# Chapter 1

## Introduction

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Muscle is one of the most extensively studied tissues in biological and medical science. Our fascination with how muscle is responsible for movement in humans and other animals has existed for thousands of years. Erasistratus (315-240BC) was one of the first Greek practitioners to examine the structure of skeletal muscle. He proposed that muscle was like an inflatable bag surrounded by inextensible but flexible cords. A few hundred years later another Greek physician, Galen, came to prominence and his findings on the anatomy of various mammals were widely publicized. However, it was not until the sixteenth century that a complete investigation on the anatomy of the human body was performed by the Belgian physician, Andreas Vesalius. In 1543 he published one of the landmark books in the history of medical science, De Humani Corporis Fabrica (On the Structure of the Human Body), which proved to be extremely influential in inspiring further research on many different organs of the body, including muscle. Today muscle research is carried out in a wide range of species from invertebrates, such as *Drosophila*, to mammals. Recently, the teleost *Danio rerio* (more commonly known as the zebrafish) was discovered as an ideal model organism for studying many aspects of development, including myogenesis.

In this chapter I begin by introducing the different muscle types that exist in vertebrates and highlight some common human myopathies that arise from mutations in muscle genes. I then explain why zebrafish is an ideal model organism for studying muscle development and myogenic disorders, before describing how mesoderm eventually gives rise to differentiated skeletal myocytes in the zebrafish embryo. Finally, I describe the main components of striated muscle, in addition to the mechanics of actin thin filament and sarcomere assembly.

## 1.1 General overview of muscle

### 1.1.1 Muscle types

The principal function of muscle is to convert chemical energy into mechanical energy. The force generated from this process is essential for the function of multiple internal organs and body movement in all animals. In humans, almost half of the total body mass is comprised of muscle tissues that are involved in a variety of functions such as regulating organ volume, generating heat and moving fluids and nutrients through various systems. In vertebrates, muscle is classified into three types; smooth, skeletal and cardiac. Smooth muscle is made of short, spindle shaped fibres packed together in layers (Fig. 1.1A) and is found in the walls of internal organs such as the intestines, blood vessels and stomach. Skeletal muscle is composed of bundles of long, almost cylindrical shaped fibres that are multinucleated and are attached to the skeleton (Fig. 1.1B). Skeletal muscle is composed of two fibre types: slow (Type I) and fast (Type II). Slow twitch fibres contract slowly and have a higher resistance to fatigue than fast twitch fibres which are used for short bursts of speed and power. Cardiac muscle is located in the walls of the heart and is made of short interconnecting fibres that are mononucleated (Fig. 1.1C). Although the structure of skeletal and cardiac muscle fibres are not identical, they are both striated (i.e. the fibres contain alternating light and dark bands (striations) that are visible under a light microscope). These striations are due to the ordered arrangement of sarcomeres that make up all cardiac and skeletal muscle fibres.

Striated muscle cells are composed of a multitude of interconnected components that form the complex dynamic framework which is integral to muscle function. The bulk of each muscle cell (myotube) is comprised of bundles of myofibrils which are made up of highly ordered arrays of sarcomeres that are the basic contractile units within striated muscle (Fig. 1.2). The myofibrils

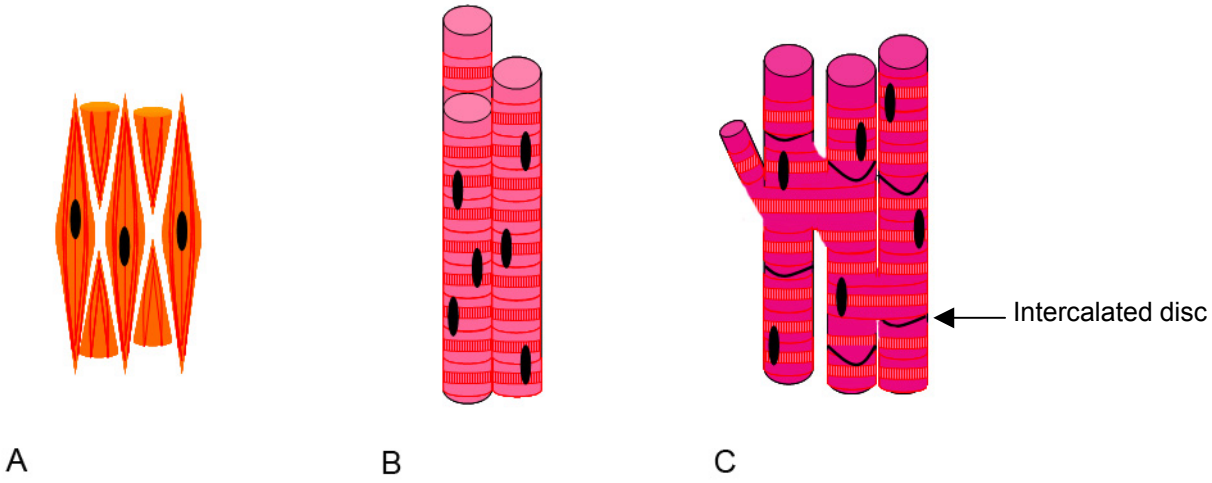


Fig. 1.1. Illustration of smooth, skeletal and cardiac muscle fibres. A) Each smooth muscle fibre is smaller than a cardiac or skeletal fibre and contains a single, centrally located nucleus. B) The skeletal muscle fibre has a very long and roughly cylindrical shape. Fibres run parallel to each other within the muscle and are multinucleated. C) Cardiac fibres have a similar shape to skeletal muscle fibres, however, are also branched and tend to join with each other end to end via intercalated discs (transverse thickenings of the membrane), which are unique to cardiac muscle. Additionally, each muscle fibre tends to be mononucleated. Cardiac and skeletal muscle fibres are composed of sarcomeres (the basic contractile unit) which give rise to the striated pattern observed in these fibres. Smooth muscle is not made up of sarcomeres and thus is not striated. However, it does contains the same elements for contraction i.e. myosin and actin filaments, but they are not regularly organized.

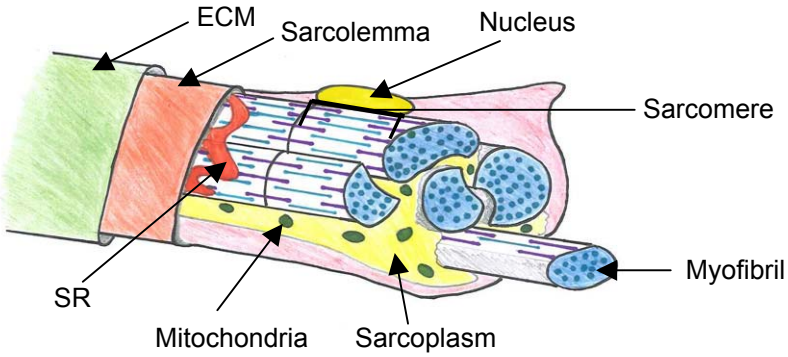


Fig.1.2. Schematic of a stereotypical striated muscle fibril. The striated fibril is composed of many bundles of myofibrils that are surrounded by a specialized plasma membrane (sarcolemma) and an overlaying ECM. The sarcoplasm (muscle cell cytoplasm) that surrounds the myofibrillar bundles contains the primary energy source (glycogen) for muscle contraction. Additionally, it is composed of organelles such as nuclei, the golgi apparatus, mitochondria and sarcoplasmic reticulum (SR). The SR is a fluid filled system of membranous sacs that encircle each myofibril and is the main store of calcium ions. Each myofibril is composed of repeating contractile units called sarcomeres which are primarily composed of myosin and actin filaments that are integral to generating force.

are surrounded by a sarcolemma (plasma membrane) and an overlaying extracellular matrix (ECM). The sarcolemma acts as an adherent substratum that the myofibrils are attached to via a costameric network that is comprised of intermediate filament and cytoskeletal elements. These structures facilitate the transduction of force between the myofibrils and the sarcolemma. The sarcolemma in turn is important for transducing the force along the entire length of the fibril by its connections to the ECM. These connections are also crucial for signaling between the underlying myofibrils and ECM, and primarily occur via the dystrophin glycoprotein complex (DGC) and focal adhesions. It is clear that an immense number of components are involved in the formation and function of striated muscle. It is not surprising therefore, that a large number of human genetic diseases have been attributed to mutations in muscle components. These diseases are debilitating and often fatal.

### 1.1.2 Human muscular disorders

Human myopathies (diseases of the muscle) are classified into a number of subgroups. Two major categories are dystrophies and congenital myopathies. Over 30 different inherited diseases have been classified within the muscular dystrophy subgroup, and are characterized by a progressive weakness and degeneration of skeletal muscle. Mutations in structural elements of the myofibre, in addition to regulatory components such as enzymes and signaling molecules give rise to various dystrophies (reviewed in Davies and Nowak, 2006). Duchenne muscular dystrophy is one of the most common forms of muscular dystrophy that affects 1 in 3500 males. This fatal, hereditary disorder is due to mutations in the *dystrophin* gene (Hoffman et al., 1987) and is characterized by gradual muscle weakness and degeneration. The absence of dystrophin disrupts the connection between the costameric network and the sarcolemma. Thus the link

between the force generating myofibrils and the membrane is weakened, making the myofibres susceptible to damage during contraction (Beam, 1988; Petrof et al., 1993).

Originally, any muscle disorder present at birth was classified as a congenital myopathy. Today, the accepted definition of a congenital myopathy describes a subset of disorders that include nemaline myopathy, actin myopathy and intranuclear rod myopathy (Sparrow et al., 2003). Congenital myopathies are heterogeneous disorders characterized by loss of muscle tone and muscle weakness at infancy, however, unlike dystrophic diseases there are no signs of muscle fibre degeneration, cell death or inflammation. Instead, characteristic changes in muscle morphology such as the presence of rods and aggregates of protein are used to distinguish congenital myopathies from other neuromuscular disorders.

Nemaline myopathy is one of the most common forms of congenital myopathy. It is an autosomal dominant or recessive disorder and is characterized by muscle weakness, loss of muscle tone and delayed motor development. Additionally, muscle tissues contain nemaline rods or bodies, which are electron dense structures emanating from Z-lines of the sarcomere that extend along the axis of thin filaments. Nemaline rods are thought to be primarily composed of the Z-disc protein α-actinin as well as other Z-line proteins. Genetic mutations responsible for this disease have been found in *α-actin*, *nebulin*, *α-slow tropomyosin*, *β-tropomyosin*, and *slow troponinT* (Donner et al., 2002; Johnston et al., 2000; Laing et al., 1995; Nowak et al., 1999; Pelin et al., 1999). How nemaline rods form is unknown, however, they are thought to arise from abnormal ratios of functional actin and actin binding proteins, and alterations in the interactions between these proteins (Karpati, 2002).

Currently there is no treatment for sufferers of the various forms of muscular dystrophy or congenital myopathy. However, gene therapy trials are proving somewhat successful (Foster et al., 2006). To establish better treatment strategies for these disorders, further understanding of their pathology is required. A number of mammalian models for muscular dystrophies do exist; most notably the mdx mouse and the golden retriever Duchenne muscular dystrophy dog (Bulfield et al., 1984; Cooper et al., 1988; Sicinski et al., 1989; Watchko et al., 2002). These models have been used to investigate potential treatments, including gene replacement and cell transplantation (Nowak and Davies, 2004). Zebrafish is becoming an attractive model to study the pathology of muscular disorders. Moreover, this organism is ideally suited for studying various aspects of muscle development. The advantages of using zebrafish as a model organism are described in the following section.

## 1.2 The advantages of using zebrafish to study muscle development and muscular disorders

Zebrafish are a freshwater species that originated from the Ganges. They are commonly found in home aquariums and are widely available in pet stores around the world. It was not until the 1970s that a scientist in Oregon, George Streisinger, discovered that this small teleost could be an extremely powerful model organism for studying development. Zebrafish have many qualities that make them an ideal model organism for research on various aspects of development, including myogenesis. They have a short generation time (two to three months) and hundreds of embryos are produced per mating making them highly amenable to genetic analysis. The embryos develop *ex utero* and are transparent, allowing all stages of development to be visualized and the fate of all cells in the embryo to be mapped. For example, single cells can be injected with a lineage tracer dye and their movement monitored over time (Kimmel and Law, 1985a). Moreover, zebrafish embryos are anatomically simpler than other vertebrate model organisms and a relatively small number of cells contribute to each tissue type. These advantages have made zebrafish a popular organism to study numerous developmental processes, and in the last decade an overwhelming amount of research has been performed using this species.

Zebrafish are particularly useful to study the development of muscle. As they are aquatic organisms, zebrafish do not require a robust skeletal system and as a result muscle tissue contributes to 60% of their total body weight. Therefore, a large proportion of cells in the developing embryo give rise to muscle tissues. Moreover, zebrafish have a relatively simple muscle system comprised of separate slow and fast myofibres. In higher vertebrates slow and fast myofibres are mixed. This feature, coupled with the transparency of the zebrafish embryo has

made studying the formation, migration and differentiation of each muscle fibre type particularly amenable to researchers.

Large scale mutagenesis screens were performed in the early 1990s to identify zygotic-effect embryonic lethal mutants and subsequent studies of these mutants have enriched our understanding of zebrafish development (Driever et al., 1996; Haffter et al., 1996). In particular, mutants with defects in somitogenesis and muscle subtype specification have been invaluable in understanding how the muscle develops (Stickney et al., 2000). An additional group of 63 mutants with defects in muscle motility were also isolated (Granato et al., 1996), however, less than 10% have been positionally cloned and characterized (Table 1.1). Research on the remaining zebrafish muscle mutants will be beneficial in extending our knowledge of muscle development and disease. Indeed, a number of the currently characterized zebrafish mutants are now being used as models to study muscular disorders.

Many of the genes associated with muscular dystrophy in humans are also found in zebrafish, and mutations in these genes cause phenotypes similar to what is observed in the skeletal muscle of human patients (reviewed in Bassett and Currie, 2003). The first dystrophic zebrafish mutant, *sapje*, was mapped to the *dystrophin* gene in 2003 (Bassett et al., 2003). It was demonstrated that the muscle in *sapje* mutant embryos degenerates due to weak somitic muscle attachments at the myotendinous junctions. Antisense morpholino oligonucleotides (MOs) have also been used to knockdown other zebrafish sarcolemmal proteins such as Caveolin and δ-Sarcoglycan (Guyon et al., 2005; Nixon et al., 2005) and similar skeletal muscle defects are observed to human patients with defects in these genes (McNally et al., 1998; Nigro et al., 1996). A number of research groups and biotechnology companies are now using MO injected embryos and mutants in high-throughput screens to discover novel pharmacological

Table 1.1 List of zebrafish muscle motility mutants identified by Granato et al., 1996. Asterisk indicates the positionally cloned mutants described in this thesis.

| Zebrafish mutant | Number of alleles | Mutated gene | Related Human Muscular disorder |
|---|---|---|---|
| *sloth* | 2 | | |
| *frozen* | 1 | | |
| *fibrils unbundled* | 2 | | |
| *turtle* | 22 | | |
| *buzz off* | 5 | *nebulin** | Nemaline myopathy [1] |
| *faulpelz* | 2 | | |
| *slow motion* | 1 | | |
| *schnecke* | 1 | *capzα1** | |
| *hermes* | 2 | | |
| *Duesentreib* | 1 | | |
| *mach two* | 1 | | |
| *slop* | 1 | | |
| *jam* | 1 | | |
| *slinky* | 1 | | |
| *sapje* | 2 | *Dystrophin*[2] | Duchenne and Becker muscular dystrophy [3] |
| *softy* | 3 | | |
| *schwammerel* | 2 | | |
| *runzel* | 1 | *Titin*[4] | Titinopathy [5] |
| *candyfloss* | 2 | *laminin α2*[6] | Merosin-deficient muscular dystrophy [7] |
| Unresolved | 10 | | |

1. **Pelin, K. et al**. (1999). Mutations in the nebulin gene associated with autosomal recessive nemaline myopathy. *Proc. Natl. Acad. Sci.* **96**, 2305-2310.

2. **Bassett, D. I., Bryson-Richardson, R. J., Daggett, D. F., Gautier, P., Keenan, D. G. and Currie, P. D.** (2003). Dystrophin is required for the formation of stable muscle attachments in the zebrafish embryo. *Development* **130**, 5851-60.

3. **Koenig, M., Monaco, A. P., and Kunkel, L. M.** (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* **53**, 219-226.

4. **Steffen, L. S., Guyon, J.R**., **Vogel, E.D**., **Howell, M.H**., **Zhou, Y**., **Weber, G.J**., **Zon, L.I**. **and Kunkel, L.M**. (2007). The zebrafish runzel muscular dystrophy is linked to the titin gene. *Dev Biol.* **309** (2), 80-92.

5. **Hackmann, P. et al**. (2002). Tibial muscular dystrophy is a titinopathy caused by mutation in TTN, the gene encoding the giant skeletal-muscle protein titin. *Am. J. Hum. Genet.* **71**, 492-500.

6. **Hall, T.E., Bryson-Richardson, R.J., Berger, S., Jacoby, A.S., Cole, N.J., Hollway, G.E., Berger, J. and Currie, P.D. (**2007). The zebrafish *candyfloss* mutant implicates extracellular matrix adhesion failure in laminin α2-deficient congenital muscular dystrophy. *Proc. Natl. Acad. Sci.* **104** (17), 7092-7.

7. **Helbling-Leclerc, A. et al.** (1995). Mutations in the laminin α2-chain gene (LAMA2) cause merosin-deficient congenital muscular dystrophy. *Nature Genet.* **11**, 216-218.

and genetic therapies for muscular dystrophy . Zebrafish are advantageous for these types of screens as a large number of compounds can be tested on many embryos and the chemicals easily diffuse into the embryo. A number of drug screens have been successfully carried out in zebrafish and have shown that small molecules are able to alter the development of several organ systems such as ear, central nervous system and eye (den Hertog, 2005). Moreover, two screens have been carried out on mutants with cardiac and cell cycle defects and compounds that ameliorated the mutant phenotype were identified (Peterson et al., 2004; Stern et al., 2005). These drug screens demonstrate the feasibility of using zebrafish models of human disorders to screen for potential pharmacological treatments. Additionally, they could dramatically reduce the time and cost of drug discovery and assist in identification of novel treatments of human diseases, including myopathies.

## 1.3 Skeletal muscle development in zebrafish

### 1.3.1 Mesoderm induction and formation of the three germ layers

Striated muscle in vertebrates arises from mesoderm. Mesoderm is one of three germ layers that give rise to specific tissue types within the embryo. Mesoderm develops into a diverse variety of organs such as the notochord, muscle, blood, heart and kidneys. Ecotoderm and endodermal layers differentiate into epidermis and gut epithelia respectively. In zebrafish, cells fated to become mesoderm arise prior to gastrulation and are located in a ring on top of an extra-embryonic structure called the yolk syncytial layer (YSL) (Fig. 1.3). The YSL separates the yolk from the cells that form the embryo proper and has several important functions, including inducing the formation of the mesoderm and endoderm (Mizuno et al., 1999; Ober and Schulte-Merker, 1999; Rodaway et al., 1999). The cells closest to the YSL develop into the mesendodermal layer (a mixture of cells fated to become mesoderm and endoderm). Cells slightly further away from the YSL develop into mesoderm and cells furthest away from the YSL are fated to become ectoderm (Fig. 1.3) (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999).

The ability of the YSL to induce the formation of germ layers was initially discovered by transplantation of the yolk cell into the animal region of the blastoderm. This procedure ectopically induced genes normally expressed in the mesendoderm (Mizuno et al., 1999). Also, addition of RNAse to the YSL blocked expression of ventral and lateral mesendodermal markers (Chen and Kimelman, 2000). These findings lead to a hypothesis that secretion of signaling molecules from the YSL induces the formation of mesendoderm and mesoderm, and cells furthest away from the YSL, which are not affected by these signals, become ectoderm. In

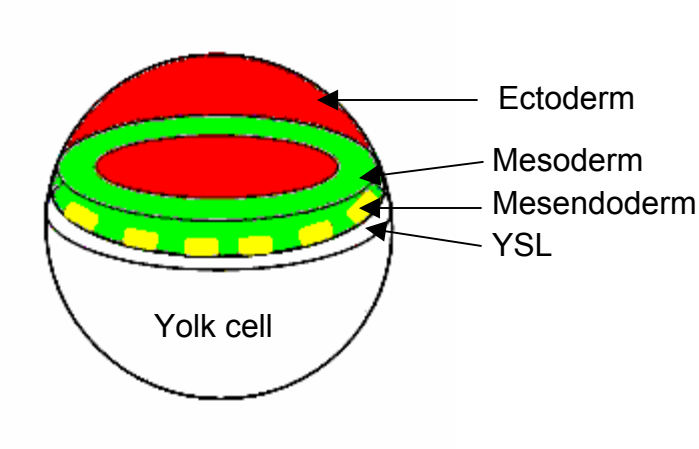


Fig. 1.3. Fate map of the three germ layers prior to gastrulation (30% epiboly). Signals from the YSL induce the formation of cells fated to become mesoderm (green) and endoderm (yellow) (mesendodermal layer) immediately adjacent to the YSL. Cells on top of the mesendodermal layer are fated to become mesoderm only, and cells furthest away from the signaling emitted by the YSL are fated to become ectoderm (red). The cells in the centre of the ring also develop into ectoderm.

*Xenopus* the maternal transcription factor VegT is able to induce the formation of germ layers.VegT is localized to the vegetal region of the embryo and is able to activate members of the Nodal gene family and induce mesoderm and endoderm formation (Zhang and King, 1996). Interestingly, in zebrafish the orthologue of *vegT* (*spadetail*) is only expressed zygotically. Moreover, mutations in this gene do not prevent formation of mesendoderm (Kimmel et al., 1989). The putative signal emitted by the YSL in teleosts remains to be determined.

Once mesoderm, ectoderm and endodermal fates are established they are organized into distinct layers in a process known as gastrulation. Gastrulation relies on the coordinated movement of cells so that the endoderm and mesoderm are located within the interior of the embryo and ectoderm forms the outer layer of the embryo. In zebrafish, gastrulation extends from approximately 5.5 hours post fertilization (hpf) to 10 hpf and can be divided into three movements: epiboly, involution and convergence and extension. These movements occur concurrently during this period.

Epiboly begins prior to gastrulation (4-4.5 hpf) and is characterized by the spread and movement of the blastoderm cells (which include cells that form the embryo proper) over the yolk cell towards the vegetal axis (Kimmel and Law, 1985b; Trinkaus, 1993)(Fig. 1.4A). The continual movement of these cells across the surface of the yolk enables gastrulation to proceed. Gastrulation begins just after 50% epiboly (when half the yolk is covered by blastoderm) and is marked by the formation of the germ ring.

The germ ring arises from involution (the internalization of cells). This type of cell movement induces the rim of the blastoderm (the margin adjacent to the YSL) to thicken as the mesodermal and endodermal cells start to migrate from the outside to the inside of the embryo

(Fig.1.4B). The internalization of these cells results in the division of the blastoderm into an outer layer of epiblast (ectoderm) and an inner layer of hypoblast (mesoderm and endoderm) (Warga and Kimmel, 1990). Involution occurs throughout the gastrula period and somitogenesis until all cells are internalized (Kanki and Ho, 1997).

A thickening on one side of the germ ring (the shield) is also observed almost simultaneously to germ ring formation (Fig. 1.4B), and arises from involution and convergence of mesodermal cells to the dorsal side of the embryo (Warga and Kimmel, 1990). The shield is the first morphological structure that indicates the establishment of a dorsoventral (DV) axis and is composed of cells of the dorsal YSL and part of the dorsal blastomere. It is equivalent to the Spemann-Mangold organizer in the blastopore lip of amphibians, the node in the mouse and Henson's node in the chick (Oppenheimer, 1936; Saude et al., 2000; Shih and Fraser, 1996). An organizer is present in all vertebrates and eventually differentiates into dorsal mesodermal structures such as the notochord, the defining feature of the vertebrate phylum. The organizer functions as a signaling centre and secretes multiple signaling factors that can change the fate of the surrounding cells. This property enables the organizer to initiate a secondary axis when transplanted to an ectopic position within the embryo (Ho, 1992; Saude et al., 2000; Shih and Fraser, 1996; Spemann, 1924).

Gastrulation movements persist after formation of the shield. The medosermal cells continue to converge to the dorsal side of the embryo and intercalate with dorsal blastomeres, resulting in narrowing of embryonic tissues mediolaterally. These cells then undergo extension movements and spread along the animal-vegetal axis, driving elongation along the anteroposterior (AP) axis (Fig. 1.4C). By the end of gastrulation the progenitors of all cell types are correctly positioned and arranged along the fully established DV and AP axis.

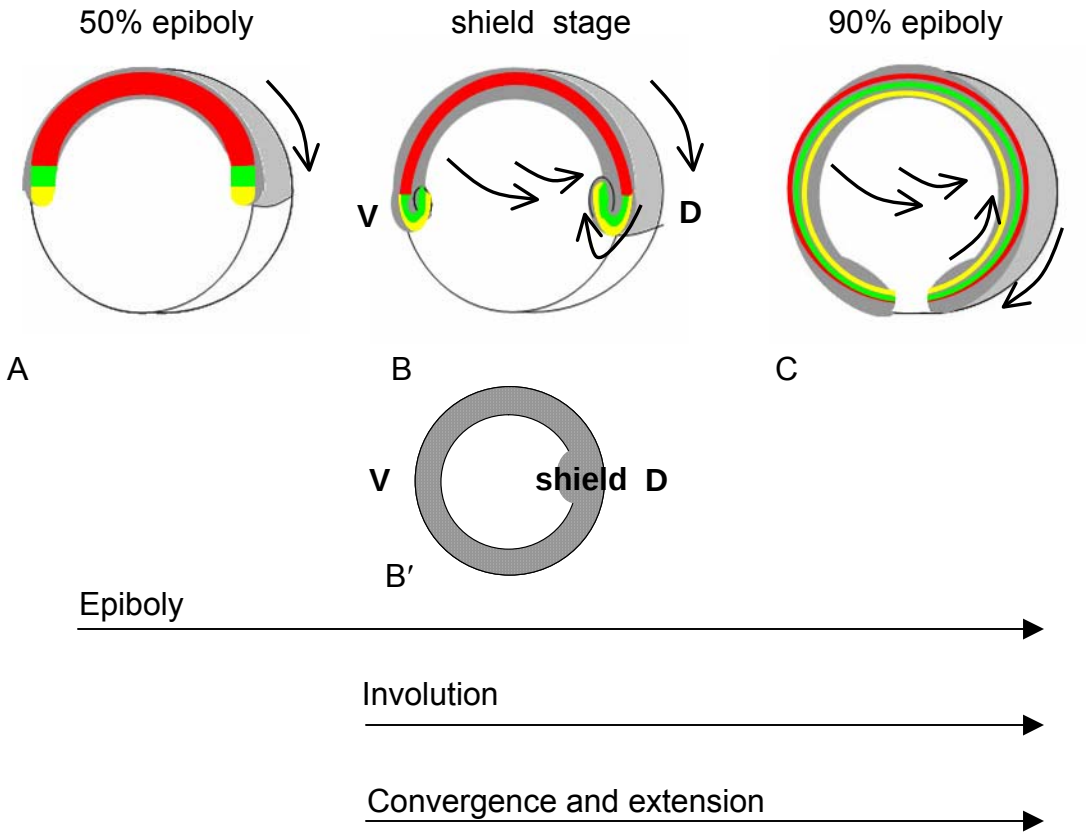


Fig. 1.4. Diagram of the three types of cell movement involved in gastrulation. A) Epiboly movements begin prior to gastrulation and move cells over the yolk towards the vegetal axis. At this stage cells fated to become endoderm (yellow), mesoderm (green) and ectoderm (red) are located on the outer surface of the embryo. B) Once just over half the yolk is covered (60% epiboly) gastrulation begins with involution and the formation of the germ ring (animal pole view shown in B′) as mesodermal and endodermal cells move from the outer edge of the embryo to the inside of the embryo. Concurrently, convergence movements result in a thickening on the dorsal side of the germ ring known as the shield. C) Epiboly, involution and convergence and extension movements continue until all three germ layers are positioned correctly along the DV and AP axis. Arrows indicate the movement of cells at each stage and D and V indicate the dorsal and ventral axis respectively.

### 1.3.2 Origin and specification of muscle progenitor tissues

#### 1.3.2.1 Paraxial mesoderm

The predominant feature along the AP axis is the segmental plate, which is comprised of paraxial mesoderm that flanks either side of the axial mesoderm (derived from the shield) and forms towards the end of gastrulation (Fig. 1.5A). Axial mesoderm differentiates into notochord and paraxial mesoderm eventually gives rise to somites. One of the main questions that has intrigued developmental biologists is how mesoderm is patterned into these two different tissues. This question was primarily addressed by using various zebrafish mutants: the T-box transcription factor mutant *spadetail* (*spt*) and the Not-type homeobox transcription factor *floating head* (*flh*) were found to be the main instigators in patterning of the mesoderm. *spt* mutants lack trunk somites (Kimmel et al., 1989), while in *flh* mutants, a notochord does not form and instead axial mesoderm is converted into somitic tissues (Halpern et al., 1995; Melby et al., 1996). The loss of *spt* in *flh* mutants rescues many aspects of the *flh* phenotype and in these double mutants the axial mesoderm is still able to differentiate into notochord. These results indicated that *spt* controls paraxial mesoderm formation while *flh* represses *spt* expression in the axial mesoderm. Thus muscle differentiation is blocked at the midline, however, not in the surrounding paraxial mesoderm (Amacher and Kimmel, 1998).

Once the axial and paraxial mesoderm are established, axial mesoderm forms prechordal plate and notochord while paraxial mesoderm segregates into two cell types: adaxial cells and lateral presomitic mesoderm cells (Fig. 1.5B). Adaxial cells form a monolayer adjacent to the

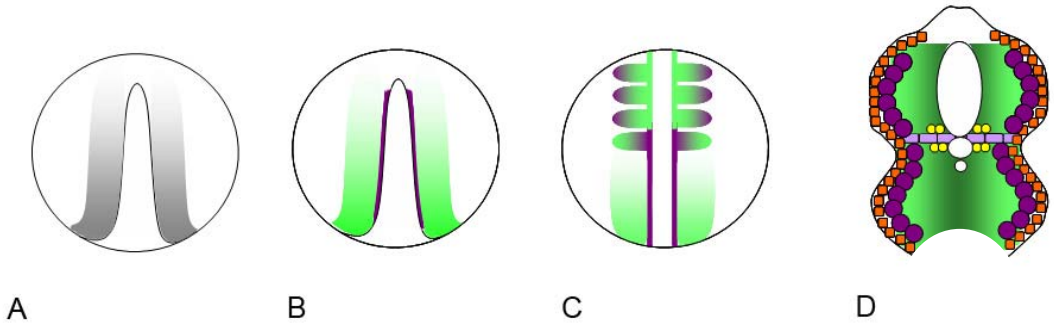


Fig. 1.5. Illustrations depicting the position of muscle precursors during zebrafish embryonic development. A) Dorsal view of embryo at 90% epiboly illustrating the notochord precursor tissue (axial mesoderm) flanked by the segmental plate containing paraxial mesoderm (grey). B) Dorsal view of bud stage embryo (~10hpf). Slow muscle precursors (adaxial cells) are positioned in a single layer (purple stripe) adjacent to the notochord while fast muscle precursors reside in lateral paraxial mesoderm on either side of the notochord (green). C). During somitogenesis, the majority of the adaxial cells migrate to the lateral edge of each somite and differentiate into superficial slow fibres. D) Transverse section through the trunk of a 24 hpf embryo showing all the differentiated muscle fibre types. Adaxial cells that do not migrate to the surface of the muscle become muscle pioneers (light purple). The bulk of the fast muscle cells differentiate into fast muscle fibres (green). A subset of the fast muscle cells located adjacent to the muscle pioneers become medial fast fibres (yellow). Superficial slow fibres (purple) are located between the fast muscle fibres and the dermomyotomal like layer of external cells (orange).

notochord. They are a specialized group of cuboidal mesodermal cells that are the precursors to all slow muscle lineages (Devoto et al., 1996; Weinberg et al., 1996). Adaxial cells arise from Sonic hedgehog signals emitted by the notochord (Barresi et al., 2000; Du et al., 1997; Wolff et al., 2003) before paraxial mesoderm segments into somites.

#### 1.3.2.2 Somitogenesis

One of the first most striking morphological muscle structures that can be visualized in the developing zebrafish embryo are the somites. Somites are the main progenitors of skeletal muscle in vertebrates and are formed when groups of paraxial mesodermal cells on either side of the notochord separate, and form blocks of epithelial tissue at regular intervals along the AP axis. The first pair of somites are observed at 10.5 hpf and each subsequent pair forms approximately every 30 min in an AP wave until 26-30 somites have formed. Each somite is composed of an epithelioid sheet surrounded by a core of mesenchymal cells. The boundary of each somite acts as a positional template for the formation of the myotome boundary (the myoseptum).

One of the unsolved mysteries of somitogenesis is how the somites are formed in such a regulated and precise manner. This topic has been subject to extensive research and mounting evidence is emerging that supports the clock and wavefront model (for review see Pourquie, 2001; Pourquie, 2003). Briefly, this model firstly proposes the existence of a molecular clock or oscillator (e.g. a cyclic wave of gene expression) that functions with temporal periodicity. Therefore groups of paraxial mesodermal cells are only able to form a segment during a brief period of time within each cycle of the somite clock. Secondly, the wavefront, a gradient of either positional or developmental information, induces the formation of each somite when it encounters a group of cells that are cycling in the permissive phase of the clock. The wavefront

first emerges in the anterior presomitic mesoderm and gradually moves at a constant speed as somitogenesis proceeds, thus the somites form and differentiate in an anterior to posterior manner along the primary axis of the embryo. The stepwise interaction between the clock and the wavefront leads to regularly spaced furrow formation in the anterior presomitic mesoderm. Moreover, somite length and the rate of formation are dependent on the frequency of the clock or oscillator and the velocity of the wavefront.

Once each somite has formed, it is subsequently patterned by signals emitted from the adjacent notochord, neural tube, lateral plate mesoderm and surface ectoderm. Each somite in vertebrates is initially subdivided into sclerotome, myotome and dermomyotome, which give rise to the vertebral column, skeletal muscle and dermis respectively. In all vertebrates, muscle is derived mostly from the myotome. Myotome is specified by dermomyotome in chick and mouse embryos, however, a consensus has not been reached as to how this occurs (for review see Hollway and Currie, 2003). In zebrafish, the existence of a dermomyotome was only recently established when laterally located cells between the slow muscle and the dermis (known as external cells) were found to express the paired type homeobox transcription factors *pax3* and *pax7* (Devoto et al., 2006). The transcripts for both these genes are found in the dermomyotomal cells of chick and mouse (Gros et al., 2005; Relaix et al., 2005), therefore the external cell layer in zebrafish is proposed to be homologous to the dermomyotome. Moreover, the external cell layer expresses the myogenic regulatory factors *myf5* and *myogenin*, which are indicative of myogenic precursor cells in other vertebrates (Groves et al., 2005; Steinbacher et al., 2006). These properties suggest that the external cell layer may contain myogenic precursors that are able to migrate and differentiate into muscle cells. Indeed, a recent study has shown that external cells are able to incorporate and differentiate into fast muscle fibres (Stellabotte et al., 2007).

#### 1.3.2.3 Myogenic regulatory factors: Myf5 and MyoD

The first significant indication of a commitment of mesoderm to a muscle specific lineage is detected with the expression of MyoD and Myf5. MyoD and Myf5 are myogenic regulatory factors (MRFs) of the basic-helix-loop-helix transcription factor family. They are expressed in proliferative myoblasts and have been implicated in the establishment and maintenance of muscle progenitors, as well as being involved in the terminal differentiation of myofibres (Choi et al., 1990; Davis et al., 1987; Pownall et al., 2002; Weintraub et al., 1991). In zebrafish, *myoD* is first expressed in the presomitic mesoderm (paraxial mesoderm) at 70-75% epiboly (Weinberg et al., 1996) and is closely followed by *myf 5* at 80% epiboly (Coutelle et al., 2001). The monolayer of adaxial cells that arises adjacent to the notochord exclusively expresses *myoD*. Subsequently, *myoD* is expressed in the posterior region within each newly formed somite until the cells begin to differentiate into fast muscle at the 15 somite stage. *myf5* is initially expressed in the posterior presomitic mesoderm in presumptive fast muscle cell precursors. Its expression declines in anterior presomitic mesoderm but is transiently re-expressed in the posterior border of each somite (Chen et al., 2001; Coutelle et al., 2001).

The expression of *myf5* and *myoD* are regulated by a host of factors that are expressed in the surrounding tissues. Experiments performed on two T-box transcription factor mutants *no tail* (*ntl*, homologue of *brachyury*) and *spt* indicate that both these factors are required to initially activate the expression of *myoD* in the paraxial mesoderm. As previously mentioned, *spt* is important in formation of the paraxial mesoderm and loss of this factor results in a lack of trunk somites. In *ntl* mutants the notochord and tail do not form. In each of these mutants *myoD* expression is delayed, but recovers during segmentation (Amacher et al., 2002; Weinberg et al., 1996). In *ntl* and *spt* double mutants *myoD* expressing cells are never detected (Amacher et al.,

2002), indicating the importance of these transcription factors in the induction of myogenesis. Many other factors including signalling molecules, such as Hedgehogs, Wnts, Noggin and BMPs work in concert to regulate transcription of MRFs and are essential for myogenesis to proceed (reviewed in Pownall et al., 2002).

### 1.3.3 Muscle differentiation

Formation of differentiated skeletal muscle in vertebrate embryos is a tightly regulated process involving cell migration and differentiation of muscle precursor cells, followed by myoblast fusion. Zebrafish contain four different types of muscle fibre: slow muscle fibres (superficial slow fibres), muscle pioneers, fast muscle fibres and medial fast fibres (Fig.1.5D). Each fibre type has unique morphological and developmental properties and is located in distinct regions within the muscle.

#### 1.3.3.1 Slow and fast muscle fibre formation

All slow muscle fibres are derived from the adaxial cells adjacent to the notochord and fast muscle fibres are derived from lateral paraxial mesoderm. However, fate mapping studies have shown that prior to gastrulation, slow and fast muscle precursor cells occupy distinct locations within the mesoderm that is located at the margin (Hirsinger et al., 2004; Kimmel et al., 1990). At 50% epiboly slow muscle precursors are located close to the shield (on the dorsal side) and fast muscle precursors are located towards the ventral side. At this stage it was found that muscle precursor cells can still readily change fate if transplanted into another domain. Therefore they are not committed to forming a particular muscle subtype until after they enter the segmental plate and become exposed to signals emitted from the notochord (Hirsinger et al., 2004).

Shortly after somite formation, most of the adaxial cells elongate before migrating to the lateral part of the myotome to form a subcutaneous layer, where they differentiate into mononucleated slow twitch myofibres that span the somite (Blagden et al., 1997; Devoto et al., 1996; Roy et al., 2001). The migration of adaxial cells is dependent on the differential expression of the cell adhesion molecules M- and N-Cadherin (Cortes et al., 2003). The subset of adaxial cells that do not migrate, differentiate into medially located muscle pioneer cells, which are a distinct population of slow twitch fibres. They are the first cells to become terminally differentiated and are responsible for producing the horizontal myoseptum that subdivides the myotome into dorsal and ventral halves. All slow twitch fibres are characterized by the expression of the *slow myosin heavy chain* (*slow myhc*) and the homeodomain gene *prox1* (Blagden et al., 1997; Roy et al., 2001). Muscle pioneers also express genes of the *engrailed* (*eng*) homeobox protein family (Ekker et al., 1992; Felsenfeld et al., 1990; Hatta, 1992; Hatta et al., 1991).

The remaining bulk of the muscle cells differentiate into fast muscle fibres that are localized medially to the overlaying slow muscle. A subset of the fast fibres that surround the adjacent slow muscle cell pioneers form medial fast fibres. Intriguingly, although these cells express *fast muscle myosin heavy chain* (*fast myhc*) they also express *eng*, which is a characteristic of the muscle pioneers (Wolff et al., 2003). The function of these fibres remains unknown.

#### 1.3.3.2 Hedgehog signalling

The primary signaling factor responsible for the differentiation of the four muscle subtypes, and in particular the slow myofibres, is Sonic hedgehog (Shh). *shh* and the two related *hedgehog* (*hh*) genes, *echidna hedgegog* (*ehh*) and *tiggywinkle hedgehog* (*twhh*) are expressed in axial structures. *ehh* and *shh* are expressed in the notochord (Currie and Ingham, 1996) and *twhh* is expressed in the overlying floorplate of the neural tube (Ekker et al., 1995). Hedgehog (Hh) proteins are essential for inducing the formation of slow muscle and muscle pioneers *in vivo* and *in vitro* (Du et al., 1997; Norris et al., 2000; Weinberg et al., 1996; Wolff et al., 2003). Pioneering studies in chick first demonstrated that *myoD* is activated by both Shh and Wnt signaling in the pre-segmental plate (Munsterberg et al., 1995). In zebrafish, Hh signals have also been found to drive the expression of MRFs in slow muscle precursors (Coutelle et al., 2001; Lewis et al., 1999).

Hh signalling acts through two receptor transmembrane proteins, Patched (Ptc) and Smoothened (Smo). Ptc negatively regulates Hh signalling by inhibiting Smo activity. However, once Hh binds to Ptc, Smo dissociates from Ptc and is able to transduce the Hh signal (Ingham and McMahon, 2001). In zebrafish *smoothened* mutants (*smooth muscle omitted* (*smu*)), adaxial cells do not express *myoD* and slow muscle fibres and muscle pioneers do not form. All somitic tissues differentiate into fast muscle fibres (Chen et al., 2001). It has been proposed that Hh signalling is required for slow fibre specification and acts as a gradient, whereby different levels of Hh induce the formation of different muscle subtypes.

The gradient model for Hh activity was proposed by treating zebrafish embryos with cyclopamine, a small molecule inhibitor of Hh signaling, in a dose dependent manner (Wolff et al., 2003). When Hh signaling is completely inhibited by high concentrations of cyclopamine, muscle pioneers, slow muscle myofibres and medial fast fibres are eliminated. At intermediate concentrations muscle pioneers and medial fast fibres are absent, and at the lowest concentrations only the muscle pioneers are disrupted. These findings indicate that Hh signals act as a gradient i.e. those cells that are closest to the source of Hh emanating from the notochord are exposed to high levels of Hh activity and commit to form muscle pioneers and *eng* positive medial fast fibres. Those cells that are located further away from the source of the gradient are exposed to lower levels of Hh which induces the specification of the superficial slow muscle fibre cell fate.

Fast muscle fibres derived from lateral paraxial mesoderm are still able to form in the absence of Hh signaling (Chen et al., 2001). This indicates that Hh signals are not the only governing factor in specifying muscle cell fate. The gradient of activity and threshold response to specific levels of Hh may be provided by a combination of the spatial locations of precursor cells, the timing of exposure to Hh signalling and other factors that interact with Hh signals. Indeed, a number of accessory proteins have been identified that may be required to fine tune Hh signaling and specify the differentiation of slow precursor muscle fibres (e.g. Dzip1 (DAZ interacting protein 1) (Wolff et al., 2004) and Blimp1 (Baxendale et al., 2004)). Blimp1 is one of the key Hh target genes that controls slow muscle differentiation and is mutated in the *u-boot* (*ubo*) mutant. In this mutant all slow muscle fibre cell types are lost (Roy et al., 2001). Blimp 1 is a transcription factor that is specifically expressed in adaxial cells (Baxendale et al., 2004). It is necessary for slow muscle induction and is required for the repression of *fast myhc* and activation of the *slow myhc* and *prox1* genes. It has therefore been proposed that Blimp1 may set the transcriptional status of key genes required for differentiation of the slow muscle lineage.

It is still not clear what molecules induce the formation of fast muscle fibres. However, signaling factors such as Tgf-β family members (which can inhibit slow fibre formation) and Wnts may be involved (Du et al., 1997; Makita et al., 1998). Although Hh signals are integral to the specification of the muscle fibre subtypes many other components are involved in patterning of the myotome. Further exploration of this field is necessary to conclusively determine how paraxial mesoderm differentiates into the four main classes of myofibres.

#### 1.3.3.3 Fast fibre elongation

Once the fast fibres have been specified they elongate prior to fusion. Surprisingly, the morphogenesis of these fast fibres is triggered by the migration of slow fibres (Henry and Amacher, 2004). As the slow twitch cells migrate laterally they induce fast fibre elongation in a medial-to-lateral wave (Henry and Amacher, 2004). So although fast muscle fibres do not directly require Hh signals to elongate they need the slow muscles to migrate, which is induced by Hh signaling. The molecular mechanisms responsible for fast fibre elongation are unknown, though it has been postulated that the slow muscle cells could secrete a factor that promotes elongation. Alternatively, direct cell-to-cell contact may induce the elongation of some of the fast fibres, which subsequently activates elongation in adjacent fast muscle fibres. After the fast fibres elongate they fuse to form around 80 multinucleated fast twitch fibres per somite.

#### 1.3.3.4 Myoblast fusion and terminal differentiation of myofibres

During myoblast fusion mononucleated myoblasts fuse to form multinucleated muscle fibres. In zebrafish, only the fast muscle cells fuse while the slow muscle fibres remain mononucleated. This process has been most extensively studied in mammalian cell cultures

where fusion can be synchronized (Knudsen, 1992; Wakelam, 1985). These studies have identified a number of cell adhesion molecules (e.g. calmodulin and protein kinases) that may be involved in myoblast fusion. However, the role of these proteins *in vivo* has still not been thoroughly investigated and how myoblast fusion is regulated in vertebrates is poorly understood.

Mechanisms of myoblast fusion *in vivo* have primarily been elucidated by studies in *Drosophila*. In *Drosophila*, founder myoblasts prefigure the position, orientation and identity of individual muscles, and fusion competent myoblasts fuse with founders and convert them into syncytia (for review see Chen and Olson, 2004) ). Central to the fusion process is a signaling pathway involving *dumbfounded* (*kirre*) and *roughest*, which are both Ig-domain containing membrane receptors (Ruiz-Gomez et al., 2000; Strunkelnberg et al., 2001). A recent report has identified the first vertebrate homologue of *kirre* in zebrafish, *kirrel* (Srinivas et al., 2007). *kirrel* is required for muscle precursor fusion in zebrafish and is expressed in all fast myoblasts. Knockdown of Kirrel with a MO produced embryos with unfused fast muscle cells. However, MRFs were still expressed in these cells, suggesting that myoblast differentiation is unaffected (i.e. formation of sarcomeres and myofibrils). Thus muscle differentiation and fusion appear to be independent of each other. Inhibition of the most downstream intracellular transducer of the fusion signal in *Drosophila*, Rac, also compromised fast muscle fusion in zebrafish. However, unlike *Drosophila*, constitutively active Rac led to hyperfused giant syncytia. Therefore Rac could potentially be important for limiting the number of fusion events and the polarity of the fast muscle cells.

Caveolin-3, a sarcolemmal protein expressed in skeletal and cardiac muscle (Tang et al., 1996; Way and Parton, 1995) may also be involved in myoblast fusion. Studies of muscle cell lines extracted from transgenic mice found that overexpression of Caveolin-3 reduces fusion,

while loss of Caveolin-3 enhances myoblast fusion (Volonte et al., 2003). In contrast, in the Caveolin-3 mouse knock out myoblast fusion is not defective (Galbiati et al., 2001; Hagiwara et al., 2000). In zebrafish, myoblast fusion is reduced in muscle fibres isolated from caveolin-3 MO injected embryos (Nixon et al., 2005). Due to the contradictary results of Caveolin-3 to date, further studies are required to elucidate its function. However, it will be interesting to determine how this protein is involved in controlling myoblast fusion.

Both slow and fast muscle cells terminally differentiate into striated muscle fibres which must contain all the basic elements to enable muscle contraction to take place. Very little is known about these last stages of muscle development, especially *in vivo*. However, it is likely that the master MRFs MyoD and Myf5, as well as Myogenin and Mrf4, play roles in regulating the expression of myofibril components (Edmondson and Olson, 1989; Lassar et al., 1989; Rhodes and Konieczny, 1989; Wright et al., 1989). Retinoic acid is also thought to be involved in differentiation of fast muscle fibres, and has been shown to inhibit proliferation and induce fast muscle fibre differentiation (Alric et al., 1998). Moreover, it has been shown that zebrafish retinaldehyde dehydrogenase 2, the main retinoic acid synthesizing enzyme, is expressed in the somites where it activates FGF8 expression and consequently fast muscle differentiation (Hamade et al., 2006).

As myoblasts become terminally differentiated, a wide range of different transcription factors are expressed (Tapscott, 2005) e.g. Mef2 and SRF. Mef2 has been shown to be co-expressed with MyoD to enhance the conversion of non-muscle cells into myogenic cells *in vivo* (Molkentin et al., 1995) and both Mef2 and SRF have been shown to regulate many heart and skeletal muscle genes (Balza and Misra, 2006; Black and Olson, 1998). In addition, it has been

demonstrated that Mef2 family proteins are required to upregulate thick filament components, such as myosin, in the zebrafish embryo (Hinits and Hughes, 2007).

The exact mechanisms that drive terminal differentiation of myofibres in zebrafish and other vertebrates are still unknown. Preliminary antibody stainings of some sarcomeric components in zebrafish embryonic muscle indicate that sarcomeric assembly begins around 24 hpf and myofibres are fully differentiated by 48 hpf (Costa et al., 2002). However, how the muscle components are temporally and spatially regulated to result in the formation of a fully functioning contractile unit is yet to be elucidated. Thus further detailed analysis of sarcomere and myofibril formation in zebrafish embryos will be beneficial in understanding the later aspects of muscle development in vertebrates. Moreover, this information will be invaluable to unraveling the mechanisms underlying many human muscular disorders.

## 1.4 Components of striated muscle

### 1.4.1 The sarcomere

The sarcomere is the fundamental contractile unit of all striated muscles and is composed of an elaborate array of elements that are precisely assembled and maintained. The ultrastructural arrangement of sarcomeric components is clearly illustrated by transmission electron microscopy (TEM), and defined regions of the sarcomere including the A-band, I-band, Z-band and M-band are readily visualized (Fig. 1.6). The thick and thin filaments that are integral to the generation of force are located within the A- and I-bands. The thick filament is comprised of bundles of myosin which are located primarily within the A-band. Within this region, the globular head of the myosin chains are able to interact with actin containing thin filaments, which interdigitate with the thick filaments. The small region where the thick filaments no longer contact the thin filament is called the bare zone. Within the middle of this region, the M-band (M-line) is where the myosin bundles are anchored to the middle of the sarcomere. The M-band ensures the regular packing of myosin filaments and is important in maintaining sarcomeric stability during contraction. The Z-band (also known as the Z-line or Z-disc) forms the lateral boundary of each sarcomere and contains proteins that crosslink components of adjacent sarcomeres, such as titin, nebulin and antiparallel thin filaments.

Muscle contracts by a sliding filament mechanism. This mechanism was first hypothesized from TEM images of the sarcomere, which showed that the lengths of the thick and thin filaments did not change during muscle contraction. Instead, the sarcomere appeared shortened, suggesting that the thick and thin filaments slide past each other (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). This movement draws the Z-discs to the middle

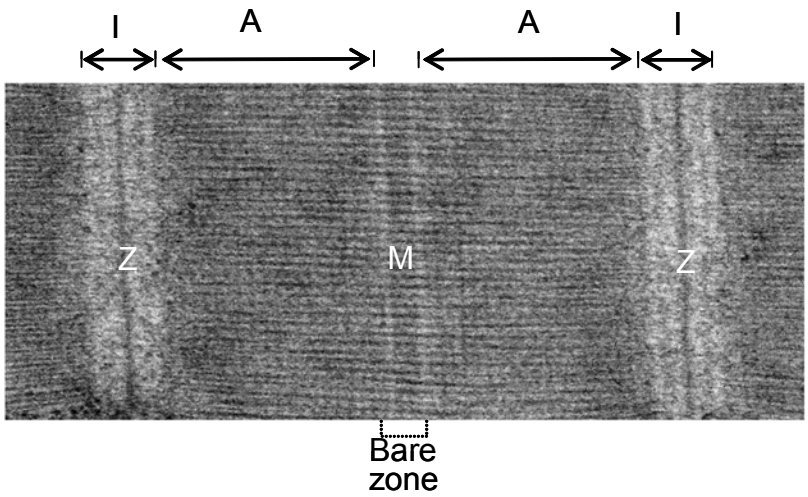


Fig. 1.6. Transmission electron microscope image of a zebrafish sarcomere. The Z-lines (Z) form the boundary of each sarcomere. The M-band (M) is located within the bare zone at the centre of the sarcomere. The I-band (I) is positioned between sarcomeres and spans the region of the thin filament attached to the Z-line. The A-bands (A) form the bulk of the sarcomere and consist of interdigitated thin and thick filaments.

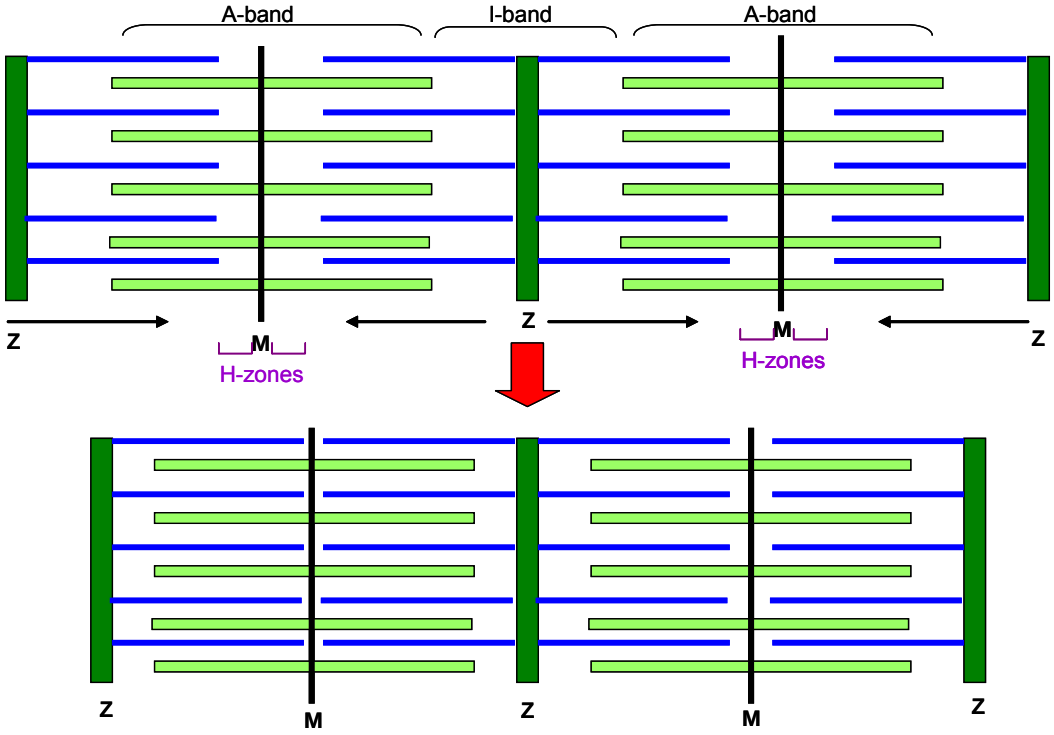


Fig. 1.7. Schematic illustration of the sliding filament mechanism for sarcomere contraction. As the thick (light green rods) and thin filaments (blue rods) slide past each other, the Z-lines (dark green bars) move closer together, thereby decreasing the width of the I-bands and H-zones (region between the M-band and the edge of the thin filament), however the width of the A-band remains constant. M indicates the M-line.

of each sarcomere so that the I-bands and H-zone narrow, however, the A-band remains constant (Fig. 1.7).

It is apparent that large forces are placed on the sarcomere during contraction, therefore it is not surprising that this contractile unit is composed of a multitude of interconnected structural elements. The major components of the sarcomere are illustrated in Fig. 1.8 and will be described herein.

#### 1.4.1.1 The actin thin filament and its associated proteins

Filamentous actin (F-actin) is involved in numerous functions including cell motility, cytokinesis, formation of stress fibres and muscle contraction (Carlier, 1998). In different cell types actin filaments form specialized structures, such as thin filaments within the sarcomeres of striated muscle, internal stiffening rods in the microvilli of intestinal brush borders and the membrane skeleton within erythrocytes. There are at least six isoforms of actin in mammals and birds, and these are grouped into three classes by their isoelectric points: α-actin is found in muscle and the β- and γ-actins are primarily found in non-muscle cells. Each actin monomer is tightly associated with a molecule of ATP, enabling it to polymerize by ATP hydrolysis. Actin filament formation begins with nucleation. This involves the association of three or four monomers that form a stable oligomer (nucleus) from which polymerization can proceed. Once polymerized, each actin filament appears as two twisted strands of actin monomers and has a barbed and pointed end. The designation of barbed and pointed is derived from TEM images of actin filament decorated with myosin heads, which give the appearance of arrowheads along the length of the filament.

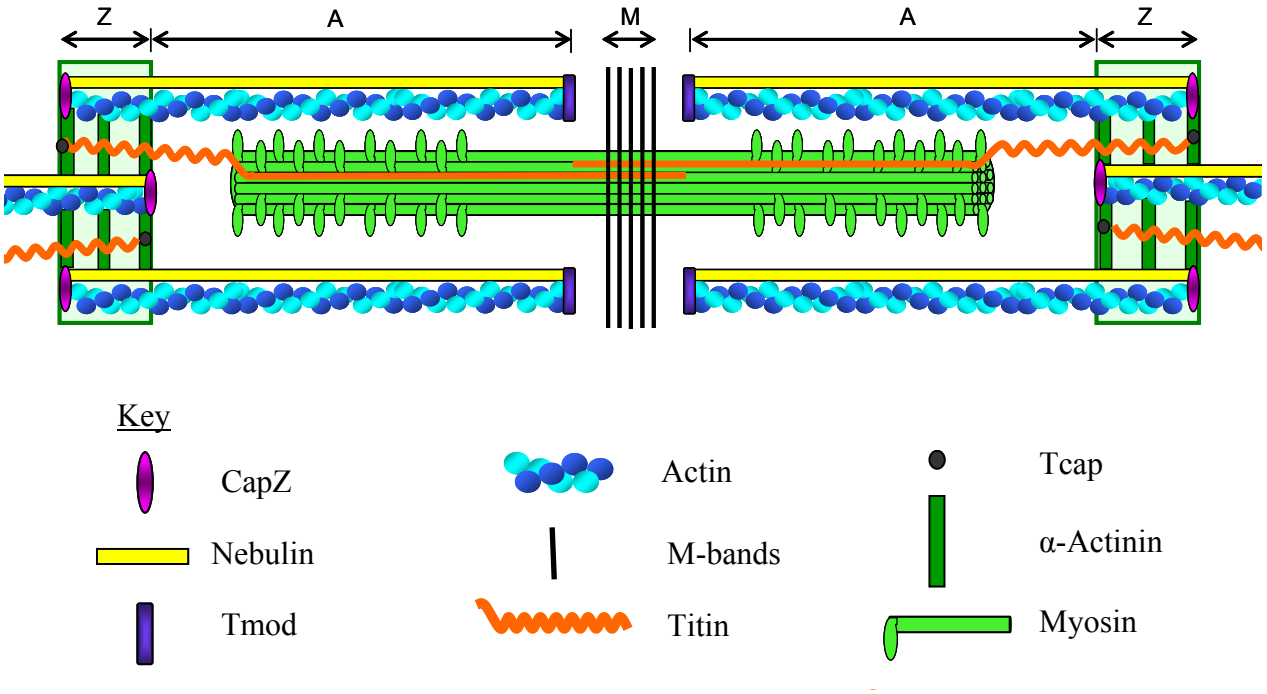


Fig 1.8. Diagram of the sarcomere illustrating the major components. The thin filament is made up of filamentous actin which is capped at each end by capZ and Tmod. Nebulin spans the length of the thin filament. The thick filament is composed of myosin bundles which are cross-linked at the M-band. Titin centres myosin to the middle of the sarcomere and is anchored to the Z-line by telethonin (Tcap). α-Actinin links thin filaments between adjacent sarcomeres and is one of the major components of the Z-line.

Within the sarcomere, each actin filament spans half of the I-band and part of the A-band. The pointed end extends towards the M-line and interdigitates and associates with the thick filament within the A-band region, while the barbed end is anchored to the Z-line (Fig. 1.8 light and dark blue helix). Regulation of actin filament formation, dynamics, maintenance and stability is very tightly controlled and this is reflected by the vast number of distinct actin binding proteins that cap, sever and crosslink the actin filament (dos Remedios et al., 2003).

CapZ and tropomodulin (Tmod) are the major capping proteins that cap the barbed and pointed end of the actin thin filament in striated muscle respectively (Fig. 1.8, capZ is represented as a pink disc and Tmod is represented as a purple rectangle). They are both important factors in actin dynamics and are able to regulate and maintain the uniform length of the actin filament. CapZ is a heterodimer composed of α and β subunits and is present in all eukaryotic cells (Cooper and Schafer, 2000). In skeletal muscle, capZ is localized at the Z-line of the sarcomere. A number of biological roles for capZ have been reported. Firstly, it can nucleate actin filament assembly *in vitro* (Fowler, 1996; Schafer et al., 1995). Secondly, its binding affinity for actin filament ends appears to be regulated by Phosphatidylinositol 4,5-bisphosphate ($PIP_2$) and increases in $PIP_2$ levels causes rapid disassociation of capZ from actin filament barbed ends *in vitro* (Schafer et al., 1996). Another candidate for regulating capZ's affinity for the barbed end is S100, a Z-line associated protein that binds to capZ in a calcium dependent manner (Ivanenkov et al., 1995; Kilby et al., 1997). S100 has been proposed modulate the function of capZ indirectly, by affecting its interaction with other Z-line components and regulating barbed end alignments at the Z-line (Littlefield and Fowler, 1998). Thirdly, capZ is thought to directly regulate isometric tension of sarcomeres in cardiomyocytes and is involved in the binding of protein kinase C to the myofibrils (Pyle et al., 2002; Pyle et al., 2006). Fourthly, capZ binds to α-actinin (Papa et al.,

1999) and nebulin (Witt et al., 2006) and is thought to anchor the actin thin filament to the Z-line. Finally, from *in vitro* studies, capZ is proposed to organize and align the barbed end of thin filaments at the Z-line (Schafer et al., 1995; Schafer et al., 1993).

At present four vertebrate Tmod isoforms (Tmod-1-4) are known and are referred to as E, N, U and Sk-Tmod respectively (Conley et al., 2001; Cox and Zoghbi, 2000; Fowler, 1990). Each isoform is encoded on a separate gene and all isoforms have unique expression profiles. Two of the Tmod isoforms are expressed in muscle: Tmod-1 is expressed in the heart and slow-twitch skeletal muscle and Tmod-4 (Sk-Tmod) is expressed in fast-twitch skeletal muscle. In addition to capping the actin filament, Tmod can simultaneously bind to nebulin (the thin filament ruler, see below) and Tropomyosin (Tm) (Greenfield and Fowler, 2002; Kostyukova et al., 2006; Vera et al., 2000). Within striated myofibrils Tmod is regarded as one of the key elements in maintaining sarcomeric structure and regulating actin filament length (Fischer and Fowler, 2003; Gregorio et al., 1995; Littlefield et al., 2001; Sussman et al., 1998; Ursitti and Fowler, 1994).

Nebulin is one of the largest proteins identified in vertebrates. It is encoded on one gene and is extensively alternately spliced to produce isoforms varying in size between 500 – 900 kDa (McElhinny et al., 2003). Over 95% of the protein consists of 35 residue repeat modules that function as actin binding domains (Horowits et al., 1996; McElhinny et al., 2003; Wang, 1996). Each group of seven 35 residue repeat modules are arranged into a higher order repeating unit termed a super-repeat. Nebulin is anchored to the Z-line at its C-terminus and extends the length of the thin filament (Fig. 1.8, yellow rod). It binds to many different components of the sarcomere such as myopalladin and the actin capping proteins capZ and Tmod (Bang et al., 2001a; McElhinny et al., 2001; Witt et al., 2006). Modules that span the A-I junction bind to actin, myosin and calmodulin (Jin and Wang, 1991; Lukoyanova et al., 2002; Root and Wang,

2001; Wang, 1996). Nebulin (also known as nebulette in the heart) is expressed in the heart but at much lower levels than in skeletal muscle (Kazmierski et al., 2003). It appears to assemble early in myofibrillogenesis before actin filaments attain their mature lengths and organization (Ojima et al., 1999).

Nebulin is predicted to act as a molecular ruler for defining the length of thin filaments in striated muscle (Wang and Wright, 1988). A number of properties of this protein make it an ideal candidate for a ruler. Firstly, the entire protein spans the length of the actin filament. Secondly, it binds to both actin thin filament capping proteins (capZ and Tmod) and thirdly it is able to associate with the actin filament along its entire length. Indeed, loss of nebulin in cultured rat cardiomyocytes resulted in longer actin filaments (McElhinny et al., 2005). However, in contrast to this finding, two separate studies that generated a nebulin targeted mouse mutant suggested that actin filaments were shorter as a result of a lack of nebulin (Bang et al., 2006; Witt et al., 2006). Although nebulin has a role in maintaining the length of the thin filament, the precise mechanism of how it contributes to this process remains unknown. However, it is likely to involve a number of other sarcomeric components which act in concert to precisely regulate actin dynamics and thereby control the length of actin filaments. The role of nebulin and other proteins associated with thin filaments in actin dynamics will be discussed at the end of this chapter.

Tropomyosin (Tm) and Troponin (Tn) are key elements in the interaction between thin and thick filaments and thus the generation of force. Tm forms homodimers and heterodimers to produce two chained parallel coils that extend the length of both sides of the actin thin filament (Perry, 2001). In resting muscle Tm blocks the myosin head from binding to the actin filament. Tn is a trimer composed of three subunits C, I and T. Upon activation of muscle contraction calcium binds to TnC, altering its confirmation and thus the interaction between TnC and TnI

(McKay et al., 1997). As a result Tm shifts its position and therefore a binding site becomes available for the myosin head to dock to the actin filament. Once the myosin head has bound to actin, myosin ATPase is activated and causes further movement of Tm along the rest of the thin filament, thereby allowing myosin to 'walk' along the entire length of the filament and generate force (Craig and Lehman, 2001). Tm is also thought to function in stabilizing the thin filaments as it increases the stiffness of the actin polymer and inhibits fragmentation of the filament (Wegner, 1982). It is also able to affect polymerization and depolymerization at the pointed ends due to its interaction with the N-terminus of Tmod (Broschat, 1990; Kostyukova and Hitchcock-DeGregori, 2004). The interaction of Tmod with Tm increases the binding affinity between Tmod and the pointed ends of the actin filament, and in the absence of Tm it has been shown that Tmod caps the pointed end much more weakly (Babcock and Fowler, 1994).

#### 1.4.1.2 The myosin thick filament and its associated proteins

Myosin is the second most abundant protein in striated muscle after actin and is regarded as a molecular motor that drives muscle contraction. Different muscle types generate forces at different strengths. This is achieved by having many different classes of heavy and light chain myosin isoforms that have varying activity rates and are tightly regulated in terms of their expression. Indeed, 15 classes of myosin heavy and light chain isoforms have been identified (reviewed in Sellers, 2000). The thick filament within striated muscle is composed of members of the myosin II family. Each member of this family is comprised of a long rod like tail domain and two head domains. The rod domain is composed of two identical heavy chains that are twisted into a coiled coil at their C-terminal regions. The head domain is composed of the N-terminal regions of each heavy chain from the rod domain and two light chains. Each head domain has ATPase and motor activity and binds to actin (forming a cross-bridge, reviewed Milligan, 1996),

as well as containing binding sites for nucleotides (Rayment et al., 1993). The rod enables two myosin filaments to join in a bipolar orientation (Fig. 1.8, light green golf shaped rods), so that the thick filament is able to move groups of oppositely oriented actin filaments past each other and induce muscle contraction. Briefly, muscle contraction involves changes to the conformation of the myosin head derived from ATP binding and hydrolysis. Attachment of ATP to the myosin head changes the conformation of the actin binding site, thus reducing the affinity of the myosin head for the actin filament. Moreover, ATP binding allows the myosin head to move along the actin filament. ADP and inorganic phosphate (Pi) remain attached to the myosin head after ATP hydrolysis.At this stage the myosin head binds weakly to a new site on the actin filament, however, release of the Pi results in tighter binding and induces the head to undergo a large angular rotation (the power stroke). Following rotation, ADP dissociates and the actomyosin complex returns to the relaxed state.

Titin is the largest protein identified to date (Maruyama et al., 1977; Wang et al., 1979). Two titin molecules are associated with each thick filament and span the entire sarcomere (Fig. 1.8, orange line). The C-terminal end of each titin molecule overlaps at the M-line, and at the Z-line the N-termini of titin from adjacent sarcomeres overlap. Titin appears to have a number of roles in sarcomeric function and assembly. Firstly, the portion of titin spanning the I band has elastic properties and therefore it has been labeled as a molecular spring and is thought to maintain the position of the thick filaments during contraction and resting phases. Secondly, it is a primary candidate for acting as the template for sarcomere assembly. Indeed, ablation of titin in rat cardiomyocytes disrupted myosin incorporation into myofibrils (Person et al., 2000) and similar results were obtained in a myofibroblast cell line lacking titin (van der Ven et al., 2000). Therefore it has been hypothesized that titin is required for thick filament incorporation into the sarcomere. Titin may also play a role in assembly of the Z-lines. The N-terminus of titin is

capped and anchored to the Z-line by telethonin (Tcap) (Gregorio et al., 1998; Mues et al., 1998). In addition to this anchorage point the N-terminal region of titin also contains a number of repeats (Z-repeats) that interact with α-actinin, the predominant scaffolding element of the Z-line. Intriguingly, the number of titin Z-repeats varies between muscle types that differ in Z-line width. The current hypothesis is that titin can influence the width of the Z-line by regulating the number of cross-links generated between anti-parallel titin Z-repeats and α-actinin dimers (Ohtsuka et al., 1997; Sorimachi et al., 1997). Finally, it has been suggested that titin is also involved in signaling pathways as the C-terminus contains a serine/threonine kinase domain.

Myosin binding proteins C and H (MyBP-C and MyBP-H) bind to myosin at the bare zone of the thick filament in a series of transverse stripes. MyBP-H is also found outside this zone (Bahler et al., 1985; Bennett and Bagshaw, 1986; Craig and Offer, 1976). To date only MyBP-C has been found to contain a titin binding site (Gilbert et al., 1996; Gruen et al., 1999) and it has therefore been speculated that this protein links titin to the myosin filament and aligns the thick filaments within the A-band. It has also been reported that both these proteins are important in aiding the assembly of the myosin polymer to the correct length, as myosin filaments polymerized in the absence of MyBP-C are shorter and less uniform (Davis, 1988; Gregorio et al., 1998; Koretz, 1979; Seiler et al., 1996). They may also be involved in regulating muscle contraction (for review see Winegrad, 1999).

#### 1.4.1.3 Z-line

The boundaries of each sarcomere are defined by Z-lines that are precisely aligned within and between myofibrils. This structure is thought to act like a scaffold and anchors all the major components of the sarcomere including actin thin filaments, titin and nebulin. An increasing

number of novel proteins associated with the Z-line have also been discovered such as myotilin, cypher, MuRF1 and myopalladin (Frank et al., 2006). For many of these novel proteins their function at the Z-line is still unknown, however, a few key proteins will be discussed below. In addition to integrating the sarcomeric components, the Z-line is also laterally associated with intermediate filament proteins such as desmin. These associated proteins are thought to link the peripheral myofibrils to costameres at the sarcolemma, and also to mitochondria and the nuclear membrane. The Z-line has been likened to a biomechanical sensor that can respond to changes in tension in the sarcolemma. Indeed, Z-lines are responsible for transmitting tension generated by individual sarcomeres along the length of the myofibril, allowing for efficient contractile activity (Squire, 1997). Due to the multiple functions of the Z-line it is not surprising that many different proteins associate with it and assist in maintaining its structural stability and signalling properties. Deciphering the molecular interactions of the proteins involved in the formation and maintenance of Z-lines will be pivotal for understanding the regulation of myofibril assembly, sarcomeric organization and the mechanical properties of striated muscle.

The major component of the Z-line is the actin thin filament cross linking protein α-actinin (reviewed Blanchard et al., 1989). The rod domains of actinin monomers interact to form anti-parallel dimers which cross link actin and titin from adjacent sarcomeres. Moreover, they are capable of transmitting and distributing the force generated by the sliding filaments (Vigoreaux, 1994). Intriguingly, in invertebrates α-actinin is not essential for proper assembly of the sarcomere (Fyrberg et al., 1998). In *Drosophila* the muscle still forms in the absence of α-actinin and although there are defects in Z-line organization and muscle attachment to the tendon matrix, the muscle is still able to contract albeit poorly. α-Actinin therefore appears to be required for sarcomere stability once contraction begins. Many other Z-line proteins are able to bind to α-actinin and therefore it is understandable that even in its absence a Z-line can still form. The

modular nature of α-actinin enables it to bind to many proteins simultaneously. In addition to actin, α-actinin interacts with muscle LIM proteins (MLPs), α-actinin associated LIM proteins (ALPs), FATZ-1 (also known as calsarcin-2/myozenin) and myopalladin.

MLPs are members of the cysteine rich protein (CRP) family and localize to the periphery of the Z-line and the intercalated disc in cardiomyocytes (Ehler et al., 2001). In addition to binding to α-actinin, MLPs can also bind to zyxin and nebulin related anchoring protein (NRAP) (Ehler et al., 2001). The multiple binding properties of this protein reinforce the hypothesis that MLPs act as scaffold or linker proteins. Loss of this protein in cardiac muscle results in disorganization of the myofibrils suggesting that its primary role is in stabilizing the Z-line (Arber et al., 1997). It may also link the Z-line to the membrane via costameres through its interaction with NRAP. In *Drosophila*, MLPs are localized to the nucleus and in vertebrates over expression in cultured myocytes enhanced muscle differentiation (Arber et al., 1994). Moreover, MLPs have been shown to bind to MyoD *in vitro* (Kong et al., 1997), therefore they may also play a role in directly regulating transcription of muscle components.

Several members of the enigma protein family are expressed in muscle and localize to the Z-line including enigma, ALP and cypher. All these proteins bind to α-actinin and enigma is also able to bind to Tm. One of the functions of the enigma protein may be to serve as a docking site for other signaling molecules at the Z-line. For example enigma and cypher bind to protein kinase C. Moreover, mouse knock outs of *cypher* and *ALP* indicate that they stabilize the muscle cytoskeleton (Pashmforoush et al., 2001; Zhou et al., 2001).

Filamin, α-actinin and telethonin binding protein of the Z-disc-1 (FATZ-1), as its name suggests, binds to α-actinin, telethonin and filamin and is exclusively located at the Z-line

(Faulkner et al., 2000; Takada et al., 2001). FATZ-1 is part of a novel family of proteins named calsarcins. They are able to interact with calcineurin, a calcium/calmodulin dependent phosphatase which is involved in the regulation of genes affecting muscle differentiation and the formation of different fibre types (Delling et al., 2000). FATZ-1 has been proposed to anchor calcineurin to the Z-line and may regulate its activity.

Myopalladin tethers together the C-terminal domain of nebulin to α-actinin at the Z-line (Bang et al., 2001b). The N-terminal region of myopalladin also specifically binds to cardiac ankyrin repeat protein (CARP). CARP is a nuclear protein involved in control of muscle gene expression and has been shown to negatively regulate the expression of cardiac genes. Myopalladin may link the regulatory mechanisms involved in Z-line structure (i.e. α-actinin and nebulin) to those involved in muscle gene expression (Bang et al., 2001b).

#### 1.4.1.4 M-line components

The formation of the M-line or M-band is proposed to be the final step in myofibril assembly (Markwald, 1973). It is thought to arrange and anchor thick filaments within the sarcomere (Agarkova and Perriard, 2005; Knappeis and Carlsen, 1968; Luther and Squire, 1978). Indeed, myosin filaments are crosslinked at the M-line by M-bridges (Van Der Ven et al., 1996). As yet it has still not been confirmed what these M-bridges are made of, however, the two primary candidates are myomesin and M-protein. Myomesin is arranged in an anti-parallel staggered fashion at the M-line (Obermann et al., 1996) and is able to bind to titin (Obermann et al., 1997) and myosin. Due to these properties myomesin has been proposed to integrate the thick filament into the sarcomere (Ehler 1999). M-protein has only been found at the M-line of fast muscle fibre types and cardiac muscle (Grove et al., 1984; Noguchi and Tanaka, 1982; Obermann

et al., 1997). Like myomesin it is also able to bind to titin and myosin (Masaki and Takaiti, 1974; Nave et al., 1989), however, its function remains unclear. It is possible that it may interact with myomesin and MyBP-C to anchor thick filaments to titin in fast skeletal and cardiac muscle (Clark et al., 2002).

Three members of the Muscle specific RING finger protein family (MuRFs) have been identified (Centner et al., 2001; Dai and Liew, 2001). MuRF1 is the only member that directly binds to titin at the M-line (Centner et al., 2001). Recent studies have implicated MuRF-1 in regulating muscle protein degradation (Bodine et al., 2001). Intriguingly, this protein has also been associated with the nucleus and interacts with transcription factors as well as other nuclear components. Therefore there is speculation that this protein is in fact dynamic and is involved in regulating myofibril signaling pathways.

### 1.4.2 The Sarcolemma

The sarcolemma is the plasma membrane of the muscle cell. It is connected to the underlying myofibrils and organelles by a cytoskeletal network that is essential for stabilizing the muscle fibre and transducing the force across each cell. Intermediate filaments and microtubules are the main cytoskeletal elements that link the sarcomere to anchorage points associated with the sarcolemma (e.g. the costameric network and myotendinous junction in skeletal muscle and the intercalated disc in cardiac muscle (Fig. 1.9)).

#### 1.4.2.1 Intermediate filaments and microtubules

The intermediate filament cytoskeleton of mature muscle is composed predominantly of desmin (Lazarides, 1980; Lazarides, 1982). It binds to a number of intermediate filament proteins such as skelemin, desmuslin and syncoilin (Bilak et al., 1998; Granger and Lazarides, 1980; Newey et al., 2001). In addition, it also interacts with sarcomeric proteins such as α-actinin and nebulin (Bang et al., 2002) and is thought to link individual myofibrils laterally via the Z-lines (Fig. 1.9). Moreover, it has been shown to connect the sarcomeres with the mitochondria, nucleus, sarcolemma, microtubules and is also concentrated at myotendinous junctions of skeletal muscle. Knock out mice studies indicate that although desmin is not essential for muscle function it contributes to maintaining the integrity and alignment of the mature and regenerating myofibrils (Carlsson et al., 1999; Li et al., 1997). Less is known about other intermediate filament proteins such as vimentin, nestin, synemin and paranemin, however, it is thought that they may be important in the assembly of the muscle cytoarchitecture and may link contractile apparatus to costameres or adjacent myofibrils (reviewed in Clark et al., 2002). Additional intermediate filament proteins such as cytokeratins are thought to cooperate with desmin and its associated molecules in organizing and stabilizing the sarcolemma (O'Neill et al., 2002). Unexpectedly, nuclear lamins also appear to be involved in contributing to muscle cytoarchitecture and function. Moreover, mutations in a number of *lamins* are the cause of several types of muscular dystrophy (Morris, 2001).

Microtubules are polymers of α and β tubulin and are involved in numerous cell processes such as mitosis, cell motility and intracellular transport. Relatively little is known about their role in striated muscle, however, mounting evidence is emerging that microtubules are involved in muscle differentiation, morphology and contractile activity. In skeletal muscle microtubules are

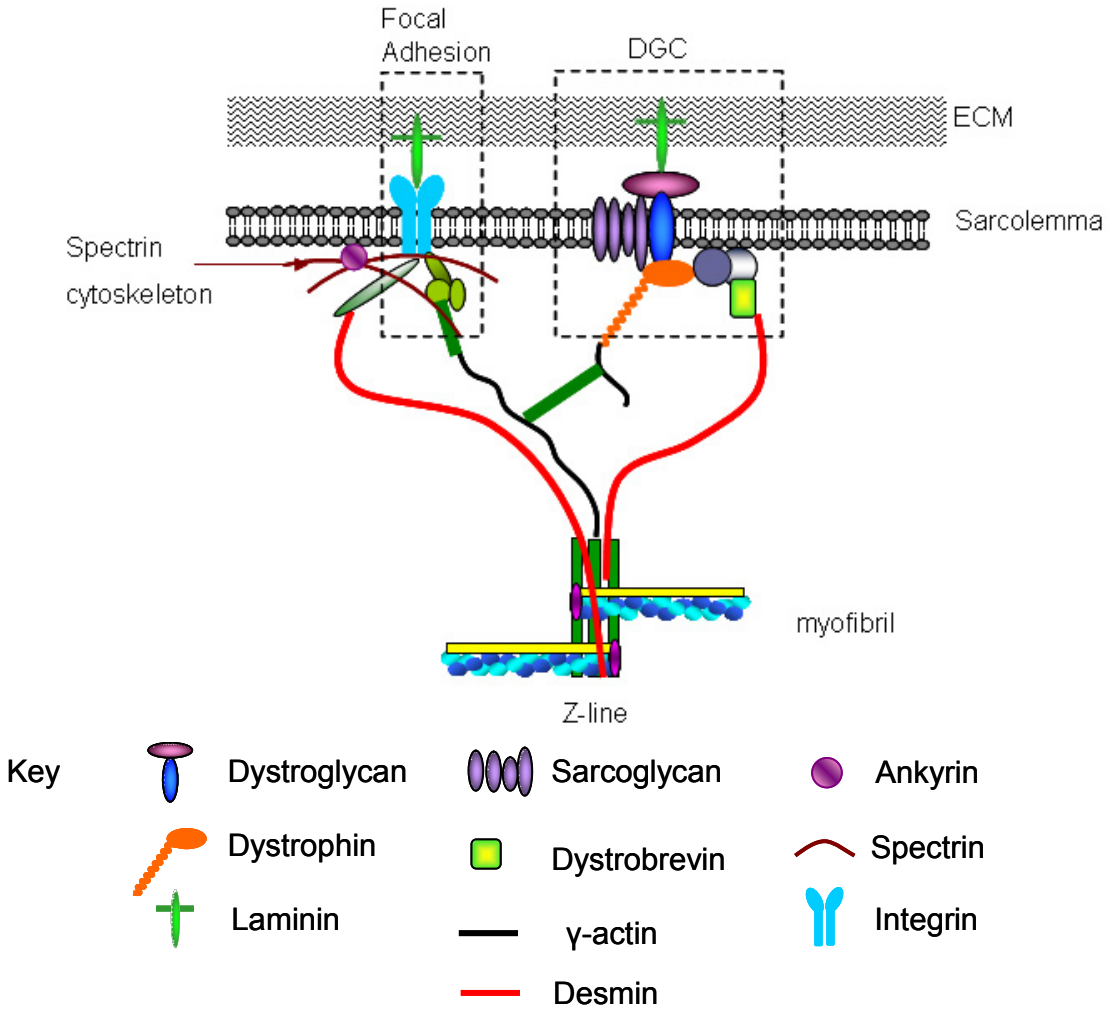


Fig. 1.9. Illustration of the costameric network and the components that link the myofibrils to the sarcolemma. The focal adhesion / integrin kinase complex and the dystrophin glycoprotein complex (DGC) are boxed. The spectrin cytoskeleton (brown lines) is anchored to the sarcolemma via ankyrin (purple circle). Desmin (red lines) and γ-actin (black lines) link the costameric network to the myofibrils by attaching to Z-lines. Most intermediate filament proteins, including desmin associate with the costamere through dystrophin and dystrobrevin.

positioned between myofibrils and are also associated with the sarcolemma, the golgi complex and nuclei. When microtubule dynamics is disrupted in skeletal myocytes, myoblast fusion and differentiation are disrupted (Antin et al., 1981; Saitoh et al., 1988). Moreover, the loss of microtubules in heart muscle affects contractile function (reviewed in Hein et al., 2000).

#### 1.4.2.2 The Costameric network

Costameres are subsarcolemmal protein assemblies that surround and align with each Z-line and M-line of the peripheral myofibrils and anchor the sarcomere to the sarcolemma (Craig and Pardo, 1983; Danowski et al., 1992; Pardo et al., 1983; Porter et al., 1992). Costameres are composed of at least three structures: integrins /focal adhesion complexes; the dystrophin glycoprotein complex (DGC) and the spectrin based cytoskeleton (Fig. 1.9). All these elements work together to promote the stability of the muscle fibre and to efficiently transduce the force from the Z-line to the membrane, where the force is transmitted laterally to the muscle termini (Danowski et al., 1992).

Integrins are transmembrane proteins that mediate attachment of the actin cytoskeleton to the ECM (Adams and Watt, 1993). Over 20 distinct integrin isoforms have been identified so far and each possesses unique functional properties (Bouvard et al., 2001). β1 Integrin is enriched within costameres and studies indicate that it plays a critical role in maintaining the organization of sarcomeres in cardiomyocytes (Fassler et al., 1996). In addition to playing a structural role within striated muscle, integrins are also important signaling molecules and are able to sense mechanical stress and activate signaling pathways to induce changes in gene transcription and cytoskeletal reorganization (reviewed in Shyy and Chien, 1997). Their prominent location makes them ideal candidates for biomechanical sensors that respond to changes in force.

The DGC functions to anchor the sarcolemma to the costameres and stabilize the sarcolemma against the physical forces transduced through costameres during muscle contraction. Defects in a number of molecules that make up this complex such as dystrophin, dystroglycan, laminin 2 and sarcoglycans result in human muscular dystrophies (reviewed in Davies and Nowak, 2006). Dystrophin is the core component of this complex and mediates the critical connection between the ECM, the muscle membrane and the costameric network by linking β dystroglycan to costameric actin. Indeed, as previously described in section 1.1.2, mutations in *dystrophin* result in DMD, the most common form of human muscular dystrophy. Moreover, dystrophin is also important in the organization of other components of the costameric network such as vinculin and spectrin (Williams and Bloch, 1999).

The involvement of spectrins in the costameric network is just beginning to be elucidated. Spectrin is primarily known for organizing the membrane cytoskeleton of erythrocytes and in conjunction with ankyrin, also plays a prominent role in anchoring integral membrane proteins to the membrane cytoskeleton (reviewed in Thomas, 2001). In striated muscle α/β spectrin heterodimers are found in the costameric network surrounding the Z-line, whereas β homodimers are found at the M-line (Porter et al., 1997). All populations of spectrin are linked to ankyrin, which limits the spectrin cytoskeleton to the sarcolemma (Williams et al., 2001).

#### 1.4.2.3 Myotendinous junction

The myotendinous junction (MTJ) is the structure in skeletal muscle where the myofibrils terminate and is equivalent to the vertical myoseptum in zebrafish. The MTJ is especially

reinforced as it is the main structure that bears the brunt of the force transmitted during muscle contraction. The thin filaments terminate at a dense subplasmalemmal layer that appears to mediate thin filament attachment to each other and to the muscle membrane (reviewed in Tidball, 1991). Focal adhesion-like adhesion plaques containing integrins, vinculin and talin are found at the MTJ along with the DGC, and implicates these structures in connecting the terminal thin filaments to the muscle membrane.

## 1.5 Myofibrillogenesis

Formation of myofibrils requires the assembly of thin and thick filaments, Z and M-lines and the association of many other components that are all arranged into a highly organized structure to form the sarcomere. Due to the complexity of the sarcomeric structure deciphering the mechanisms that lead to its assembly have proved to be challenging, however, a number of models have been proposed.

### 1.5.1 Models for sarcomere assembly

It is generally agreed that myofibrillogenesis occurs in a stepwise process and initially occurs in association with the sarcolemma. One of the first models described was the template model (Dlugosz et al., 1984). In this model thin filaments, thick filaments and Z-band proteins are recruited to stress fibre-like structures (temporary bundles of actin) in the developing muscle cell. These structures serve as a template for assembly of the sarcomeres. This model has since been disregarded as the stress fibre-like structures are composed of sarcomeric proteins such as α-actinin, tropomyosin, troponins and tropomodulin (Almenar-Queralt et al., 1999; Rhee et al., 1994). Thus it has been concluded that the stress fibre-like structures are in fact the newly formed myofibrils themselves. It has also been observed that Z-bodies containing α-actinin fuse to form mature Z-lines in myofibrils of muscle cells (Dabiri et al., 1997; McKenna et al., 1986). This finding is inconsistent with the template model, where it would be expected that no fusion would occur as the stress fibre-like structures should already act as a template to position all the components of the sarcomere.

The independent subunit assembly model (the stitching model) proposes that I-Z-I bodies (dense bodies of Z-band proteins associated with thin filament) and thick filaments assemble randomly throughout the muscle cell (Holtzer et al., 1997). Titin then joins the I-Z-I bodies and thick filaments together to form sarcomeres. This model was based on reports where myosin was not detected between the I-Z-I bodies (Schultheiss et al., 1990). Moreover, it was supported by studies of *Drosophila* mutants that lacked muscle myosin, where the I-Z-I brushes in flight muscles were still able to align (Beall et al., 1989). The caveat of this model is that it omits evidence for the presence of non-muscle myosin II, detected in differentiating cardiomyocytes (Dlugosz et al., 1984; Fallon and Nachmias, 1980). However, the third model (the premyofibril model) does explain the presence of non-muscle myosin II in myofibrillogenesis.

The premyofibril model incorporates aspects of the two previously described models. In this model premyofibrils containing 'mini sarcomeres' (I-Z-I bodies which are distributed throughout the myofibril) interdigitate with non-muscle myosin II in the absence of titin. Premyofibrils then become nascent myofibrils, where non-muscle myosin II is replaced by myosin and titin. Moreover, the thick filaments become fairly well aligned between adjacent clusters of Z-bodies, which begin to align and cluster into groups and often form irregular Z-lines. Actin and titin may contribute to aligning the thick filaments (that have been assembled separately) into the sarcomere. At this stage the M-line becomes apparent, however, the I-bands are still of a variable length. These nascent myofibrils then become mature myofibrils by further addition of myosin, titin, MyBP-C, and other elements that localize to the M and Z-lines. Finally, the thin filaments within the myofibrils mature and grow in length resulting in complete alignment of all sarcomeric components (Rhee et al., 1994).

One of the main controversies of this current model is the function and presence of non-muscle myosin IIb. It has been demonstrated that non-muscle myosin IIb is present in association with I-Z-I bodies in cardiomyocytes (Rhee et al., 1994), and it has been proposed that the function of this protein would be to interdigitate and organize the mini-sarcomeres prior to the incorporation of muscle myosin and titin. Indeed, inhibition of phosphorylation activity of non-muscle myosin results in the loss of premyofibrils and nascent myofibrils in cardiomyocytes (Du et al., 2003; Ferrari et al., 1998). However, sarcomeres are still formed in the mouse knock out of non-muscle myosin IIb (Tullio et al., 1997). It is also unclear how non-muscle myosin is replaced by muscle myosin.

The caveat of all the models proposed is that they are based on observations and experiments performed in cultured cells, which may not truly reflect what occurs *in vivo*. Cultured muscle cell extracts may already have some mature myofibrils at the time of isolation, therefore it cannot be excluded that what is observed is not *de novo* myofibril assembly. The *in vitro* two dimensional environment might favour specific structures to form which aren't found *in vivo*. Indeed, several *in vivo* studies do not support many aspects of the premyofibril model. Costa and colleagues (2002) reported no intermediate premyofibril stages in zebrafish. Ehler and colleagues (1999) were also not able to detect any premyofibril like elements in developing chicken hearts, and although non-muscle myosin IIb was expressed in the heart it did not arrange between Z-bodies as was observed in cardiomyocytes. It is possible that premyofibrils are not detected as this event occurs much more quickly *in vivo* compared to within a cultured muscle cell. It is clear that although studies of myofibrillogenesis in cultured cells have been important in unraveling aspects of sarcomere assembly, to gain a more realistic understanding of events these processes need to be examined *in vivo*.

All the models proposed so far do not elaborate on how the final length of the thin filament is determined. However, two different proposals have been documented, the variable length model and the fixed length model. In the variable length model, which supports the Sanger premyofibril model, nascent thin filaments that are attached to the Z-bodies are either shorter or longer than their final mature length and in the final stages of maturation they grow to the correct size by polymerization from the barbed or pointed ends (Rhee et al., 1994). In the fixed length model proposed by Fowler and colleagues (1999) the thin filaments are pre-assembled to defined lengths by nebulin, the capping proteins are then integrated into non-striated premyofibrils (I-Z-I bands) and the thin filament is subsequently rearranged and aligned to form the proper length. The fixed length model proposes that the thick and thin filaments are organized into striated myofibrils by a process of filament sliding, where interactions with titin, nebulin, M-line and Z-line proteins align the barbed and pointed ends of the thin filament at the Z-line and central bare zone. Titin is one of the main candidates for aligning the filaments and has been proposed to act as a template to organize the thin and thick filaments into mature sarcomeres based on its size, its interacting partners and the presence of different isoforms i.e. different templates.

The length of the thin filament is likely to be defined by the dynamics of actin polymerization, which is regulated by nebulin and the capping proteins capZ and Tmod. Significantly, the C-terminus of nebulin and capZ are detected in discontinuous punctuate patterns in non-striated actin filament bundles at the tips of skeletal myotubes (Holtzer et al., 1997; Schafer et al., 1993). One could therefore speculate that nebulin acts as a template for the formation of actin filaments in the premyofibril with capZ and Tmod. This aspect of sarcomere assembly has still not been addressed fully in any of the models proposed to date, however, it is an integral part of myofibrillogenesis and investigation of this field may reveal new insights into sarcomere assembly.

### 1.5.2 Actin thin filament dynamics and assembly

Actin dynamics are crucial in not only assembly of the actin filament but maintaining the structure and length of each filament. Actin filaments differ in length between different cell types, however within each cell type the length is precisely controlled. A number of different components are required to maintain the length of the actin filament in the sarcomere, including the template protein (nebulin), capping proteins (Tmod and capZ) and severing proteins (e.g. gelsolin and severin), in addition to molecules that sequester monomeric actin or promote polymerization/depolymerization (Cooper and Schafer, 2000).

In most cells actin filaments are always in a continuous state of assembly and disassembly. Elongation of the actin filaments can occur from either the pointed or the barbed end. The predominant site of actin monomer incorporation in non-muscle actin occurs at the barbed ends of the actin filament (Dome et al., 1988; McKenna et al., 1985; Shimada et al., 1997) and disassociation occurs predominantly at the pointed ends. In striated muscle the dynamics of actin polymerization are quite different. Studies by Littlefield and colleagues in 2001 indicated the pointed ends were the major sites of actin monomer incorporation, and the dynamics of Tmod capping maintained and determined the length of the actin filament. Moreover, Tmod dynamics is regulated by tropomyosin and is able to exist in low and high affinity binding states (Fowler et al., 2003). While capZ only exists in a high affinity state, as the levels of exogenous CapZ in muscle cells are such that the barbed end is rapidly and securely capped (Schafer et al., 1996; Wear et al., 2003).

Nebulin has long been classified as the template that defines the length of the actin thin filament within sarcomeres. However, there has been much debate on exactly how this process

occurs (Fowler, 1997). The ruler mechanism is currently the most widely accepted theory for how actin filament length is defined. In this model the actin filament periodically binds to nebulin and extends along the length of the nebulin filament. Once the actin filament has reached each end of the nebulin filament it is capped by the capping proteins (capZ and Tmod), which prevent further monomer addition or loss and securely tether the ruler to the actin filament. In light of the recent findings that Tmod is integral to actin dynamics, the cap locator model has been proposed (Fowler et al., 2006). In this model the nebulin N-terminus is important in localizing Tmod to the desired length of the actin filament, and as nebulin interacts with Tmod at the N-terminus this end could create a localized concentration of Tmod. This model is consistent with findings that thin filament length is dependent on Tmod concentrations (Littlefield et al., 2001; Sussman et al., 1998), however, further experiments will be required to resolve which of the two models occurs *in vivo*.

The mechanisms involved in actin thin filament length regulation are complex and involve multiple sarcomeric components. Determining the molecular roles of each length determining factor will be important in defining how this process occurs. Moreover, it may provide further insight into how the actin filament is assembled into sarcomeres during myofibrillogenesis.

## 1.6 Objectives and outcomes of this thesis

The aim of this PhD project was to positionally clone and characterize the zebrafish muscle motility mutant *schnecke* (*sne*). I identified a mutation at the *sne* locus in *capzα1*, a subunit of the actin capping protein, CapZ. Analysis of the *sne* mutant and MO knockdown of other CapZ subunits suggests that CapZ is essential for the myofibril and sarcomeric integrity within zebrafish skeletal muscle. Additional double knockdown studies with associated components of the actin thin filament (Nebulin and Tmod) indicate that CapZ is required for maintenance of the Z-line and thin filament formation. Moreover, localization of the intermediate filament protein Desmin is altered in *sne* mutants, thus CapZ may be involved in linking the myofibrils with the costameric network. Finally, motoneuron development is affected in capZα1 MO injected embryos. This raises the possibility that CapZ may be directly involved in movements that facilitate motoneuron formation.

The results presented in this thesis provide new evidence for an additional role of CapZ in maintaining the stability of the myofibril within skeletal muscle. The phenotype produced from the mutation in *capzα1* illustrates the importance of sarcomeric proteins in maintaining muscle integrity, and although the loss of this protein does not completely abolish sarcomere formation, the architecture and integrity of the muscle is disrupted. The progressive degeneration of the muscle structure in the *sne* mutants is characteristic of human muscular dystrophies, therefore dysfunctional CapZ could potentially be a novel candidate for this type of myopathy. Further analysis of *sne* mutant will benefit our understanding of the complex interactions within the myofibre and will ultimately assist in our knowledge of many aspects of muscle function and development, which will enable us to design new methods in treating myogenic disorders.