Chapter 4

Expression patterns of the zebrafish *jam* family during development

Summary

In this chapter I describe the embryonic expression patterns of all six zebrafish *jam* family genes during the first 48 hours of development post fertilization, as determined by RNA *in situ* hybridisation. Both *jamb* and *jamc* are co-expressed by myoblasts in the developing myotome. The expression patterns of the other family members do not overlap with those of *jamb* and *jamc* in space or time, suggesting no functional redundancy between paralogues. Jamb protein is present on the cell surface of myoblasts and muscle fibres during development. Expression of *jamc* is repressed by *prdm1*, a transcription factor that regulates slow muscle fate, suggesting a function for the gene that is specific to fast muscle myogenesis.

4.1 Introduction

Spatio-temporal data concerning the embryonic expression of *JAM* family genes in any model organism is sparse and disorganised. All three *JAM* genes are expressed during murine development, as determined by RT-PCR and real time PCR, in whole embryos at embryonic stages between 9.5 – 16.5 days post conception (d. p. c.; Sakaguchi *et al*, 2006; Gitton *et al*, 2002). Expression of the *JAM* genes does not appear to be restricted to any of the germ layers.

Embryonic *Jam-A* expression has been documented by use of a *LacZ* knock-in reporter line (Parris *et al*, 2005). Throughout embryogenesis, *Jam-A* expression is observed in the vasculature, inner ear and nasal placode, brain and choroid plexus, kidney, lung, gut and skin. RNA *in situ* hybridisation at 14.5 d. p. c. also demonstrates widespread expression of *Jam-A*. Expression in the pancreas has been detected between 11.5 – 18.5 d. p. c. by RT-PCR (Hoffman *et al*, 2008). Immunohistochemistry studies at very early stages of embryonic development reveal expression of Jam-A from as early as the 8-cell stage, where it is thought to play a role in the timing of blastocoel cavity formation (Thomas *et al*, 2004).

Both Jam-B and Jam-C are known to be expressed in testes and spermatogonia, respectively, during development as demonstrated by immunohistochemistry and *Jam-C LacZ* reporter line (Gliki *et al*, 2004). RNA *in situ* hybridisation has detected expression of both genes in a wide range of tissues at 14.5 d. p. c., including, but not limited to, brain, spinal cord, retina, gut, liver, kidney, pancreas, ear, heart and of particular interest, tongue, diaphragm and skeletal musculature (Visel *et al*, 2004). Jam-B and Jam-C expression was also described in a recent mouse knockout screen for transmembrane and secreted proteins (Tang *et al*, 2010). Both genes are expressed in neural tissues between 8.5 and 11.5 d. p. c. and in the somites between 10.5 and 12.5 d. p. c., as determined by wholemount RNA *in situ* hybridisation. Before commencement of this project, wholemount *in situ* hybridisation performed by members of the laboratory demonstrated co-expression of *jamb* and *jamc* in the somites and developing myotome of zebrafish embryos, specifically, fast muscle precursor cells.

I sought to further characterise the expression of the zebrafish *jam* family genes over several stages of development through wholemount RNA *in situ* hybridisation, to identify potential sites of interaction. Better understanding of the biological context of expression of each of the identified *jam* family orthologues (Chapter 3) highlighted differences between the regulation of the family members and their respective roles

in development. Comprehensive analysis of embryonic expression reiterates the observation that *jamb* and *jamc* are likely to interact during muscle development and also suggests that the respective paralogues do not function redundantly.

4.2 Expression patterns of the *jam* family

To get a comprehensive understanding of the expression of each of the zebrafish genes during early development, I performed wholemount RNA *in situ* hybridisation of riboprobes for each gene at several stages representative of the consecutive periods of development: gastrulation (shield, 6 h. p. f.; figure 4.1), segmentation (10 – 13 somites, approximately 14 h. p. f. and 21 somites, approximately 19½ h. p. f.; figures 4.2 and 4.3 respectively), pharyngula (24 h. p. f.; figure 4.4) and hatching (48 h. p. f.; figure 4.5). The riboprobes used were derived from the immunoglobulin domain-encoding regions of each gene (see Chapters 2 and 3). Tissue annotations for each gene and developmental stage are listed in table 4.1.

The expression patterns of *jama* and *jama2* are nearly identical, both spatially and temporally. This may be a result of cross-hybridisation between riboprobes and their respective targets because there is a high level of identity between the coding sequence of the immunoglobulin domains of both genes (table 4.2). However, the *jama2* riboprobe does not detect expression in any tissue of the 10 - 13 somites stage embryos and only weakly in tissues of the later developmental stages. Another possible explanation is that both genes may be regulated by the same promoter and enhancer elements because of the close proximity of *jama* and *jama2* within the zebrafish genome (see Chapter 3).

There is little similarity between the expression patterns of the remaining closely-related paralogues. *jamb* is predominantly expressed in the somites during segmentation (figures 4.2 and 4.3). This is attenuated in rostral myotomes during the pharyngula period (figure 4.4), and is undetectable in the myotomes of hatching period embryos, but strongly expressed in craniofacial mesoderm and hypaxial, epaxial and pectoral fin muscles (figure 4.5). In contrast, *jamb2* is expressed most strongly in the epithelia over the yolk ball, lateral to the main axis of the embryo, from early segmentation through to the pharyngula period (figure 4.6). By the hatching period, *jamb2* expression is restricted to pectoral fin muscles and branchial/mandibular arch mesoderm. There is possible overlap between expression of *jamb* and *jamb2* in craniofacial mesoderm at this stage.

Similarly, there is little overlap of expression of *jamc* and *jamc2* observed using this technique during early development. Expression of *jamc* is similar to that of *jamb*.

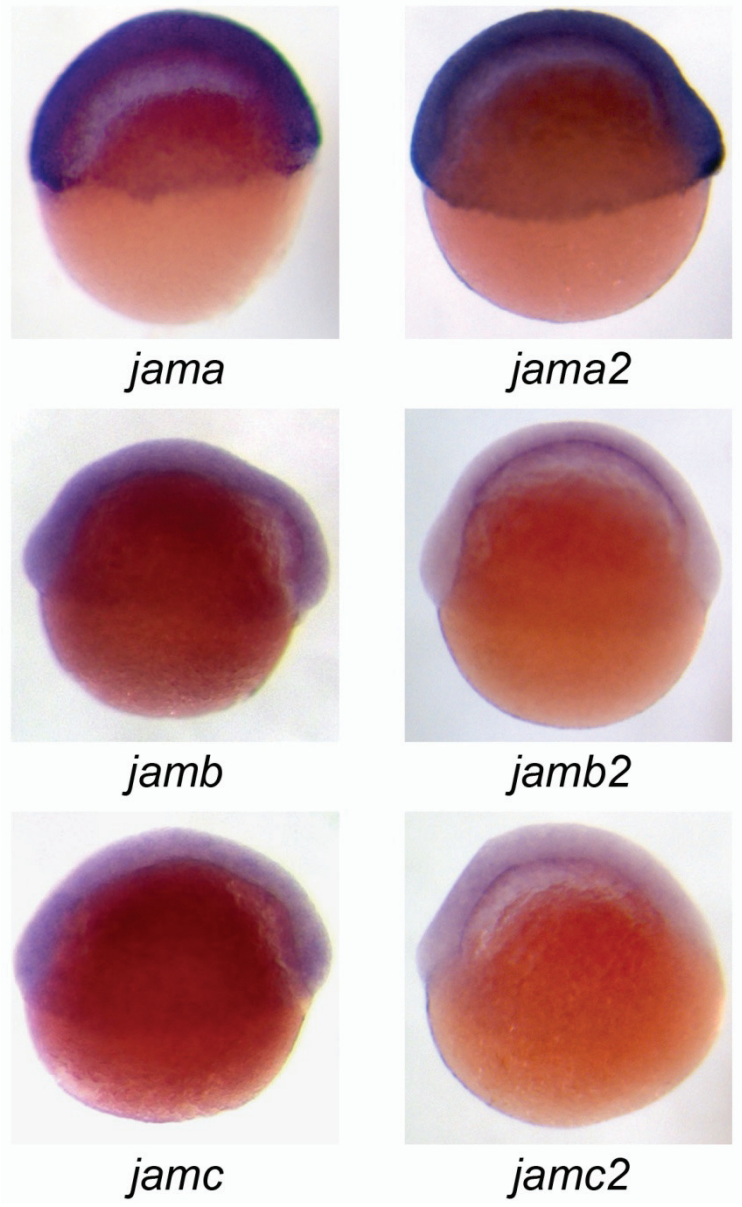

Figure 4.1 Expression patterns of *jam* family genes in the gastrula.

Wholemount RNA *in situ* hybridisation of shield stage embryos (6 h. p. f.), animal pole top, using riboprobes derived from the extracellular domain-encoding region of each *jam* family gene. There is uniform expression of *jama* and *jama2*, but no detectable expression of *jamb*, *jamb2*, *jamc* and *jamc2*.

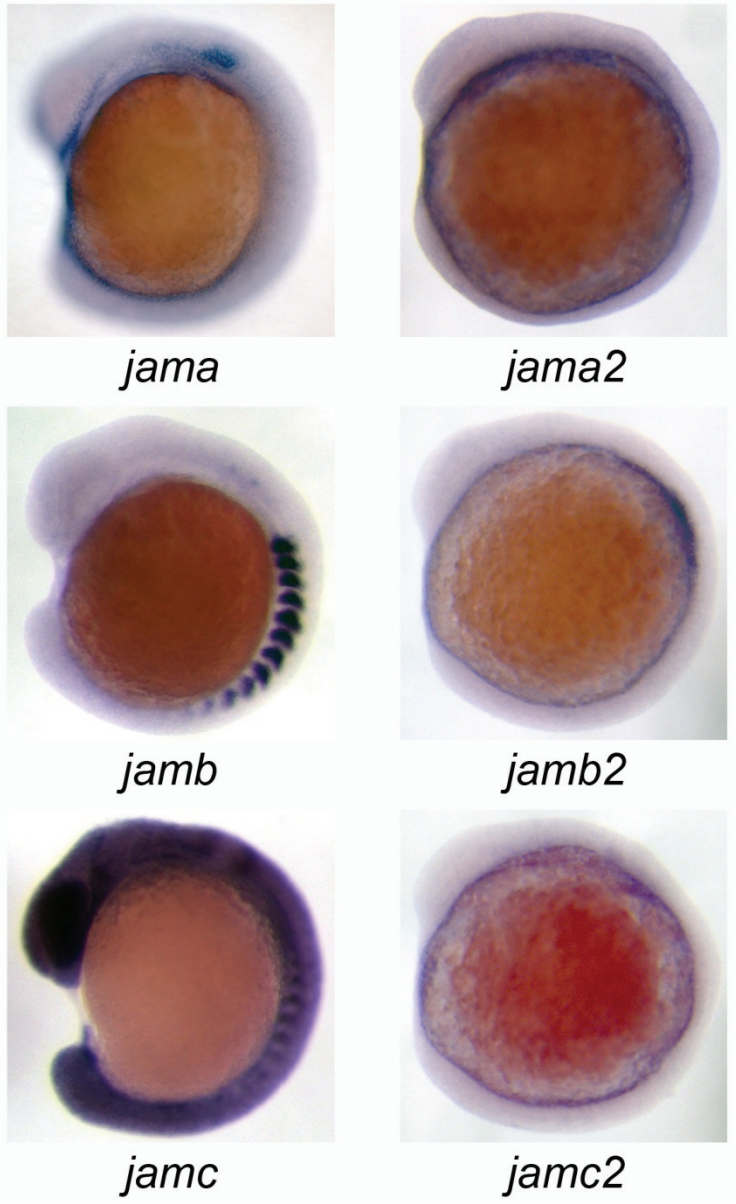

Figure 4.2 Expression patterns of *jam* family genes during early segmentation.

Lateral views of wholemount RNA *in situ* hybridisation expression patterns of 10 – 13 somites stage embryos (approximately 14 h. p. f.); anterior top. There is diversity between the regions of expression of each gene, with the exception of *jamb* and *jamc*. Both genes are expressed in mature somites. The closely-related paralogues are not expressed in similar regions within the developing embryo.

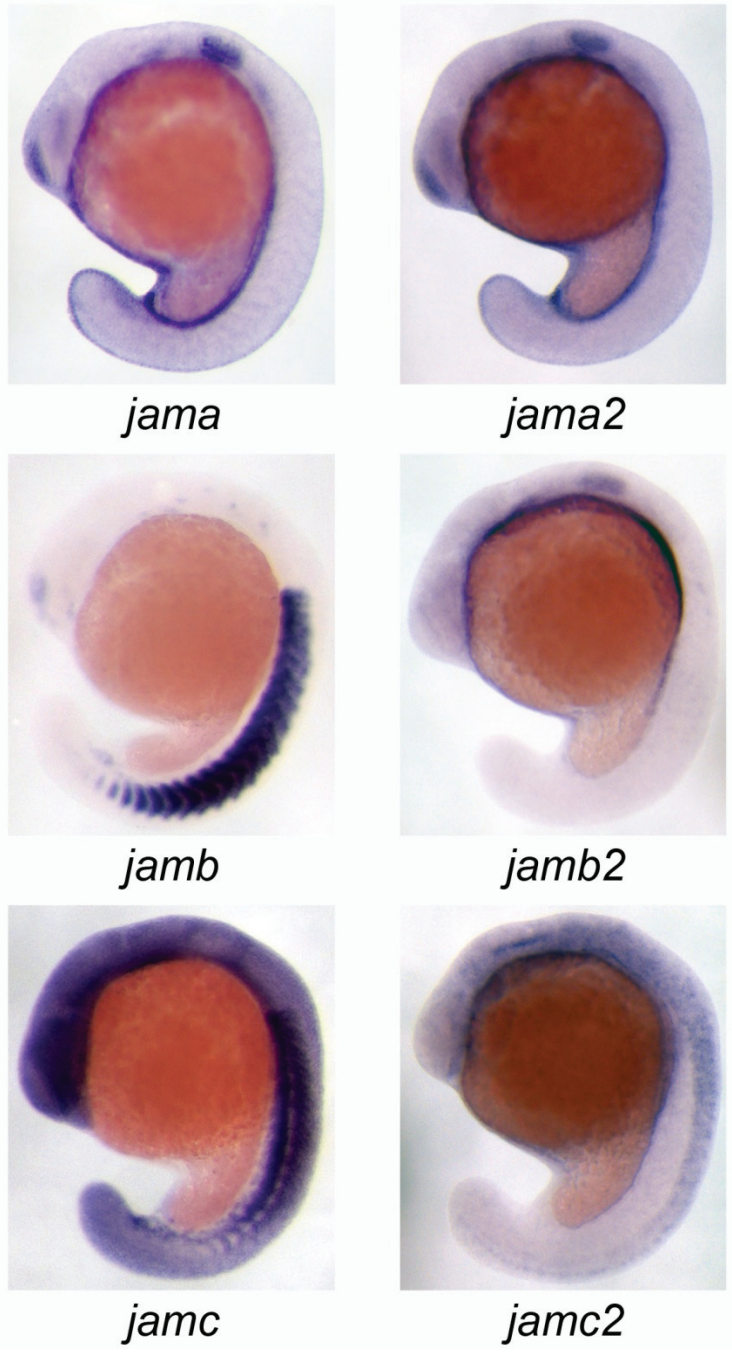

Figure 4.3 Expression patterns of *jam* family genes during late segmentation.

Lateral views of wholemount RNA *in situ* hybridisation patterns of embryos at 21 somites stage (19½ h. p. f.); anterior top. There are diverse, but overlapping, expression patterns of *jam* family genes. Both *jamb* and *jamc* continue to be expressed in anterior myotomes and caudal somites. Closely-related paralogues are expressed in different tissues of the embryo, with the exception of *jama* and *jama2*.

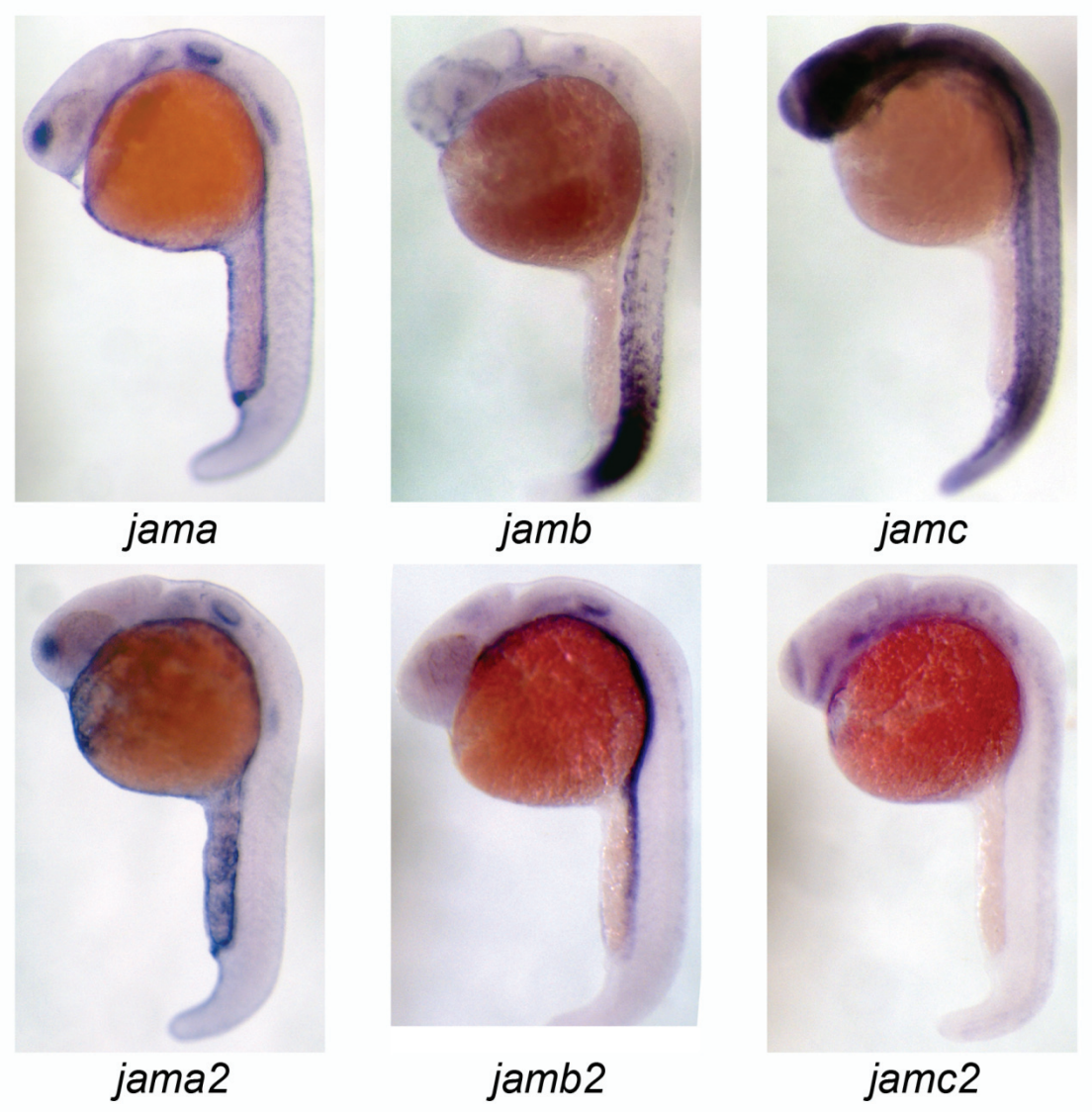

Figure 4.4 Expression patterns of *jam* family genes during the pharyngula period.

Lateral views of wholemount RNA *in situ* hybridisation of 24 h. p.f. embryos; anterior top. Co-expression of *jamb* and *jamc* is attenuated in all but the most caudal somites. The closely-related paralogues are expressed in different tissues, except for *jama* and *jama2.*

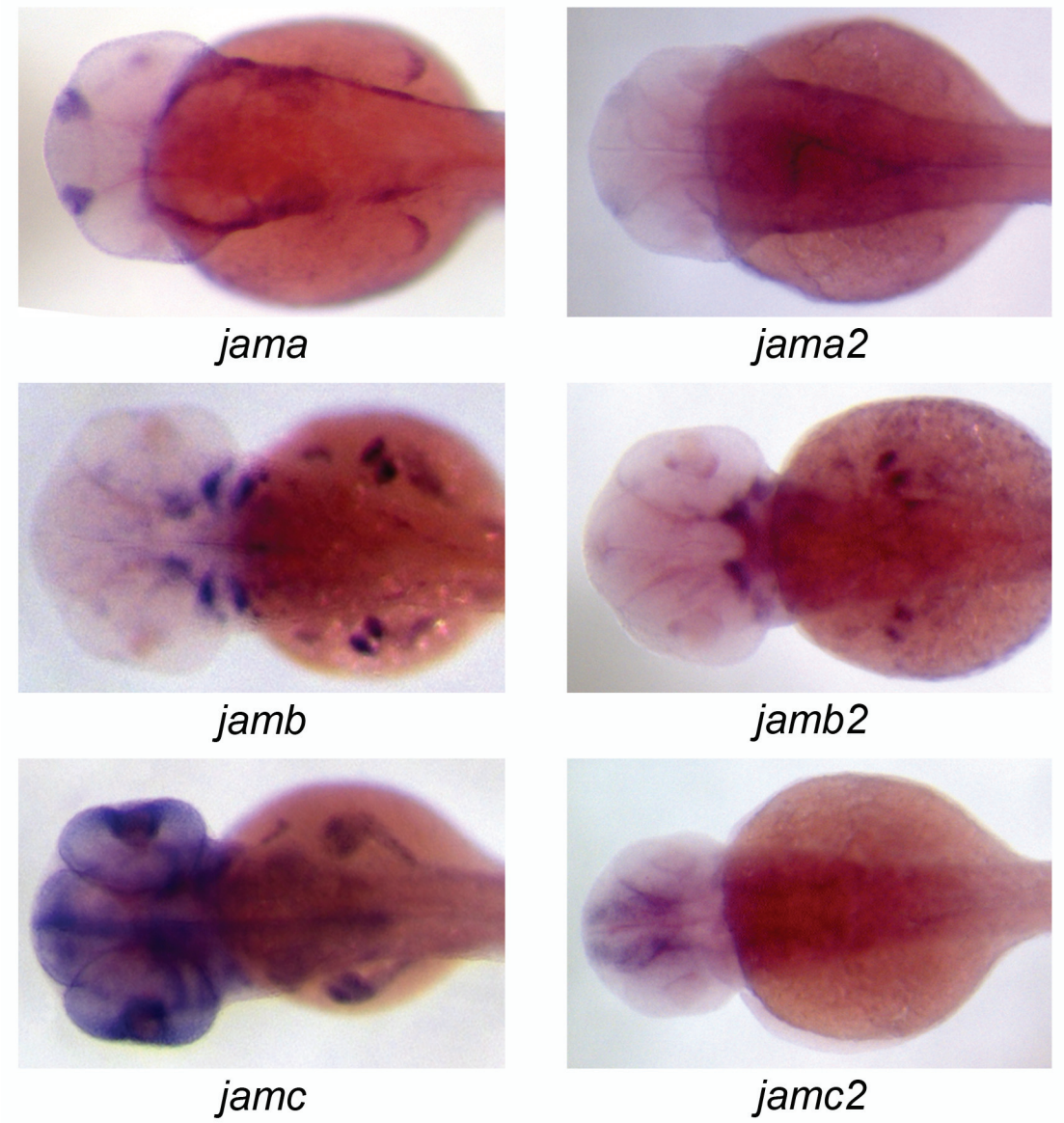

Figure 4.5 Expression patterns of *jam* family genes during hatching.

Dorsal views of head and trunk regions of wholemount RNA *in situ* hybridisation of 48 h. p. f. embryos; anterior left. Both *jamb* and *jamc* are expressed in craniofacial mesoderm, hypaxial, epaxial and pectoral fin muscles. *jamb2* is also expressed in pectoral fin muscles, and may overlap in expression with *jamb* in the head.

Figure 4.6 Expression pattern of *jamb2* between early segmentation and pharyngula periods.

Dorsal views of wholemount RNA *in situ* hybridisation of *jamb2* during development. There is strong expression of *jamb2* detected in the epithelia over the yolk ball, lateral to the main axis of the embryo, and yolk extension.

Table 4.1 Expression patterns of zebrafish *jam* family genes determined by wholemount RNA *in situ* hybridisation.

Gene	Stage									
	Shield		10 – 13 somites		21 somites		24 h. p. f.		48 h. p. f.	
jama	uniform	+++	**otic placode** nasal epithelium	+++ ++	epidermis eye lateral line primordium **otic vesicle** nasal epithelium pronephric ducts	+ + ++ +++ +++ +++	epidermis lateral line primordium **otic vesicle** nasal epithelium pronephric ducts	+ ++ ++ +++ +++	epidermis lateral line primordium **otic vesicle** nasal epithelium pronephric ducts pectoral fin	+ +++ ++ +++ +++ ++
jama2	uniform	+		-	epidermis eye **otic vesicle** nasal epithelium pronephric ducts	+ + + + +	epidermis lateral line primordium **otic vesicle** nasal epithelium pronephric ducts	+ + + ++ ++	**otic vesicle** nasal epithelium pectoral fin	+ + +
jamb		-	**anterior and posterior poles of otic placode** **posterior epithelium and mesenchyme of mature somites**	++ +++	**anterior and posterior poles of otic placode** **myotome/mature somites** **forebrain**	++ +++ ++	**otic vesicle** **caudal somites** **brain**	+ +++ +	**pectoral fin muscles** **hypaxial/epaxial muscles** **craniofacial mesoderm**	+++ +++ +++
jamb2		-	epithelium over yolk ball	++	**otic vesicle** epithelium over yolk ball	+ ++	**otic vesicle** epithelium over yolk ball	+ ++	**pectoral fin muscles** branchial/mandibular arches	++ ++
jamc		-	ubiquitous expression **rostral somites, posterior-medial foci** hindbrain	++ +++ +++	ubiquitous expression **dorsal/ventral myotome, caudal somites, posterior-medial foci**	++ +++	ubiquitous expression **caudal somites** **hindbrain/forebrain** eye	++ +++ +++ +++	ubiquitous expression **pectoral fin muscles** **hypaxial/epaxial muscles** **craniofacial mesoderm** eye	++ +++ +++ +++ +++
jamc2		-		-	neural tube anterior lateral line primordium	+ +	neural tube **brain**	+ +	**brain**	+

Some sites of expression are colour-coded by anatomical feature: **blue** - somites/myotome/muscle **green** – otic placode/vesicle **orange** - brain
Qualitative descriptors: '-' Absent '+' Weak (light staining after 4 hours or requiring overnight development) '++' Strong (clear, heavy staining after 2-4 hours of development) '+++' Very strong (very heavy staining after 2 hours of development)

It is strongly expressed in the somites throughout segmentation (figures 4.2 and 4.3) and is attenuated in the myotomes of the pharyngula (figure 4.4), remains absent in the trunk and tail of hatching period embryos but is strongly expressed in craniofacial muscle mesoderm and hypaxial, epaxial and pectoral fin muscles. There is also strong expression of *jamc* in the hindbrain at 10 – 13 somites stage (figure 4.2) and 24 h. p. f. (figure 4.4). In contrast, *jamc2* expression is not detected until late stages of segmentation (figure 4.3) and is restricted primarily to the neural tube until at least 24 h. p.f. (figure 4.4). *jamc2* is expressed in the brain throughout pharyngula (figure 4.4) and hatching periods (figure 4.5). There is strong ubiquitous expression of *jamc* evident in all developmental stages tested. I believe this represents the true pattern of *jamc* transcription because RNA *in situ* hybridisation performed using an independent riboprobe, derived from the 3′ UTR of *jamc*, yielded the same results.

There is overlap in expression of the *jam* family genes in the otic placode/vesicle from segmentation through to the pharyngula period, where *jamb*, *jamb2* and *jama*, *jama2* are expressed. Curiously, the expression of *jamb* seems to be limited to small foci at the anterior and posterior poles of the otic vesicle. There is also co-expression of *jamb*, *jamc* and *jamc2* within the brain of the pharyngula (figure 4.4). There is a strong and sustained co-expression of *jamb* and *jamc* within the somites from early segmentation stages (figure 4.2), continuing through late segmentation stages as the caudal-most somites transition into myotome (figure 4.3) before the attenuation of expression of both genes in all but the most rostral somites of the phyrangula (figure 4.4). During the hatching stages, both genes are co-expressed in the craniofacial, limb and abdominal musculature (figure 4.5).

4.3 Detailed expression patterns of *jamb* and *jamc*

While there is clear co-expression of *jamb* and *jamc* in the somites from early segmentation, there seems to be a more dynamic and spatially-restricted expression of *jamc*. Whilst *jamb* is expressed in all myoblasts within nearly all somites of the 10 – 13 somites stage embryo, *jamc* seems to be initially expressed in only a small sub-population of cells in the most rostral somites (figure 4.2). Flatmounts of 10 – 13 somites stage embryos further highlight the limited expression domain of *jamc* to medio-posterior myoblasts along the dorso-ventral axis (figure 4.7) in comparison to the expression of *jamb* in apparently all myoblasts. To better understand the dynamic nature of their expression, I observed expression of both *jamb* and *jamc* throughout early stages of segmentation by wholemount RNA *in situ* hybridisation (figure 4.7). The expression of *jamb* in the somites was first observed at 3 somites stage and

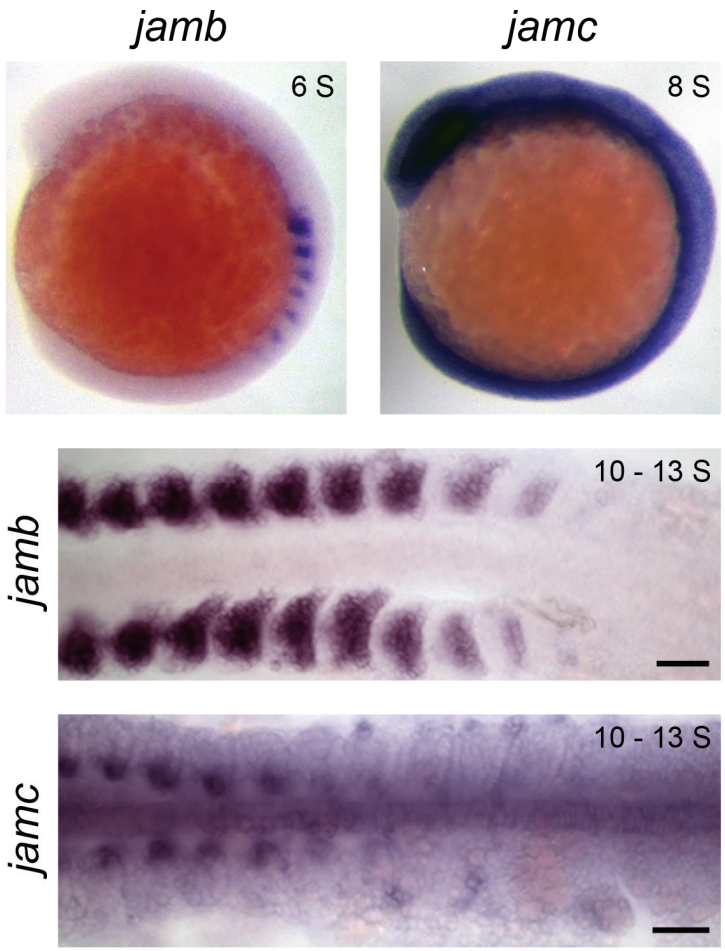

Figure 4.7 Detailed observation of *jamb* and *jamc* expression in somites.

Wholemount RNA *in situ* hybridisation of *jamb* and *jamc* during early segmentation reveals a difference in the timing of expression of both genes. *jamb* is expressed in each somite shortly after formation (top left) and is expressed in all myoblasts (middle). In contrast, *jamc* is not expressed in the somites (top right) until approximately the 10 – 13 somites stage, when it is expressed in a subpopulation of myoblasts in the rostral somites simultaneously (lower panel). Lateral views (top; anterior top) and flatmounts (middle and lower panel; dorsal, anterior left) of wholemount RNA *in situ* hybridisation against *jamb* and *jamc* during early segmentation, stages as indicated in panels. Scale bars represent 50 µm.

continued to be expressed in newly formed somites shortly after their formation. In contrast, *jamc* was not observed in the somites until approximately 10 – 13 somites stage. It was apparently expressed in the first few rostral somites simultaneously, in only a small medio-posterior sub-population of myoblasts. Thereafter, expression of *jamc* seems to appear in each somite shortly after its formation, in a similar fashion to *jamb*.

4.4 Regulation by *prdm1* suggests a fast muscle-specific function for *jamc*, but not *jamb*

Given the apparently static nature of *jamb* expression and the dynamic nature of *jamc*, I sought to find a cause of differential regulation between these genes in the fast muscle myoblasts. Upon examination of the literature, the muscle fate regulatory switch formed by *sox6* and *prdm1* seemed a likely source of regulation of *jamb* and *jamc*, principally because of the similarity of expression pattern between *sox6* and *jamc* (von Hofsten *et al*, 2008). Briefly, *prdm1* is a transcriptional repressor expressed in the adaxial cells of the zebrafish embryo. It represses expression of *sox6*, which would otherwise repress the expression of slow muscle-specific genes, and also directly represses fast muscle-specific genes in the adaxial cells. This combination of activity allows the adaxial cells to adopt a slow muscle fate. In the absence of *prdm1*, adaxial cells express fast muscle-specific genes and adopt a 'mixed' muscle fate.

To test whether either *jamb* or *jamc* are directly regulated by *prdm1*, I performed wholemount RNA *in situ* hybridisation of both genes in wild-type and *prdm1*^{tp39} mutant embryos (kindly provided by Dr Stone Elworthy). I also included a riboprobe against another fast muscle-specific gene, *kirrel*, which encodes a cell surface protein known to play an important role in myoblast fusion (Srinivas *et al*, 2007). Only *jamc* was misexpressed in the adaxial cells of *prdm1*^{tp39} mutants; both *jamb* and *kirrel* were only expressed in fast muscle myoblasts (figure 4.8). These results suggest that only *jamc* expression is repressed in slow muscle by *prdm1*.

4.5 Jamb is located on myoblast and myofibre membranes

To further characterise the expression of Jamb, I made use of a polyclonal antibody raised against the recombinant extracellular domain of Jamb (figure 4.9). Zebrafish muscle differentiation proceeds in a medial-to-lateral wave within each somite in relation to the migration of slow muscle fibres (Henry and Amacher, 2004). Accordingly, Jamb protein was detected on the cell surface of myofibres (medial) and

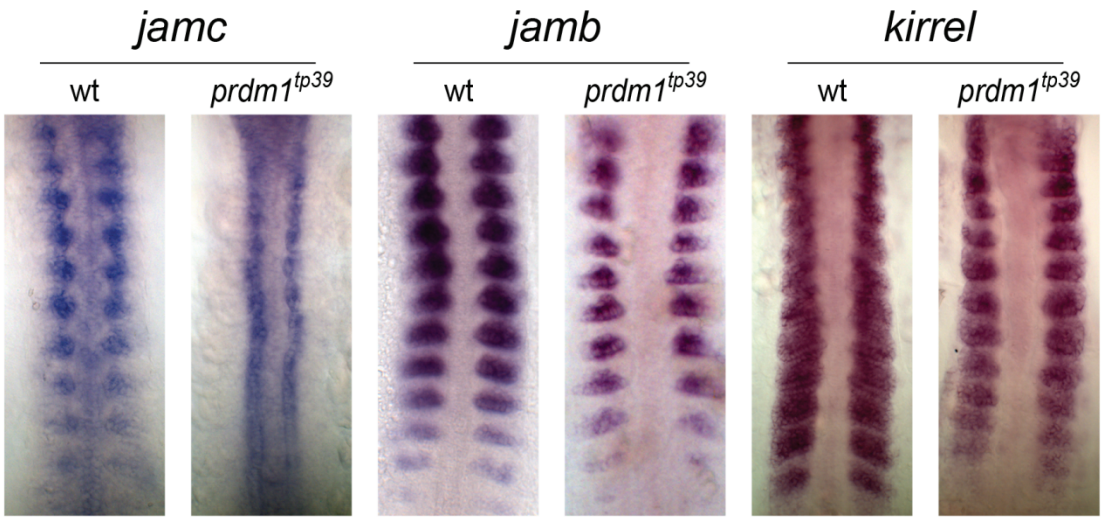

Figure 4.8 *jamc*, but not *jamb* or *kirrel*, is misexpressed in *prdm1*^{tp39} mutants.

Flatmounts of wholemount RNA *in situ* hybridisation of 10 – 13 somites stage wild-type and *prdm1*^{tp39} embryos for *jamc*, *jamb* and *kirrel*. *jamc*, but not *jamb* or *kirrel*, is misexpressed in the adaxial cells of *prdm1* mutant embryos, suggesting it is regulated by *prdm1*, a known repressor of fast muscle-specific genes.

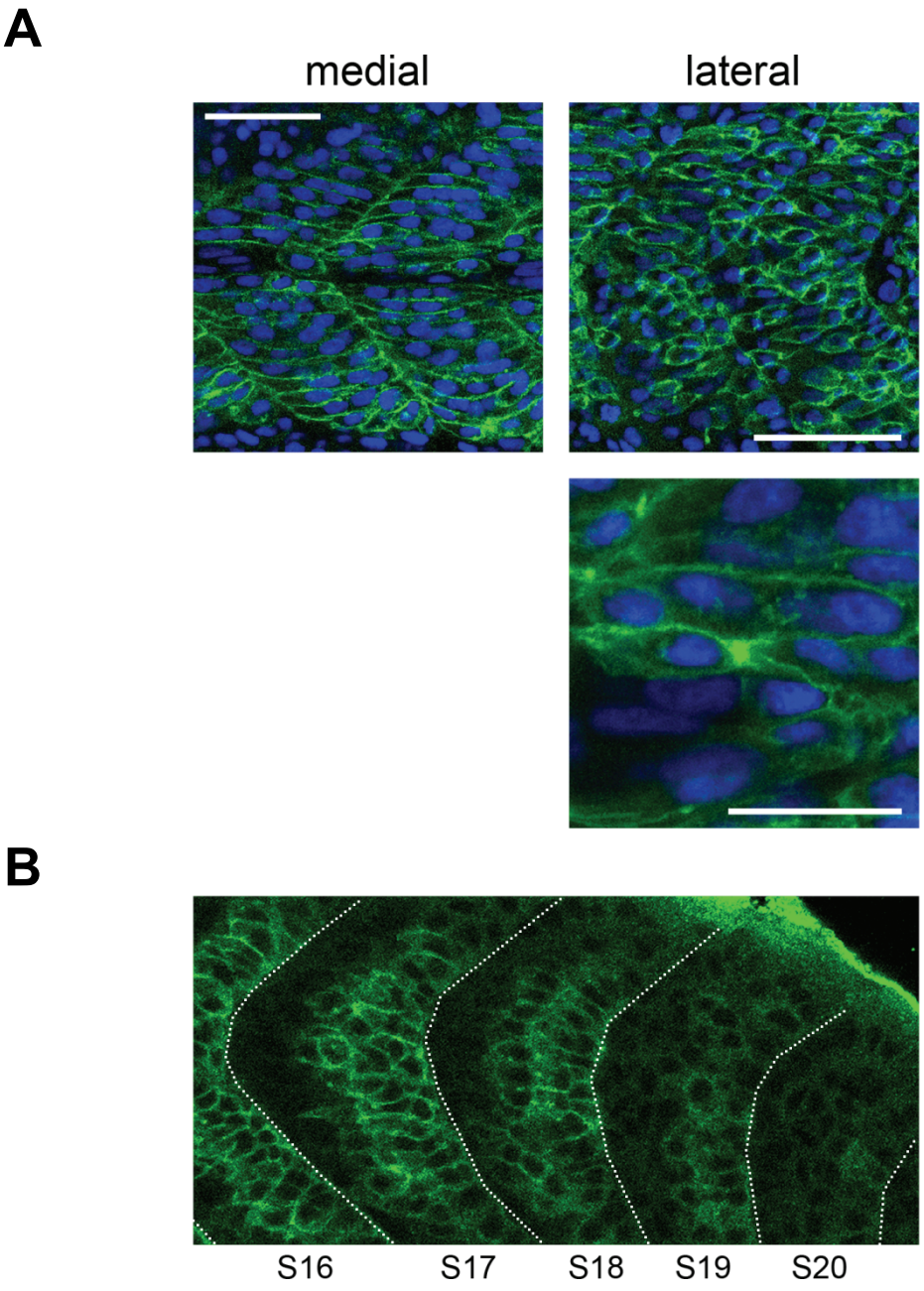

Figure 4.9 Jamb is expressed on the cell surface of myotubes and myoblasts.

A. Immunohistochemistry against Jamb (green) shows the presence of the protein on the cell surface of multinucleated medial myofibres (left) and lateral myoblasts (right). There is considerable enrichment of Jamb at sites of contact between myoblasts (bottom). Somites 8-9 of a 21 somites stage embryo counterstained with DAPI to highlight nuclei (blue), anterior left. Scale bars represent 50 μm in top panels, 20 μm in bottom panel. **B.** Jamb protein (green) is present on the cell membranes of myoblasts within somites shortly after their formation. Caudal somites of 21 somites stage embryo, anterior left. Dotted lines indicate somite boundaries.

myoblasts (lateral) in wild-type embryos during segmentation (figure 4.9). Jamb did not appear to be spatially restricted within the plane of the cell membrane, but was notably enriched at sites of contact between myoblasts. Detection of Jamb in the caudal somites demonstrate little time difference between the transcription and translation of *jamb* and confirm the observation that *jamb* is expressed in myoblasts shortly after the formation of each somite.

4.6 Discussion

To identify potential sites of interactions between Jam proteins and assess redundancy of expression between paralogues, I determined the expression patterns of all members of the zebrafish *jam* family during development by wholemount RNA *in situ* hybridisation.

The predominant example of spatio-temporal co-expression of *jam* family members is that of *jamb* and *jamc* in the somites, between early segmentation and pharyngula periods, coincident with primary myogenesis. In addition, both genes are later expressed in craniofacial, limb and abdominal musculature. Furthermore, Jamb protein was detected on the cell surface of myofibres and myoblasts during segmentation. Taken together, these results strongly suggest a function for the interaction between both proteins in muscle development.

Careful observations of *jamb* and *jamc* expression in the somites during early segmentation reveal interesting differences in the spatio-temporal nature of their regulation. Expression of *jamb* is stable throughout segmentation, beginning in each somite shortly after its formation and attenuated after it has matured into a myotome. In contrast, *jamc* is only expressed in the somites after approximately 10 – 13 somites have formed. It is simultaneously expressed in the most rostral somites and is subsequently upregulated in the remaining somites in an anterior-to-posterior wave. In addition, it is initially only expressed in a sub-population of myoblasts within the somite, a medio-posterior group of cells along the dorso-ventral axis. The expression domain of *jamc* within the somite expands medio-laterally over time. Like *jamb*, *jamc* is also attenuated as each somite matures into myotome. The dynamic expression of *jamc* in comparison to the stable expression of *jamb* suggests differential regulation between the two genes. The expression pattern of *jamc* is very similar to that of myogenin (Weinberg *et al*, 1996), an important transcription factor for terminal differentiation of muscle (reviewed in Pownall *et al*, 2002). It remains to be determined if *jamc*, but not *jamb*, is a target of myogenin.

Notably, *jamc* is misexpressed in the adaxial cells of *prdm1*^{tp39} mutants. This

suggests that *jamc*, but not *jamb* and *kirrel*, is repressed by *prdm1*. This observation is of particular interest because wild-type slow muscle is mononuclear (Roy *et al*, 2001) but *prdm1* mutant adaxial cells undergo fusion with other myoblasts (von Hofsten *et al*, 2008). Therefore, key components of myoblast fusion must be regulated by *prdm1*, directly or otherwise. Kirrel is orthologous to Dumbfounded and Roughest, cell surface proteins known to be critical for myoblast fusion in *Drosophila*. Loss-of-function of *kirrel* in zebrafish results in a near complete block of myoblast fusion (Srinivas *et al*, 2007). Given these observations, it is surprising to find that *kirrel* is not regulated by *prdm1*, but that *jamc* is. One possible hypothesis is that *jamc* is a critical regulator of myoblast fusion. Subsequent experiments demonstrate that this is likely to be the case (see Chapters 6, 7 and 8).

Previous analysis indicated a high level of conservation between the amino acid sequences of the extracellular domains of the zebrafish Jam family proteins, especially between paralogues (see Chapter 3). This suggested a possibility of cross-hybridisation between riboprobes and other *jam* transcripts, confounding the purpose of determining the expression patterns of each gene. The level of nucleotide conservation between the coding sequences of the extracellular domains is lower, as determined by clustalW alignments, but still very high between *JAM-A* paralogues, 82% (see table 4.2). The expression patterns of both *jama* and *jama2* genes are almost identical, although the relative expression of *jama2* does seem to be weaker. However, both genes are in close proximity in the zebrafish genome (see Chapter 3), and so may be regulated by the same promoter or enhancer elements. Further investigation with riboprobes derived from the dissimilar 3′ untranslated regions (UTR) of either gene would be necessary to differentiate between these possibilities. The distinct expression patterns between the *JAM-B* and *JAM-C* orthologues suggest that any overlap between them is unlikely to be a result of cross-hybridisation. It also suggests that there is little similarity in transcriptional regulation of the paralogues, with the obvious exception of *jama*/*jama2*, reducing the likelihood of redundancy amongst them.

In summary, analysis of expression of the zebrafish *jam* family indicates a role for *jamb* and *jamc* during primary myogenesis and lessens the likelihood of redundancy amongst family members that might otherwise confound analysis of the function of the interaction between Jamb and Jamc during development.

Table 4.2 Identity of coding sequence and amino acid sequence of immunoglobulin domains between *jam* family members. Percent identity of clustalW alignments; values ≥ 50% are highlighted in bold.

	jama	*jama2*	*jamb*	*jamb2*	*jamc*	*jamc2*	
jama		**75%**	35%	33%	29%	31%	Immunoglobulin domains (aa)
jama2	**82%**		33%	29%	31%	31%	
jamb	34%	7%		**50%**	36%	32%	
jamb2	30%	14%	**57%**		32%	31%	
jamc	31%	10%	49%	17%		**63%**	
jamc2	17%	22%	14%	26%	**63%**		
	CDS of immunoglobulin domains (nucleotides)						