZ is essential in the early stages of development.

7.3.3 The crystal structure of CapZ indicates that the C-terminal region of CapZα1 is important in actin binding and dimerization to the β subunit

The crystal structure of chicken CapZ (α1 and β1) has been published (Yamashita et al., 2003) and provides further support for the proposal that the CapZα1 mis-spliced products may not able to dimerize with CapZβ and cap the end of actin filaments. Analysis of the crystal structure indicated that the C-terminal region of CapZα1 is critical for actin binding and dimerization with the β subunit. Additionally, Yamashita and colleagues have also suggested that the C-termini of the CapZα1 and CapZβ1 subunits are mobile and this enables the C- terminal region to dock to the end of the actin filament. I*n vitro* studies have subsequently confirmed that the capping protein caps the barbed end using the mobile C-termini of the α1 and β1 subunits (Wear et al., 2003). Moreover, the C- terminal of the α1 subunit was found to contribute more to capping affinity and kinetics than the C- terminal of the β subunit. *In vivo* studies performed in yeast also indicate that the C-terminal region of CapZα (residues 259-286) is crucially required for actin binding (Casella and Torres, 1994; Hug et al., 1992; Sizonenko et al., 1996).

By comparing the surface area that is exposed between each subunit, the crystal structure of CapZ also revealed that interactions between the α1 and β1 subunit occur mainly between C-terminal domains. 74% of the regions that come into contact between the subunits are buried between the C-terminal domains and only 19% are buried between the N-terminal regions. The interactions between the C-termini are likely to be very important in dimerization of the α and β subunits.

In silico translation of all mis-spliced transcripts expressed in the *sne* mutants revealed that the C-terminal domain of CapZα1 would be disrupted (residues 240-286). Out of the three mis-spliced transcripts observed in the mutant, only one would be unable to translate the critical C-terminal domain. The other two transcripts were predicted to still produce the C-terminal domain, however, it is entirely feasible that the deletion and insertion of amino acids in these mis-spliced products could affect the stoichiometry of this region. As shown in chapter 4 the C-terminal region is highly conserved between vertebrates (Fig. 4.1) and therefore it is possible that even a slight change in the C- terminal domain may affect the ability for the α1 subunit to dimerize to the β subunit.

Although the MO knockdowns and immunostaining analyses have provided the initial evidence that the *sne* allele is hypomorphic, further experiments are required to confirm the functional capability of the mis-spliced CapZα1 isoforms translated in the mutant. Firstly, the predominant mis-spliced CapZα1 isoform that is translated in the mutant needs to be established and its function in terms of its ability to bind to the β subunits needs to be determined. Secondly, the existence, function and localization of a CapZβ1 subunit in zebrafish need to be verified. Finally, experiments would need to be performed that show that the α and β subunits could

combine in different ways to produce isoforms of CapZ that have different functions. Identifying how the mutation in *capzα1* induces the *sne* mutant phenotype on a molecular level will be invaluable in discerning the functions of the different CapZ subunits, and will no doubt contribute to defining the exact role of CapZ isoforms in early vertebrate development.

7.4 Potential roles for the α2 and β subunits of CapZ

7.4.1 CapZα2

MOs were designed against the α2 subunit of CapZ to determine if there was any redundancy between the two α subunits. Due to their high homology, the capZα2 ATG MO knocks down both the α1 and α2 isoforms (Fig. 5.14). Despite this, injection of this MO still produced an informative phenotype. Very small doses of capZα2 ATG MO severely delayed embryonic development and by 24 hpf many embryos had truncated axes, U-shaped somites and smaller brains (Fig. 5.11 and 5.12). As this phenotype is more severe than knock down of CapZα1 alone, it would suggest that complete ablation of CapZ severely affects development of the embryo and the α2 subunit may play a more important role in earlier stages of development than the α1 subunit. Indeed, the *capzα2* RNA *in situ* expression pattern is equivalent to the *capzα1* expression pattern until the 13-15 somite stage. However, by 24 hpf *capzα2* expression is more widespread and diffuse throughout the embryo than *capzα1*, which is strongly expressed in the somites (Fig. 4.11 and 4.12). Intriguingly, in adult mouse skeletal tissue the RNA and protein levels of the α2 subunit are much greater than the α1 subunit (Hart et al., 1997b). This finding contradicts the expression pattern I observed, however, adult tissues may have different levels of the α subunits compared to embryos.

In zebrafish, the expression pattern and double knockdown of the CapZα subunits indicate that CapZα1 has a more prevalent role in capping actin filaments within the sarcomeres of the skeletal muscle, while CapZα2 may be more important in capping actin filaments that play other essential roles within all cells. Surprisingly, no phenotypic defects were observed in the capZα2 splice morphant, therefore, assuming that the splice MO is actually knocking down CapZα2, this finding may suggest that the α1 subunit is also able to compensate for the lack of the α2 subunit throughout development. Of course there is still the possibility that the splice MO is not effective and confirmation of its function by RT-PCR is necessary.

Further characterization of the α2 subunit is required to determine its exact function in development. A MO specifically designed against a unique site of the *capzα2* 5′ UTR will be useful in determining whether loss of CapZα2 alone produces a severe defect, as is seen in the double knock down of the α subunits, or whether there is some redundancy shared with the α1 subunit. Defining the *in vivo* localization of the α2 subunit will also assist in unraveling its function in zebrafish development and this could be achieved by making a GFP fusion construct. The fusion constructs for both the subunits could subsequently be co-injected with different α1 and α2 MOs to determine whether exogenous CapZα2 can rescue the phenotype of the capZα1 morphant or vice versa.

7.4.2 CapZβ

The other key element in the function of CapZ is the β subunit. Without α and β subunit dimerization CapZ is not produced. As mentioned previously, the β1 and β2 isoforms display tissue specific expression and localization: the β1 isoform localizes to the Z lines and the β2 subunit localizes to the intercalated disc and cell to cell junctions in chicken cardiac muscle and

striated muscle cells (Schafer et al., 1994). Over expression of the β subunits in the cardiomyocytes of transgenic mice indicated that each subunit is distinctly localized *in vivo* and each isoform is unable to compensate for the other (Hart and Cooper, 1999). RNAse protection assays found that in skeletal muscle there was a much higher expression of the β1 isoform than the β2 isoform (Schafer et al., 1994) and this was also verified by 2-D immunoblots (Hart et al., 1997b). In zebrafish I was only able to identify the *β2* isoform, however, due to the unique function of each the β subunits observed in other vertebrates it seems likely that an alternate isoform of *capzβ* is expressed in zebrafish. Genomic sequencing of the intronic region encoding the exon that makes the *β1* isoform unique will be required to establish whether zebrafish do indeed express more than one *capzβ* subunit.

Low doses of capZβ ATG or splice MO produce morphants with similar phenotypes to the *sne* mutant (Fig. 5.17 and 5.18). It could be speculated that only the muscle phenotype is observed in these morphants as the MO does not completely knock down all CapZβ. Therefore in the early stages of development there is enough CapZβ for CapZ to function normally, however, upon muscle differentiation these levels are not sufficient and subsequently gives rise to a phenotype similar to the *sne* mutant.

Intriguingly, in addition to the muscle defect, the edges of the caudal fin were frayed in 5 dpf capZβ ATG morphants (Fig. 5.17). It is uncertain as to why this phenotype is only observed in the ATG morphant and not in the splice morphant but may be due to differences in effectiveness of each MO. Zebrafish embryos possess two types of fin, the caudal fin and the pectoral fins, that are composed of epithelial and mesenchymal cells. Very little is known about the development of the caudal fin, however, DiI labeling was used to show that early caudal fin

bud mesenchyme is derived from trunk neural crest cells (Smith et al., 1994). Neural crest cells are precursors to a wide variety of cell types and develop from the edge of the neural ectoderm before migrating to various regions within the embryo. In addition to the caudal fin these cells give rise to most of the neurons and glia of the peripheral nervous system, craniofacial cartilage and bone, pigment and smooth muscle (Christiansen et al., 2000; LaBonne and Bronner-Fraser, 1999; Le Dourain and Kalcheim, 1999). It could be speculated that the loss of CapZβ disrupts the migration of neural crest cells. This hypothesis is supported by the fact that defects the jaw, another neural crest derivative, is observed in capZβ morphants. Actin dynamics is essential for the formation of filopodia and thus cell movement, therefore it is possible that a depletion of CapZ may affect the movement of neural crest cells. In future experiments it may be worth exploring whether defective neural crest migration does indeed give rise to the caudal fin and jaw phenotype in these morphants.

Interestingly, the defective caudal fin phenotype was also observed in the *capzβ* retroviral insertion mutant (Amsterdam et al., 2004). The insertion in this mutant is located immediately after the start site and correlates with the capZβ ATG MO target site. No muscle phenotype was described in the *capzβ* mutant, however, close inspection of the muscle may have been overlooked as the images provided do suggest that their may be a defect in the muscle. Acquisition of this mutant line will assist in the further analysis of CapZ in zebrafish muscle and caudal fin development.

7.5 CapZ regulates thin filament assembly and integrity with nebulin and tropomodulin

One of the major mysteries in sarcomere assembly has been how the actin thin filament is able to precisely polymerize into uniform lengths within the sarcomere. For a number of years a model in which nebulin acts as a ruler for defining thin filament length has been widely accepted. Additionally, the capping proteins capZ and Tmod are also thought to be involved in maintaining the length of the actin filament. However, the exact mechanisms and interactions between these three components that results in the formation of uniform actin polymers is still not completely understood.

In collaboration with Elisabeth Busch-Nentwich the effect of loss of one or more of these components on thin filament formation and stability was examined during zebrafish muscle development. The results indicate firstly that loss of CapZ or Nebulin alone does not affect Z-line integrity or formation, however, loss of function of both these proteins destabilizes the Z-line and therefore suggests that at least CapZ and Nebulin are required for maintenance of Z-line integrity. Moreover, in the absence of CapZ and Nebulin thin filament does not form in skeletal muscle indicating that CapZ and Nebulin are required for actin filament stability (Fig. 6.4 and 6.6). Secondly, double knock down of CapZ and Tmod4 results in loss of Z-lines and almost completely ablates thin filament formation (Fig. 6.4). This indicates that both proteins are required for nucleation of sarcomeric actin filament. Loss of only Tmod4 results in severe depletion of actin filament compared to loss of only CapZ (Fig. 6.2), which confirms previous studies that suggest that nucleation of actin polymerization mainly occurs from the pointed end (Littlefield et al., 2001). Interestingly, nemaline bodies similar to that of the *nebulin* mutant (*buf*) were observed in the absence of Tmod4. Thirdly, ablation of Tmod4 and Nebulin exacerbates the

formation of nemaline bodies and as a result the sarcomere structure is severely disrupted, however, fragments of thin filament are still observed (Fig. 6.4). Finally, loss of CapZ in either the *nebulin* mutant or the Tmod4 morphant abolished the formation of nemaline bodies and implies that CapZ is involved in the formation of nemaline bodies (Fig. 6.4).

The findings from these knock down experiments illustrates the importance of CapZ, Nebulin and Tmod in maintaining the integrity and stability of the sarcomere. These thin filament associated proteins act in concert to define and maintain the length of the actin filament within striated muscle. However, a number of questions have arisen from these studies such as how are nemaline bodies formed and why do nemaline bodies only form in Tmod4 morphants and *nebulin* mutants? How does the loss of CapZ affect their formation? How are Nebulin and CapZ able to stabilize the thin filament and the Z-line? By comparing the knock down and mutant phenotypes that have been generated, in combination with what is already known about these thin filament associated proteins, it is possible to provide some explanations as to how Nebulin, Tmod and CapZ are involved in maintaining the integrity of the sarcomere and will be discussed herein.

In both zebrafish and mouse *nebulin* mutants the thin filaments still form, however, the length of the filaments is affected (Bang et al., 2006; Witt et al., 2006). Both these vertebrate mutants support the widely accepted theory that Nebulin is required to precisely define the length of the actin filament. Nebulin has also been shown to increase the affinity for Tmod to cap the pointed end of the thin filament (McElhinny et al., 2005).Therefore it is likely that in the absence of Nebulin, Tropomyosin may be able to assist in elongation of the thin filament to the approximate length, however, as the actin filament is partially uncapped at the pointed end aberrant elongation of the thin filament occurs at this end. Although the assembly of the

sarcomere is not grossly affected in zebrafish *nebulin* mutants, it could be speculated that the integrity of the sarcomere is lost once the muscle starts to function due to the longer actin filament. Witt and colleagues (2006) have proposed that the longer actin filament slips and extends further into the Z-line than it should, thus more α-actinin is recruited to the Z-line to link the extra thin filaments and eventually gives rise to nemaline bodies.

It has been well established that CapZ stably caps the barbed end of the thin filament and is thought to tether the thin filament to the Z-line. As previously mentioned from analysis of the zebrafish *sne* mutant, it has been hypothesized that loss of CapZ reduces the strength of the link between the thin filament and the Z-line. Sarcomere assembly still proceeds as Nebulin may be able to compensate for the lack of CapZ and partially cap the barbed end of the filament and secure it to the Z-line. However, as soon as muscle contraction begins, the link between the Z-line and the thin filament is not strong enough to endure the force generated resulting in loss of sarcomeric integrity. When both CapZ and Nebulin are depleted the thin filament is completely de-stabilized. Even though the actin filament should still nucleate from Tmod, at least CapZ or the C-terminus of Nebulin are required for the filament to become stable. It could be that the loss of the thin filament in these double loss of function embryos causes the Z-line to collapse: As there is no thin filament for α-actinin to cross link with, the Z-line becomes unstable and therefore disintegrates. This interpretation is supported by Tmod4 and CapZ double knockdowns, which also lack thin filaments and Z-lines.

In the Nebulin and Tmod4 double loss of function embryos thin filaments were still observed even though the sarcomere had become disorganized (Fig. 6.4). In these mutants CapZ is still present and therefore in theory a small amount of actin filament polymerization should be

able to occur, however, without the other capping protein (Tmod) or Nebulin to define the other end of the thin filament the lengths would be random. Once the muscle starts to contract, as speculated in the *nebulin* mutant, the actin filaments slip into the Z-line leading to the accumulation of α-actinin and thus the production of dense Z-line bodies. As these Z-bodies enlarge they disrupt the sarcomere structure and the titin cross-links are pulled apart, leading to Z-line disintegration and disorganization of the sarcomere.

Loss of Tmod4 on its own produces a similar phenotype to the *nebulin* mutant, however, the phenotype is more severe, probably because nucleation of the thin filament occurs predominantly from Tmod (Fig. 6.2). Those filaments that do form will be longer than normal as Nebulin cannot cap the actin filament. Therefore it is proposed that during muscle contraction, as in the *nebulin* mutant, the elongated filaments will be pushed into the Z-line and attract the accumulation of α-actinin which tries to cross-link the extra thin filament, thereby giving rise to nemaline bodies.

The lack of nemaline bodies in the Tmod4 and CapZ double knockdown, and Nebulin and CapZ loss of function embryos may be due to the loss of thin filament that in turn results in the loss of the Z-line, rather than a direct consequence of CapZ itself. As no thin filaments are produced in these double mutants α-actinin cannot cross-link either the thin filaments to each other or to titin and the Z-line is destabilized. Therefore nemaline bodies cannot form, unlike in the single mutants or the Tmod4 and Nebulin double loss of function embryos where thin filaments were observed. Although CapZ has been shown to interact with α-actinin it is unlikely that it is responsible for α-actinin localization to the Z-line, as in *sne* mutants α-actinin can localize to the Z-line in the absence of functional CapZ.

A number of key experiments are still required to support the role of CapZ, Nebulin and Tmod in sarcomere integrity and in the formation of nemaline bodies. The phenotypes of the double mutants must be examined at much earlier stages of development to confirm that the sarcomere assembles, and that its integrity is compromised due to the lack of the thin filament associated proteins. Conclusive evidence is required to establish that in Nebulin and Tmod4 double loss of function embryos the thin filaments are longer. The localization of CapZ in the mutants that form nemaline bodies will also assist in ruling out the possibility that CapZ directly induces nemaline body formation.

Studies in various model organisms have investigated how thin filament associated proteins regulate thin filament formation and integrity. Many of the results support the findings that we have observed from the knockdowns of these proteins in zebrafish, however, unlike our experiments most studies have only focused on Tmod or Nebulin. The findings of these studies compared to the results obtained in zebrafish are discussed herein.

Most of the research performed on Nebulin has shown that it is important in precisely defining the length of the thin filament. However, there is conflicting evidence in the literature regarding whether loss of Nebulin increases or decreases actin filament length and whether it is involved in assembly of the sarcomere. In 2006 two different laboratories generated nebulin knock out mice (Bang et al., 2006; Witt et al., 2006). Both sets of researchers produced knock out mice with very similar phenotypes and concluded that a lack of Nebulin results in shorter thin filaments. Witt and colleagues (2006) also observed that about 15% of the Z-lines were abnormally wide and nemaline rods had formed. They speculated that the nemaline bodies form

as actin filament capping by CapZ is perturbed upon loss of Nebulin. Therefore thin filaments polymerize into the Z-line and trigger the recruitment of more Z-line proteins, resulting in widening of the Z-lines. Our model of nemaline body formation, however, suggests that the thin filament aberrantly extends from the pointed end rather than the barbed end, from a lack of Nebulin. This theory is consistent with studies that have shown that CapZ is far more stable at capping the actin filament than Tmod, so it seems far more likely that Tmod capping is perturbed from the lack of Nebulin. Additionally, the interpretation that longer thin filaments exist in the muscle of these mutants may not be correct. To observe the structure of mouse muscle the muscle tissues are first isolated and then stretched. Therefore it is possible that the stretching of the muscle induces slight changes in the muscle architecture that are not representative of the *in vivo* structure. Indeed, in the previous year McElhinney and colleagues (2005) used siRNA to knockdown Nebulin in cultured rat cardiomyocytes and found that the filaments were longer at the pointed end. They also showed that striated thin filaments, Z-lines, Titin and thick filaments failed to assemble in fetal skeletal myocytes depleted of Nebulin and concluded that Nebulin plays a role in sarcomere assembly. Although longer filaments are observed in the zebrafish *nebulin* mutant, both mouse and zebrafish mutant models are still able to assemble sarcomeric components and do not support these latter conclusions. As these knockdowns were done in cultured myocytes they may produce very different outcomes to experiments performed *in vivo*.

Many studies on Tmod have been based in cultured cardiomyocytes and therefore have looked at Tmod1. It has been shown that overexpression of *tmod* in cardiomyocytes results in shorter actin filaments (Littlefield et al., 2001; Sussman et al., 1998). This result was replicated in *Drosophila* by transient overexpression of the *tmod* homologue *sanpodo* in indirect flight muscles (Mardahl-Dumesnil and Fowler, 2001). Conversely, reduced expression of *tmod* in

cardiomyocytes resulted in thin filament elongation. (Sussman et al., 1998). All the studies to date support a model for Tmod in regulating thin filament length by preventing polymerization and depolymerization. Recently a Tmod1 mouse knock out was generated, however, the null mice die due to heart abnormalities at E10-10.5. (Fritz-Six et al., 2003), and therefore detailed analysis of myofibril assembly and maturation could not be assessed. As an alternative to the mouse mutant, Tmod1 mouse knock out ES cells were used to examine the loss of Tmod1 in cardiomyocytes derived from embryoid bodies (Ono et al., 2005). In these embryoid bodies the sarcomere assembles normally in the null myofibrils, however, surprisingly, the actin thin filaments still have uniform lengths. These findings may be unique to embryoid bodies as they completely contradict the previous studies mentioned. Further research is therefore required to reach a consensus on the function of Tmod in regulating thin filament assembly *in vivo*.

Despite the discrepancies in the different loss of function models for Nebulin and Tmod, most of these models still support the hypothesis that Nebulin acts as a template for actin polymerization (presumably through its internal super-repeats) as well as being able to assist in terminating the end regions (McElhinny et al., 2001; Witt et al., 2006) by interacting with Tmod and CapZ. The different mutant models generated will no doubt prove to be useful tools in ultimately determining how the interactions between Nebulin, Tmod and CapZ define thin filament length.

The results from the zebrafish double loss of function experiments may also provide further insight on how the sarcomere assembles *in vivo*. As outlined in the introduction the latest model for myofibrillogenesis is the Sanger premyofibril model. This model proposes that myofibrillogenesis begins with the formation of premyofibrils consisting of I-Z-I bodies that

interdigitate with non-muscle myosin II. Titin and muscle myosin are then expressed and titin and the thin filament in I-Z-I bodies are responsible for integrating and aligning muscle myosin filaments within the sarcomere. In light of our double knockdown studies (CapZ and Tmod4 or CapZ and Nebulin double loss of function embryos) the loss of thin filament does not affect the organization of the thick filaments (Fig. 6.4). One of the most striking and unexpected results is that thick filaments are still positioned in an ordered block within the sarcomere in the absence of thin filaments. This indicates that the thin filament is not required for the incorporation and alignment of thick filaments within the sarcomere. Clearly the sarcomere is non-functional without thin filament and therefore it is likely that the thick filaments eventually disassemble in these double loss of function embryos. This phenomenon was also observed in *ex vivo* experiments on sea bass muscle. When the extracted muscle was incubated with proteases that ablated Z-lines or thin filament, the thick filament was still arranged in ordered blocks within the sarcomere after 24 hpf (Taylor et al., 1997). Of course the results described may not truly reflect what would happen *in vivo*, however, it does support the hypothesis that titin and M-line proteins may be more important in the assembly and maintenance of the thick filament than thin filament components.

The assembly and maintenance of myofibrils and sarcomeres is an extremely complex process and involves many proteins that cooperate together to make up a functioning muscle cell. It is evident that there is still intense debate on exactly how the sarcomere assembles and functions, and a consensus has still not been reached on the function of many sarcomeric proteins. Although *in vitro* studies have been invaluable in defining certain aspects of striated muscle assembly, further *in vivo* studies will be vital in gaining a greater understanding of muscle assembly and maintenance in vertebrates. Zebrafish are excellent for further investigation in this

field and there are now a number of mutants available that are defective in sarcomeric proteins. Further analysis of zebrafish muscle mutants will no doubt be beneficial to our understanding of vertebrate myofibrillogenesis and may also assist in unraveling the molecular nature of human myopathies.

7.6 CapZ and the intermediate filament protein desmin

Desmin is one of the earliest known myogenic markers that is expressed in both cardiac and skeletal muscle. Along with other costameric and intermediate proteins it is thought to be important in transducing the force generated by the sarcomere across the entire length of the myofibril and also between the sarcomere and the sarcolemma. Desmin is attached to the sarcomere at the Z-line and has previously been shown to bind to α-actinin and nebulin (Bang et al., 2001), however, a direct interaction with capZ has not been identified. Intriguingly the targeted mouse knock out of desmin produced a very similar phenotype to the *sne* mutant; TEM of different muscle tissues indicated that the early stages of muscle formation were normal, however weight bearing muscles such as the soleus and diaphragm lost their structure over time and the pathology worsened from increased muscle usage. The muscle structure degenerated and lateral alignment of myofibrils was observed (Li et al., 1997). Due to the striking similarity between the zebrafish *capzα1* mutant and the mouse desmin knockout I wanted to investigate whether CapZ and Desmin acted together to maintain the structural integrity of the muscle in zebrafish. As predicted the knock down of Desmin in zebrafish produces a very similar phenotype to the mouse mutant and the zebrafish *sne* mutant, characterized by wavy fibrils that were misaligned within each somite (Fig. 6.8). Intriguingly, antibody staining of Desmin in the *sne* mutants showed that protein levels were greatly diminished and, unlike in wild type controls, Desmin staining was absent in the myofibrils and greatly reduced in the myoseptum (Fig. 6.11).

This result clearly demonstrates that there is some connection between CapZ and Desmin, however, the exact molecular mechanisms behind this result are still unknown and many more questions need to be addressed such as: Does CapZ regulate Desmin expression or is it required for Desmin to localize to the Z-lines and the myoseptum? Moreover, does CapZ directly interact with Desmin and stabilize its attachment to the Z-line? It is tempting to speculate that the loss of CapZ at the Z-line disrupts localization of Desmin and therefore lack of this filament protein contributes to the misalignment of Z-lines between myofibrils and the gradual disintegration of myofibrillar organization upon muscle function. Further experiments will therefore be required to uncover the true nature of the interaction between CapZ and Desmin.

7.7 CapZ and motoneuron development

The involvement of CapZ in motoneuron development was investigated following the finding that the neurally expressed protein V-1 binds to CapZ (Taoka et al., 2003). Interestingly, knockdown of CapZα1 by using the ATG MO resulted in the formation of additional axonal exit points from spinal cord motoneurons at 4 dpf (Fig. 6.14). This finding indicates that CapZ is involved in axonal projection. Indeed, the movement of the growth cone at the tip of the axon relies on actin based motility and is guided along specific pathways by cues emitted from the surrounding tissues. Guidance molecules include netrins, semaphorins and slits, which act as attractants and repellants in defining the path of the axon (Dickson, 2002). They achieve this by regulating actin dynamics at the barbed end and thus affect the formation of filopodia in the growth cone. Additional axonal exit points have also been observed in the zebrafish mutant *sidetracked*. This mutant was found to have a mutation in *plexin A3*, a receptor for semaphorin guidance molecules (Palaisa and Granato, 2007).

Proteins that affect actin dynamics may be regulated by the guidance molecules. One example is the Arp2/3 complex which is a regulator of CapZ. The Arp2/3 complex consists of Arp (actin related protein) 2 and 3 along with five other subunits Arpc1-5 (Machesky et al., 1994). It is activated by a member of the WASP family (Wiscott Aldrich Syndrome Protein) and nucleates new actin filament assemblies. In addition to creating a branched network of filaments it can also cap the pointed ends of actin filaments (Mullins et al., 1998).One could speculate that loss of CapZ affects the correct formation of filopodia as the growth cone is unable to respond to the guidance cues, therefore the axons project aberrantly. It will be interesting to determine whether the axonal projection defect is also observed in other neurons at earlier stages of development.

7.8 Conclusions

In this thesis I have described the positional cloning and characterization of the zebrafish muscle mutant *sne*. The *sne* allele is hypomorphic and has a mutation in *capzα1*, which induces a muscle specific phenotype. Analysis of this mutant indicates that CapZ plays an important role in myofibrillar and sarcomeric integrity. Moreover, knockdown of the other subunits of CapZ suggest that different isoforms of CapZ have different roles and are required at very early stages of development. CapZ, Nebulin and Tmod double loss of function experiments illustrate the importance of these proteins in collaboratively instigating actin thin filament assembly and maintenance *in vivo*. Further research into these proteins in zebrafish will no doubt broaden our understanding of how the thin filament is maintained in skeletal muscle, and may also establish how the sarcomere assembles in vertebrates.

The various phenotypes of the capZ subunit morphants have revealed additional roles for CapZ in interaction with the costameric network and its importance in cell motility. To extend our knowledge on the roles of CapZ in zebrafish development, it will be essential to establish the existence of all isoforms, their localization and whether they have non-overlapping functions. The results presented in this thesis indicate that CapZ is involved in many aspects of development. Further characterization of this complex will not only provide insight into how CapZ maintains the muscle architecture but will also be necessary to unravel the additional roles of CapZ in vertebrate development.