Chapter 7

Physical interaction between Jamb and Jamc is necessary for myoblast fusion

Summary

In this chapter I describe a series of transplant experiments designed to further elucidate the function and mechanism of the interaction between Jamb and Jamc during the process of myoblast fusion. As expected, both Jamb and Jamc are found to be necessary for fusion and do not act as homophilic receptors. Cellular complementation and double deficient donor transplants demonstrate that Jamb and Jamc interact *in trans* and that it is the interaction between these receptors that is essential for myoblast fusion.

7.1 Introduction

The results from experiments described so far have strongly suggested Jamb and Jamc interact and that this interaction is necessary for myoblast fusion. Both genes are expressed by myoblasts during primary myogenesis in the zebrafish embryo (see Chapter 4); Jamb and Jamc are able to interact heterophilically and homophilically *in vitro* (see Chapter 5) and furthermore, mammalian homologues have been identified as binding partners in different biological contexts (see Introduction); *jamb*^{HU3319} and *jamc*^{sa0037} mutant embryos display the same phenotype, principally a near-complete lack of myoblast fusion, suggesting they act in the same pathway (see Chapter 6). These results have not distinguished between different possible mechanisms for the function of either gene. For example, Jamb and Jamc could act as independent homophilic receptors. To further demonstrate and characterise the importance and mechanism of the interaction between Jamb and Jamc in muscle development *in vivo*, I performed a comprehensive series of transplant experiments using wild-type and mutant genotypes.

Transplant experiments have been widely used for characterising the function of genes; for example, this approach demonstrated that the transcription factor *spadetail* was found to act exclusively in the mesoderm of developing embryos (Ho and Kane, 1990). The concept and practicalities of a transplant experiment are simple (see figure 7.1). Essentially, the experiment mixes genotypically different cells within an embryo and assays their respective properties during development. The donor cells are labelled for later analysis, in this instance, using a fluorescent dextran dye. By doing so, a transplant experiment tests whether a gene functions within the cell alone, such as a transcription factor would, or outside the cell, like a secreted growth factor. Do mutant cells continue to function abnormally in a background of wild-type cells and *vice versa*? If so, the gene is considered to be cell autonomous; that is, the phenotype of the mutant cell is solely dependent on its genotype. For example, *spadetail* mutant cells were found to be unable to converge during gastrulation in a wild-type host (Ho and Kane, 1990). Are wild-type cells affected by mutant cells, or *vice versa*? If so, the gene is considered to be non-cell autonomous; the phenotype of the mutant cell is independent of its genotype. For example, wild-type donor cells transplanted into the mesodermal region of *no tail*/*brachyury* mutants could rescue formation of paired posterior somites, despite only being present on one side of the embryo (Martin and Kimelman, 2008). Neither classical definition is useful when considering the function of some cell surface proteins, as they may affect transplanted cells and neighbouring host cells simultaneously. For example, wild-type

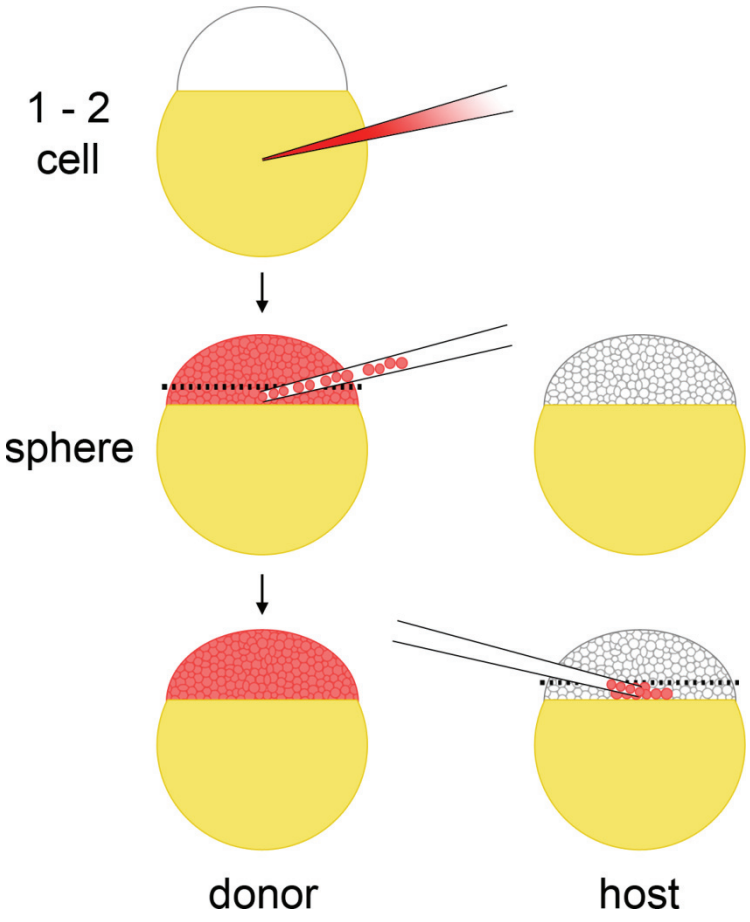

Figure 7.1 Schematic of zebrafish transplant experiments.

Schematic outlining the process of transplant experiments performed to assess the function of *jamb* and *jamc* during muscle development. Donor embryos are microinjected with a solution of fluorescently-labelled dextran at the 1-2 cell stage and allowed to develop to the sphere stage. Labelled mesoderm cells from the margin of the donor embryo (below dotted line) are then transplanted into the margin of a host embryo.

donor cells affect surrounding cells in host zebrafish embryos injected with morpholinos that target *notch1a* and *notch3*, whilst *notch* morpholino-injected donor embryo cells also affect surrounding wild-type host cells (Matsuda and Chitnis, 2009), suggesting that the Notch1a and Notch3 receptors are cell-autonomous and non-cell autonomous, simultaneously. The authors of this research use a better definition of the differing activities of these cell surface proteins: interactions *in trans* (between opposing cell membranes) and *in cis* (within a single cell membrane).

The zebrafish model organism is well-suited to this technique because of the accessibility of embryos, their rapid growth and well-documented fate-map (Kimmel *et al*, 1990; reviewed in Woo *et al*, 1995). An abundant cell type, such as somitic fast muscle precursors, are easily targeted. Rare cells are less amenable, but still possible to study; for example, specific neural cell types such as primary motor neurons that arise within the neural tube (Eisen, 1991). With respect to myoblast fusion, the phenotype of transplanted cells is easily assayed by staining host embryos with a fluorescent nuclear dye. Fluorescently-labelled myofibres are determined to be mononucleate (unfused donor cell) or multinucleate (fused donor cell) by observation using confocal microscopy. Quantification of mononucleate and multinucleate fibres gives a reasonable estimate of the degree of fusion that occurs in each combination of donor:host genotype, except where wild-type donor-donor cell fusion events could confound this. Such events are unlikely in transplants with either *jamb*^{HU3319} or *jamc*^{sa0037} donor cells, as demonstrated by the lack of fusion in *jamb*^{HU3319} and *jamc*^{sa0037} mutant embryos (see Chapter 6).

I performed a series of transplant experiments using wild-type, *jamb*^{HU3319} and *jamc*^{sa0037} embryos to characterise the function and necessity of interaction between Jamb and Jamc *in trans* for fusion.

7.2 Characterising the function of the physical interaction between Jamb and Jamc in myoblast fusion *in vivo*

To characterise the function of Jamb and Jamb during myoblast fusion, I transplanted fluorescent dextran-labelled wild-type, *jamb*^{HU3319} and *jamc*^{sa0037} mesoderm cells into the margin of wild-type, *jamb*^{HU3319} and *jamc*^{sa0037} unlabelled host embryos between high and dome stages (figure 7.1). Transplanted embryos were allowed to develop until 48 h. p. f., to ensure the completion of primary myogenesis, before fixation. Fixed embryos were then stained with DAPI to stain nuclei and analysed by confocal microscopy (figure 7.2). Fluorescently-labelled fast muscle fibres, which must be derived from donor cells, were classified as multinucleate

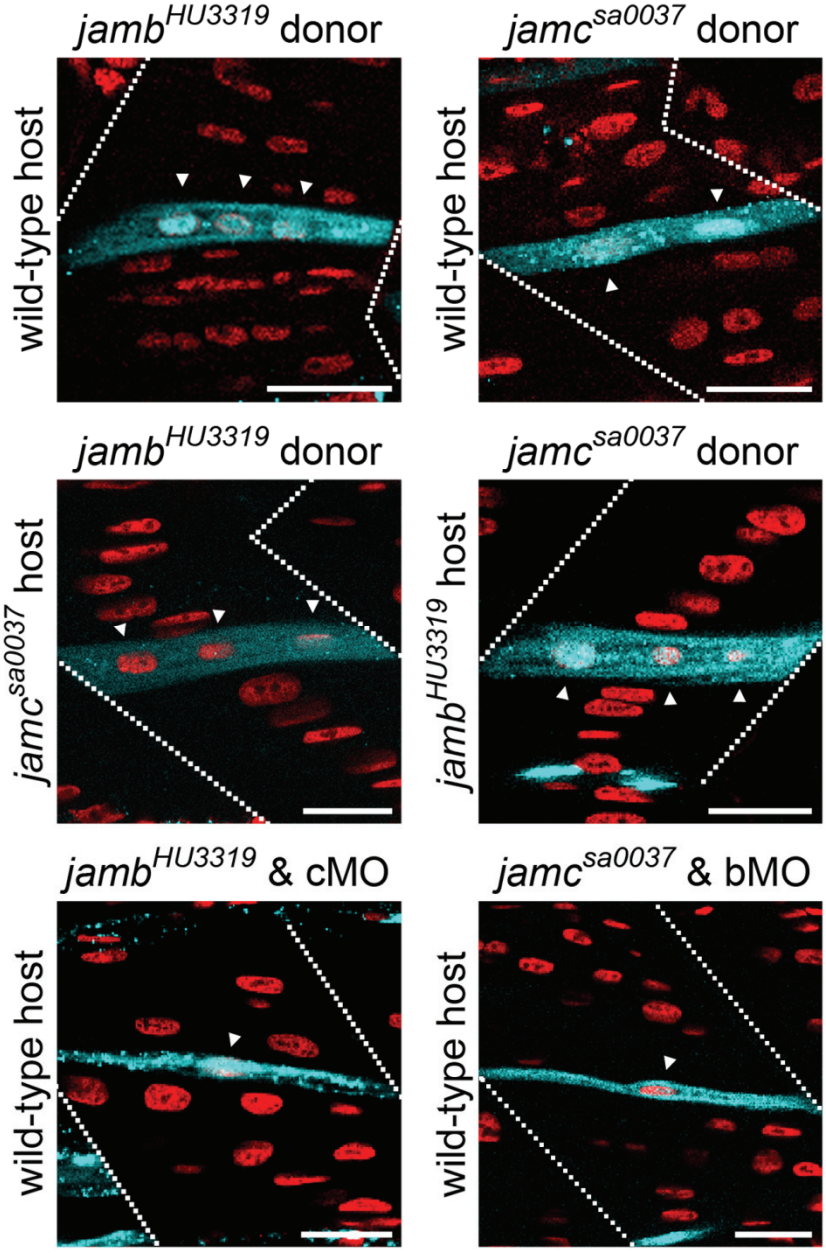

Figure 7.2 The interaction between Jamb and Jamc *in trans* is required for myoblast fusion.

Fluorescent dextran-labelled cells (blue) from *jamb*^{HU3319} (left) and *jamc*^{sa0037} (right) donors can form multinucleate fibres with wild-type (top), *jamc*^{sa0037} (middle left) and *jamb*^{HU3319} (middle right) host cells. Transplanted cells from doubly-deficient *jamb*^{HU3319}, *jamc* morpholino-injected (bottom left) or *jamc*^{sa0037}, *jamb* morpholino-injected (bottom right) donors do not fuse with wild-type host cells, suggesting both proteins are required and interact *in trans*. Confocal microscopy images from 48 h. p. f. embryos; anterior left. Dotted lines indicate myotome boundaries; arrowheads indicate nuclei within labelled fibres. Nuclei stained with DAPI (red). Scale bars represent 20 μm.

(fused) or mononucleate (unfused) and counted (table 7.1, summarised in figure 7.3).

7.3 Jamb and Jamc do not function as homophilic receptors

As expected, mutant donor cells could not form multinucleate fibres in same mutant host embryos e.g. *jamb*^{HU3319} donor cells transplanted into *jamb*^{HU3319} host embryos (table 7.1). Donor cells from *jamb*^{HU3319} embryos were able to fuse efficiently with wild-type hosts and *vice versa*, demonstrating that Jamb does not act as a homophilic receptor required for myoblast fusion (figure 7.2). Similarly, donor cells from *jamc*^{sa0037} embryos were able to fuse to wild-type hosts and *vice versa*, suggesting it also does not act as a homophilic receptor (figure 7.2).

There is a considerable decrease in efficiency of fusion when *jamc*^{sa0037} donor cells are transplanted into wild-type host embryos. The lack of efficiency was not seen in the reciprocal transplant, wild-type donor cells transplanted into *jamc*^{sa0037} host embryos, possibly because of donor-donor cell fusions (table 7.1).

7.4 Jamb and Jamc interact *in trans* during myoblast fusion

Taken together, these results suggest that expression of functional Jamb and Jamc protein *in trans* is essential for myoblast fusion. In support of this conclusion, *jamb*^{HU3319} donor cells were able to efficiently complement *jamc*^{sa0037} host cells and *vice versa* (figure 7.2), discounting the possibility of either protein acting in separate but redundant pathways. Interestingly, *jamc*^{sa0037} donor cells fuse much more efficiently to *jamb*^{HU3319} host cells than wild-type host cells. This suggests that Jamb has an additional function that is inhibitory to myoblast fusion.

To test the importance of an interaction between Jamb and Jamc *in trans*, I transplanted *jamb*^{HU3319} mesoderm cells from embryos injected with a *jamc* morpholino, or *jamc*^{sa0037} mesoderm cells from embryos injected with a *jamb* translation-blocking morpholino, into wild-type hosts. Doubly-deficient donor cells were unable to fuse to wild-type host cells (figure 7.2, table 7.1). The small amount of residual fusion events are likely a result of incomplete morpholino knockdown, as demonstrated by transplanting *jamc* morpholino-injected, *jamb*^{HU3319} donor cells into *jamc*^{sa0037} host cells and *jamb* morpholino-injected, *jamc*^{sa0037} donor cells into *jamb*^{sa0037} host cells (table 7.1). Morpholino-injected *jamb*^{HU3319} and *jamc*^{sa0037} embryos were able to undergo myogenesis (figure 7.4) suggesting no confounding effects from a synthetic phenotype.

Table 7.1 Quantification of fused (multi-nucleated) and unfused (mono-nucleated) fluorescently-labelled fast muscle fibres in transplanted hosts.

Donor genotype	Host genotype: wild-type			Host genotype: *jamb*^{HU3319}			Host genotype: *jamc*^{sa0037}		
	unfused	fused	n	unfused	fused	n	unfused	fused	n
wild-type	8 3.1%	246 96.9%	6	38 5.3%	674 94.7%	7	34 8.4%	369 91.6%	9
jamb^{HU3319}	23 6.7%	318 93.3%	8	499 94.9%	27 5.1%	6	23 4.4%	502 95.6%	9
jamc^{sa0037}	449 71.3%	181 28.7%	10	30 5.2%	552 94.8%	7	186 85.3%	32 14.7%	9
jamb^{HU3319} & *jamc* MO	648 87.8%	90 12.2%	16	n. d.			301 74.0%	106 26.0%	9
jamc^{sa0037} & *jamb* MO	190 76.0%	60 24.0%	11	128 83.7%	25 16.3%	6	n. d.		

n = number of host embryos analysed. n. d. not determined.

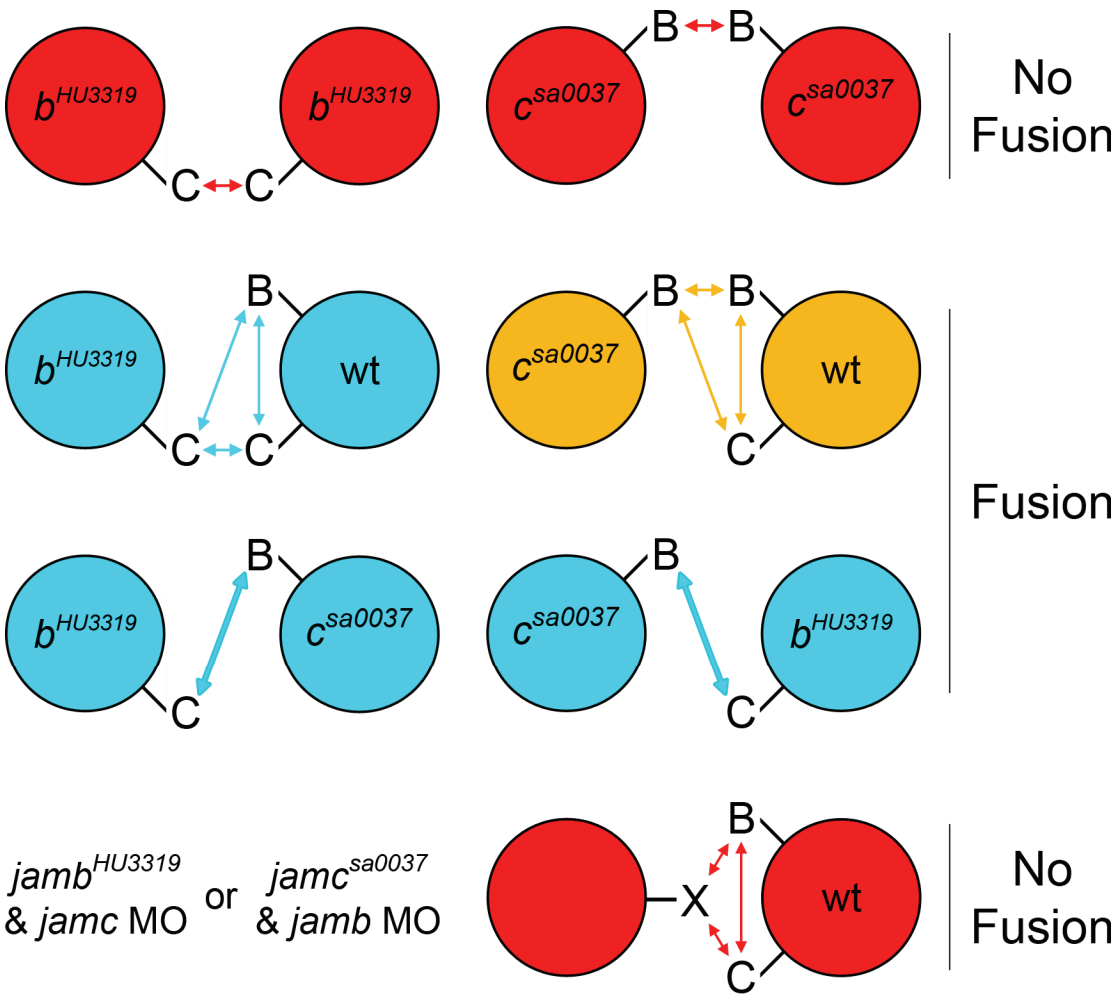

Figure 7.3 Reductive model of *jamb* and *jamc* -mediated myoblast fusion.

Diagram summarising results from transplant experiments. Each panel represents two myoblasts expressing either Jamb and/or Jamc depending upon genotype, arrows represent possible interactions. From top to bottom: myoblast fusion fails between two cells lacking the same protein (top, red), but the introduction of either protein on either cell restores fusion (cyan; second row, left), inefficiently in the case of Jamc (orange; second row, right). Loss of function of the complementary protein does not reduce the degree of fusion (third row), and restores it to wild-type levels in the case of Jamc (third row, right) suggesting interaction between Jamb *in trans* is inhibitory. Removing the function of both proteins on the same cell prevents fusion, demonstrating that the interaction between Jamb and Jamc *in trans* is necessary for myoblast fusion *in vivo* and there are no other interacting partners.

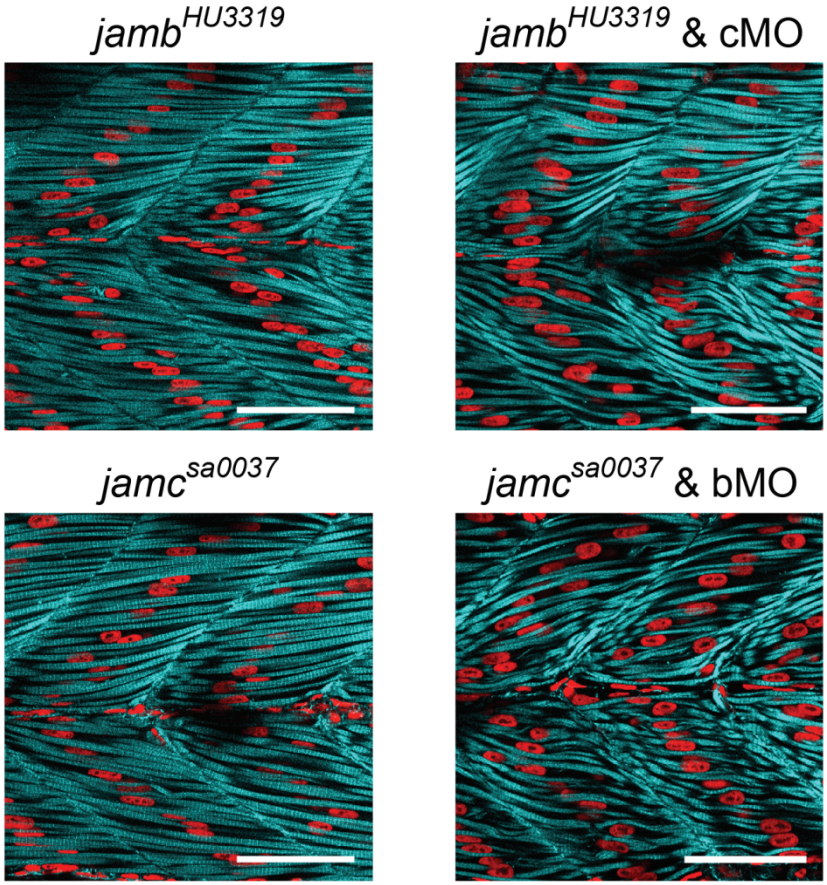

Figure 7.4 Combined knockdown of *jamb* and *jamc* does not result in a synthetic myogenesis phenotype.

Morpholino knockdown of *jamc* in *jamb*^{HU3319} embryos (top right) or *jamb* in *jamc*^{sa0037} embryos (bottom right) does not result in any further disruption of myogenesis than that observed in *jamb*^{HU3319} (top left) or *jamc*^{sa0037} (bottom left) at 48 h. p. f., suggesting no synthetic effect of combined knockdown of both genes. Confocal sections of myotomes 12-13 in 48 h. p. f. embryos, stained for F-actin (cyan) and nuclei (red). Anterior left; scale bars represent 50 μm.

7.5 Discussion

Previous experiments have demonstrated that both Jamb and Jamc are necessary for myoblast fusion to occur *in vivo*. I sought to gain further insight into the mechanism and importance of interactions between Jamb and Jamc during primary myogenesis through a systematic set of transplant experiments, making use of the mutant alleles I have characterised. The results described above indicate that the interaction between the two proteins *in trans* is a critical requirement for fusion to occur. Regulation of expression of Jamc by local signalling events (see Chapter 4) might thus contribute to controlling the process of myoblast fusion.

To draw conclusions from these experiments, I based the results on a reductive model of two myoblasts in contact, both expressing Jamb and Jamc (as established previously, see Chapter 4; see results summary in figure 7.3). Transplant experiments demonstrate that removing the function of Jamb and Jamc in the same cell prevents fusion, ruling out the possibility of other interacting partners.

The experiments show that removing the function of Jamb in either cell does not prevent fusion, but loss of Jamb in both cells does. Loss of Jamb in one cell and Jamc in the other does not prevent fusion. These results are consistent with the requirement for Jamb and Jamc to interact *in trans* in neighbouring myoblasts for efficient fusion. Removing the function of both genes in a donor cell renders it incompetent for myoblast fusion, demonstrating that Jamb and Jamc must interact *in trans*.

Loss of function of Jamc in both cells prevents fusion. Jamc activity in only one cell does not, although the efficiency of fusion is greatly reduced. Interestingly, removing the function of Jamb expression in the neighbouring Jamc deficient cell restores the efficiency of fusion, suggesting interactions between Jamb expressed by both cells inhibits fusion in these circumstances. This could suggest another level of regulation of the process; only cells that have responded to local signalling and have expressed Jamc are fully competent for fusion. However, wild-type donor cells transplanted into *jamc*^{sa0037} host embryos do not show such an effect. Refinement of the experiment to distinguish between donor-donor cell and donor-host cell fusions is necessary to test this hypothesis, before any further exploration of this putative function of Jamb is warranted. This might be acheived by labelling donor or host cell nuclei with a labelled nucleotide analogue such as bromodeoxyuridine (BrdU) or 5-ethynyl-2´ -deoxyuridine (EdU) in addition to fluorescently-labelled dextran. Another approach would be to use transgenic embryos containing a yeast upstream-

activating sequence (UAS) coupled to a fluorescent reporter as donors, and transgenic 'driver' embryos, homozygous for *jamc*^{sa0037}, in which the yeast transcription factor Gal4 is expressed in myoblasts, as host. For expression of the fluorescent reporter, the *jamc*^{sa0037}, Gal4 expressing mutant host myoblasts must fuse to the UAS-reporter transgenic donor myoblasts. Donor-donor or host-host cell fusions would not result in fluorescent fibres and would remain undetected.

The results of the transplant experiments described here do not provide any evidence to support a vertebrate equivalent of the founder cell paradigm, established through extensive studies of myoblast fusion in *Drosophila melanogaster* (reviewed in Rochlin *et al,* 2009; see Chapter 6 for further explanation). Briefly, myoblasts within the invertebrate hemi-segment are divided into a rare 'informed' population, termed founder cells, and a more numerous 'naïve' population, dubbed fusion-competent myoblasts. Fusion only occurs between the two populations – the founder cells determine the characteristics of each of the 30 muscles within the body wall, while the FCMs act as a substrate that provides bulk to each muscle by fusing to the founder cell. If vertebrate axial myotome myogenesis is based upon pre-configuration of the myotome by a small sub-population of myoblasts founder cells, then one might expect intermediate levels of fusion to be observed in wild-type donor, mutant host transplants. Only a small proportion of wild-type donor cells would differentiate into founder myoblasts and so form multinucleate muscles by fusing to the mutant host cells. The remaining donor cells would be unable to fuse to mutant myoblasts as none of the host cells could act as competent founders, and thus remain mononucleate. This was not observed in either transplant into *jamb*^{HU3319} *or jamc*^{sa0037} mutant host embryos, further supporting the hypothesis of equivalence between vertebrate myoblasts. However, as suggested above, it is possible that donor-donor cell fusions might obscure this effect. This might be resolved by differential labelling of donor and host cells, as described above.

In summary, further investigation of the function of Jamb and Jamc through transplant experiments have demonstrated that Jamb and Jamc interact *in trans* to allow fusion between myoblasts, potentially regulating the process through controlled expression of Jamc. This conclusion supports previous results demonstrating differential regulation of *jamc*, but not *jamb* or *kirrel*, by the transcription factor *prdm1* (see Chapter 4), but remains to be fully tested.