# Chapter 1

# Introduction

## Project Aims and Summary

Prior to the commencement of this project, zebrafish homologues of JAM-B and JAM-C were included in a screen designed to identify novel cell surface receptor:ligand pairs using AVEXIS, a methodology developed in the lab. The purpose of this PhD project was to determine the biological function of the interaction between Jamb and Jamc during early development of the zebrafish embryo, if any. In pursuit of this aim, I studied the evolution and homology, developmental expression and biochemistry of the zebrafish *jam* family members to better understand the developmental role of *jamb* and *jamc*. I then characterized loss-of-function mutants and identified a novel role for these proteins in myoblast fusion. Finally, I demonstrated the necessity of interaction between Jamb and Jamc *in trans* between myoblasts for fusion.

In this chapter I describe what is known about JAM family proteins in other contexts, the role of cell surface proteins in myogenesis, the process of myoblast fusion and the use of the zebrafish as a model for vertebrate muscle development.

## 1.1 The *JAM* family

The mammalian junctional adhesion molecule (JAM) family consists of three immunoglobulin superfamily domain-containing cell surface proteins that have been implicated in a wide array of functions such as, but not limited to, angiogenesis (Lamanga *et al*, 2005a; Cooke *et al*, 2006; Rabquer *et al*, 2010), cancer (Santoso *et al*, 2005; Murakami et al, 2010; Tenan *et al*, 2010), tight junction formation (Ebnet *et al*, 2003; Mandell *et al*, 2004; Rehder *et al*, 2006; Mandicourt *et al*, 2007), leukocyte extravastion and inflammation (Johnson-Léger *et al*, 2002; Cera *et al*, 2004; Chavakis *et al*, 2004; Aurrand-Lions *et al*, 2005; Ludwig *et al*, 2005; Vonlaufen *et al*, 2007) and spermatogenesis (Gliki *et al*, 2004; Shao *et al*, 2008; Wang and Lui, 2009). These studies have largely been restricted to post-natal or adult mice and cultured cells. A common thread to most of these ascribed functions is the regulation of stability, permeability, and polarity of epithelia and endothelia and subsequent interactions with leukocytes (reviewed in Weber *et al*, 2007).

The purpose of this PhD project was to assess the biological function of the interaction between zebrafish homologues of *jamb* and *jamc* during development. This interaction was identified in a screen for physical interactions between cell surface proteins *in vitro*, using a specialised methodology developed in the laboratory: avidity-based extracellular interaction screen (AVEXIS; Bushell *et al*, 2008). For this reason I will restrict the scope of discussion to the functions of Jam-B and Jam-C that have been elucidated from studies of mutant animals.

Jam-B and Jam-C were discovered by different groups in different organisms at different times and given different names (Palmeri *et al*, 2000; Cunningham *et al*, 2000; Aurrand-Lions *et al*, 2001; Arrate *et al*, *2001*; Liang *et al*, 2002), based upon their homology to Jam-A, a cell surface protein characterised as a receptor for a stimulatory platelet antibody (Naik *et al*, 1995). The nomenclature of the homologues was rationalised much later by discussion within the field (Muller, 2003), but the widely accepted new system remains unofficial (see Chapter 3).

Jam-B and Jam-C were identified as binding partners that facilitate the binding of peripheral blood leukocytes to vascular endothelia and transmigration (Arrate *et al*, 2001; Liang *et al*, 2002). *Jam-C* has subsequently been extensively studied in the context of inflammatory diseases, for example, acute pancreatitis (Vonlaufen *et al*, 2006), and the immune system (Imhof *et al*, 2007) throughout the last decade. Most recently, studies of *Jam-C* knockout mice have revealed considerable deficiencies in the development and function of the immune system (Praetor *et al*, 2009; Zimmerli *et*

*al*, 2009). *Jam-C* is highly expressed on hematopoietic stem cells (HSCs), and loss-of-function of *Jam-C* results in a large increase in myeloid progenitors, suggesting Jam-C functions in the differentiation of HSCs (Praetor *et al*, 2009). However, this phenotype might be a secondary consequence of the susceptibility of *Jam-C* knockout mice to infection (Imhof *et al*, 2007; Zimmerli *et al*, 2009), that is, upregulated differentiation of hematopoietic stem cells may be a result of unchecked bacterial challenge (Scumpia *et al*, 2010).

In further studying the function of Jam-C in the immune system, Scheiermann *et al* (2007) observed defects in the peripheral nervous system. Jam-C is expressed by Schwann cells and is localised to paranodes surrounding Nodes of Ranvier, Schmidt-Lanterman incisures and mesaxonal bands (Scheiermann *et al*, 2007). Jam-C knockout mice show decreased nerve conductivity in electrophysiological experiments using sciatic nerves, likely resulting from a loss of integrity of the myelin sheath which surrounds axons. In addition, the authors noted a general muscle weakness quantified by reduced grip strength and stride length. This peripheral nerve phenotype might also explain the dilated oesophagus present in *Jam-C* knockout mice (Imhof *et al*, 2007). Megaoesophagus results from a lack of peristalsis in the oesophagus, suggesting a neural deficit (Shiina *et al*, 2010). The authors of this study suggested a function for *Jam-C* in the contractions of the smooth muscle cells lining the oesophagus and bronchial airways (Imhof *et al*, 2007), but did not speculate on the precise role of Jam-C. It is possible that the protein maintains coherent tight junctions between smooth muscle cells, in order to allow progression of the peristaltic movement.

The first *Jam-C* knockout mice phenotype described was that of complete male sterility (Gliki *et al*, 2004) resulting from defective polarisation of spermatids and subsequent arrest in maturation. The authors proposed that this defect resulted from a loss of interaction between Jam-C, expressed on the surface of spermatids, and Jam-B, expressed on the apical surface of Sertoli cells, to which spermatids are bound. The authors demonstrated that loss of Jam-C resulted in unpolarised localisation of the Par3-Par6-aPKCγ-Cdc42 cell polarity complex. Addition of soluble Jam-B could restore this localisation *in vitro*. Both Jam-B and Jam-C had previously been demonstrated to directly interact with Par3 through the cytoplasmic PDZ domain-binding motif (Ebnet *et al*, 2003).

In contrast, relatively few reports of *Jam-B* function have been published since its identification, including very little immunological research (Ludwig *et al*, 2005). It has been used as a marker to detect an unusual retinal ganglion cell type that is

polarised and only responds to upward movements, but no functional role for Jam-B was reported (Kim *et al*, 2008). Indeed, the paucity of functional data is likely because loss-of-function of *Jam-B* has no detectable impact on the phenotype of mice (Sakaguchi *et al*, 2006). None of the phenotypic characteristics of the *Jam-C* knockout mice have been replicated in *Jam-B* knockout mice, despite extensive characterisation. It is therefore difficult to establish which functions of Jam-C *in vivo*, if any, are a direct result of interaction with Jam-B. Most functions might be mediated by homophilic Jam-C interactions (Scheiermann *et al*, 2007; Mandicourt *et al*, 2007; Santoso *et al*, 2005), by interaction or regulation of various integrins (Cunningham *et al*, 2002; Santoso *et al*, 2002; Lamanga *et al*, 2005b; Mandicourt *et al*, 2007;) or other cell surface proteins, such as CAR (Mirza *et al*, 2006). There also remains the possibility of some functional redundancy amongst family members. For example, Jam-A is also expressed in Sertoli cells, though it is restricted to basal tight junctions (Gliki *et al*, 2004). In the absence of Jam-B, Jam-A might redistribute to the surface of Sertoli cells and act as a substitute. However, no interaction between Jam-A and Jam-C has been previously described.

No developmental function for JAM-B and JAM-C has been reported previously, and neither gene has been studied with respect to muscle development, despite being known to be expressed in skeletal muscle (see Chapter 4). What potential functions might these cell surface proteins have in myogenesis?

## 1.2 The role of cell surface proteins during myogenesis

Skeletal muscle development is a complex process involving multiple, co-ordinated behaviours and interactions between myocytes in order to form an ordely array of elongated, differentiated syncytia capable of the remarkable feat of translating chemical energy into mechanical force. This property has fascinated scientists and medics for centuries, resulting in a wealth of knowledge delineating the development and function of muscles. Here I highlight some of the important phases of muscle development that must require interactions between cell surface molecules, but have not necessarily been fully characterised.

Migration of muscle cells plays a key role in the development of all muscles in every model system examined. These movements can be local, for example, fusion competent myoblasts migrating to fuse with founder cells (Ruiz-Gomez *et al*, 2000; Strünkelnberg *et al*, 2001), the positional rotation of myoblasts within the somite of the zebrafish embryo (Hollway *et al*, 2007; Stellabotte *et al*, 2007), or myocytes delaminating from the dermomyotome to form the primary myotome in chick (Kahane

*et al*, 2002). They can also be long range, for example, the migration of myoblasts from the somites into the limbs of mouse embryos (Dietrich *et al*, 1999). In some cases, migration need not depend on the differentiation state of the muscle cell; for example, elongated and differentiated slow muscle fibres migrate through the myotome in zebrafish (Devoto *et al*, 1996). These migrations must clearly depend upon interactions between cell surface molecules and the extracellular matrix and other cells to gain traction, but also short or long range signalling for pathfinding, as exemplified by the tyrosine receptor kinase c-met and secreted hepatocyte growth factor (Dietrich *et al*, 1999).

Muscles are characteristically long fibres that link distant anatomical regions. It is necessary for the muscle cells to elongate and form myotendinous junctions after contact with extracellular matrix in vertebrates (Bassett *et al*, 2003; Kudo *et al*, 2004; Henry *et al*, 2005; Snow *et al*, 2008a) or tendon cells in *Drosophila* (Steigemann *et al*, 2004; Schnorrer *et al*, 2007; reviewed in Schnorrer and Dixon, 2004). This also requires some form of polarity and directional signalling through cell surface and secreted proteins, as demonstrated by *slit* mutants in *Drosophila* (Kramer *et al*, 2001), or loss-of-function of Wnt11 in chick (Gros *et al*, 2008). The formation of a specialised junction at the attachment site of muscles is crucial for force transduction, and therefore maintenance of the fibre, as is clearly evident in the wide-spectrum of dystrophies (Conti *et al*, 2009).

Muscle fibres are syncytia formed by the fusion of muscle cells, not mitosis of a muscle cell without division (Capers, 1960). This has been clearly demonstrated recently in time-lapse studies of zebrafish embryos expressing transgenic fluorescent reporter genes (Collins *et al*, 2010). Myoblast fusion involves multiple processes that occur between two muscle cells: recognition, adhesion and controlled breakdown and union of the membranes of both cells. Each of these steps require interactions between cell surface proteins, and these may have overlapping functions. The cell surface molecules involved in this process in *Drosophila* are well characterised, but less well understood in vertebrates (see below for more detailed discussion).

For skeletal muscle fibres to be functional, each fibre needs to be directly innervated. Innervation is not required for muscles to form (Bate, 1990; Hughes *et al*, 1992; Broadie and Bate 1993), but is important for secondary myogenesis (Ross *et al*, 1987; Condon *et al*, 1990). How motor axons project from the spinal cord into muscle and innervate specific muscle groups is an active area of research (reviewed in Bonanomi and Pfaff, 2010). Initial pathfinding of axon bundles to the axial muscles in chick is thought to require chemoattractants secreted by the dermomyotome, for

example FGF (Shirasaki *et al*, 2006), with further patterning of individual axons to different muscles based upon specific target identity, likely resulting from the expression of different cell surface molecules. One interesting example is *unplugged*, a zebrafish homologue of MuSK (Zhang *et al*, 2004). Loss-of-function of a splice variant of *unplugged* (SV1) expressed by adaxial cells results in severe axon pathfinding defects. The formation of neuromuscular junctions requires interactions between the muscle cell surface proteins MuSK (Kim and Burden, 2008) and Rapsyn (Gautam *et al*, 1995) which pre-pattern acetylcholine receptors (AChR) on the surface of muscle fibres into a centralised domain, independent of nerve contact (Yang *et al*, 2001). In zebrafish, it has been proposed that Wnt signalling activates MuSK to create a centralised muscle domain that defines axon pathfinding and clustering of AChRs (Jing *et al*, 2009).

These different aspects of muscle development require careful co-ordination and tight control. Unsurprisingly, many different signalling pathways have been implicated in the regulation of muscle development, with many of them seemingly multipurpose. For example, Hedgehog signalling is known to play a role in differentiation (Feng *et al*, 2006) general specification of muscle cells and fibre-type switching (reviewed in Ingham and Kim, 2005) and, indirectly, elongation of fast muscle cells (Peterson and Henry, 2010). These signalling pathways may also be partially redundant or overlapping, for example Hedgehog and FGF signalling regulating differentiation (Coutelle *et al*, 2001; Groves *et al*, 2005). Some may also be interpreted in different contexts, for instance, WNT signalling is a directional cue for the elongation of chick myocytes (Gros *et al*, 2008), is also necessary for myogenesis in the limb (Geetha-Loganathan *et al*, 2005), and is proposed to regulate motor axon pathfinding (Jing *et al*, 2009).

The nature of the loss-of-function of both Jamb and Jamc immediately suggested that both proteins play a critical role in myoblast fusion *in vivo* (Chapter 6). For this reason, I will discuss myoblast fusion in greater detail below.

## 1.3 Current opinions in myoblast fusion

Myoblast fusion has been best characterised in *Drosophila*, through extensive genetic screens and careful morphological description. There are several recent reviews describing the state of the art (Rochlin *et al*, 2009; Haralalka and Abmayr, 2010). Vertebrate myoblast fusion is somewhat less well described and implicitly assumed to be conserved with respect to regulation, mechanism and the molecules involved. There are similarities between the vertebrate and invertebrate models, but

not much has been made of apparent differences. Here I will describe the process of myoblast fusion in *Drosophila* and compare that to vertebrate models.

The musculature of the *Drosophila* larva abdomen segments, A2-A7, is composed of a repeating pattern of 30 morphologically distinct muscles, each a single syncytial fibre (Bate, 1990). Each of these muscles is pre-figured by a founder cell, specified within *twist, sloppy paired*-expressing somatic muscle mesoderm cells by *Notch*-mediated lateral inhibition (Carmena *et al*, 1995). This rare sub-population of myoblasts migrate to different positions within the external layers of each hemisegment (Bate, 1990), express a different set of transcription factors (reviewed in Baylies *et al*, 1998) and fuse to fusion competent myoblasts (FCMs; Ruiz-Gomez *et al*, 2000; Bour *et al*, 2000; Strünkelnberg *et al*, 2001;) to form elongated muscle fibres that make contact with tendon cells of the epidermis (Steigemann *et al*, 2004; Schnorrer *et al*, 2007) and are subsequently innervated (Bate, 1990). FCMs are the more numerous sub-population of myoblasts that are identified by expression of *lameduck* (Ruiz-Gomez *et al*, 2002). Those FCMs nearest to founder cells are the first to fuse to the muscle precursors, with more internal FCMs migrating through the somatic mesoderm later (Beckett and Baylies, 2007). The process of myoblast fusion is iterative, with each muscle containing between 2 to 24 nuclei after the end of fusion at stage 15 (Bate, 1990).

The founder cells and FCMs recognise and adhere to each other through the cell surface proteins *roughest* (*rst*), *dumbfounded/kirre* (*duf*), *hibris* (*hbs*) and *sticks-and-stones (sns*; Galleta *et al*, 2004, Artero *et al*, 2001). The current paradigm posits that the founder cell-specific receptor Duf interacts with the FCM-specific receptor SNS to localise the intracellular molecular machinery necessary for fusion, to a specific site within the membrane. This results in actin-rich foci (Richardson *et al*, 2007) surrounded by a protein-dense structure, termed the fusion-restricted myogenic adhesion structure (FuRMAS; Kesper *et al*, 2007). Following this, small fusion pores occur at the site of fusion and expand over time (Doberstein *et al*, 1997). These processes require Rac signalling and actin cytoskeletal rearrangement via the Arp2/3 complex (Richardson *et al*, 2007), to regulate vesicle trafficking to and from the site of fusion (Kim *et al*, 2007; Estrada *et al*, 2007). The exact role of *roughest* and *hibris* is uncertain, as both are partially redundant with *dumbfounded* and *sticks-and-stones* (Strünkelnberg *et al*, 2001; Artero *et al*, 2001). *roughest* is expressed in both founders and FCMs (Strünkelnberg *et al*, 2001), whilst *hibris* is only expressed in a few FCMs (Artero *et al*, 2001). The functional differences between the paralogues *roughest* and *dumbfounded*, *hibris* and *sticks-and-stones*, likely result from

differences in the cytoplasmic domains of the proteins (Strünkelnberg *et al*, 2001; Shelton *et al*, 2009).

The critical receptor:ligand pair for recognition and adhesion has been identified in *Drosophila* – deletion of the partially redundant *rst*/*duf* or *sns* results in a complete block of myoblast fusion (Strünkelnberg *et al*, 2001; Bour *et al*, 2000). The FCMs persist as unfused, rounded cells that express myosin heavy chain (MyHC) and are eventually cleared by macrophages. In contrast, the founder cells elongate to form mononucleate muscle fibres. The mutant embryos subsequently die as they are unable to break free from the vitelline membrane.

No such receptor:ligand pair has been identified in any other model system. The role of vertebrate orthologues of *rst*/*duf* and *sns* have often been hinted at as drawing clear parallels between invertebrate and vertebrate myoblast fusion, but yet the applicability of this paradigm remains to be definitively proven. Loss-of-function of *kirrel,* a homologue of *rst*/*duf* in zebrafish, results in a severe myoblast fusion phenotype (Srinivas *et al*, 2007). It is unclear if the phenotypic consequences of morpholinos targeted against *kirrel* are similar to those seen in *Drosophila* mutants. It is also unclear if Kirrel acts homophilically or interacts with other receptors (see Chapter 8). The evolutionary relationship between *kirrel*, *duf* and *rst* has also yet to be fully explored. No muscle phenotype has been reported for any of the mammalian *duf* homologues (Donoviel *et al*, 2001; Tang *et al*, 2010). The *sns* homologue *nephrin* has been implicated in myoblast fusion in mouse and zebrafish, largely because of its orthology. Its role in myoblast fusion, if any, has been very poorly characterised with respect to expression or function (Sohn *et al*, 2009). Other cell surface molecules such as CDO (Cole *et al*, 2004), cadherins (Charlton *et al*, 1997; Hollnagel *et al*, 2002), neogenin and netrin (Bae *et al*, 2009) and NCAM (Charlton *et al*, 2000) have all been suggested to play a role in myoblast fusion, but none seem to have a detectable effect on myoblast fusion in mouse embryos.

In summary, it is uncertain if vertebrate myoblast fusion requires the specification of a founder cell population that prefigures muscle pattern. Identification of critical cell surface molecules that are relevant to all vertebrate models would be of great assistance to elucidating the mechanism and regulation of fusion within the developing embryo.

## 1.4 Zebrafish as a model for vertebrate myogenesis

It is not surprising that many cell surface molecules have been implicated in muscle development, given the many possible important roles they may play. The

predominant functional annotation in vertebrate studies, however, is mild reduction in myoblast fusion, often only determined in cell culture after little or no noticeable effect in animal models. There is little ability to discern between the subtly different physiological functions of cell surface proteins in cell culture, many of which may play partially redundant roles, for example, cadherins (Krauss, 2010). In mouse knockout models, no muscle phenotype has been described for cadherins (Charlton *et al*, 1997; Hollnagel *et al*, 2002), even M-cadherin which is almost exclusively expressed in developing muscle (Hollnagel *et al*, 2002). In contrast, knockdown of M- and N-cadherin in zebrafish has been shown to be essential for the migration of slow muscle fibres through the myotome (Cortés *et al*, 2003). These genes are unlikely to play exactly the same role in mammals, where slow and fast fibre types are not spatially separated (Hämäläinen and Pette, 1995), but this example does highlight the power of the zebrafish as a model system for functional analysis of genes involved in muscle development.

Use of the zebrafish model has great potential to pick apart the subtle functions of cell surface proteins during muscle development, because of a unique combination of factors. Zebrafish embryos develop externally, are translucent and available in large numbers from a single mating event. Very rapid, easy to perform, loss-of-function experiments are possible through the use of targeted morpholinos, though these reagents need to be used with extreme care to avoid spurious results. In addition, the bulk of individual zebrafish is made up of axial muscle tissue, repeated in a stereotypical pattern along the length of the animal. These elements make it very easy to visualise and quantify even subtle muscle defects caused by loss-of-function of cell surface proteins *in vivo*, and in time-lapse with transgenic reporter lines.

There are technological drawbacks currently, particularly with respect to obtaining targeted, heritable mutants in genes of interest, but improvements to loss-of-function methods are in progress (see Chapter 6). Researchers using the zebrafish as a model system should also be aware of the genetic complications resulting from an ancient genome duplication event in teleosts (see Chapter 3). The effects of gene duplication on gene orthology and function, such as sub-functionalisation, have to be assessed in each case. Nevertheless, the ease of use and powerful imaging capabilities make the zebrafish an attractive model for further in-depth study of muscle development.

In summary, there is no known developmental function for JAM-B and JAM-C, though they are known to interact and are co-expressed during development. The zebrafish is an attractive and powerful model to explore the possible functions of the

interaction between these proteins, particularly in the context of myogenesis. I determined that physical interaction between Jamb and Jamc is necessary and critical for normal muscle development. This work represents the first discovery of a vertebrate-specific receptor:ligand pair vital for myoblast fusion, opening up the possibility of a full understanding of the mechanism and regulation of the process in vertebrates.