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Review

Similarity and diversity in mechanisms of muscle fate induction between ascidian species

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Developmental processes can change during evolution at many levels of the ontogeny of an individual. Embryos of solitary ascidians have a largely invariant mode of development, with fixed cleavage patterns and fate maps. Thus the cell lineages and final body plan of the two quite distantly related species considered in this review, *Ciona intestinalis* and *Halocynthia roretzi*, are highly similar. However, close comparison of the developmental mechanisms used by these two species provide examples of evolutionary changes and help pinpoint which aspects of development are evolutionarily flexible. Examples of both similarity and diversity are observed in the mechanisms used to generate the full complement of larval muscle. We will describe the changes in muscle-cell lineage, as well as some striking differences in the intercellular signalling pathways used to induce muscle fate. The somewhat surprising conclusion is that in ascidians, as in nematode vulval development, different signalling mechanisms have been adopted to mediate similar interactions between equivalently positioned cells.

Introduction

Studies on related species allow the identification of changes in developmental mechanisms that have arisen during evolution. Such changes are ultimately responsible for speciation and variations in morphology. In this review, we will describe the mechanisms of muscle specification in two ascidian species, *Ciona intestinalis* and *Halocynthia roretzi*. Although the overall ontogeny of these organisms is highly similar, in terms of their fixed cell cleavage patterns, cell lineage and final body plan, analyses of muscle cell specification in these two species have revealed differences in both the cell lineages of the muscle cells and also some surprising differences in the use of the cell signalling molecules responsible for muscle-cell induction.

Extensive comparative studies of different nematode species have shown that many aspects of early development are subject to variation, such as cell lineage, cell interaction, cell competence and equivalence, timing of cell divisions, axis establishment and the orientation of cell divisions (reviewed in Goldstein, 2001). In particular, extensive studies on the development of the nematode vulva among species have provided much information about what kind of evolutionary changes can take place. In all species of nematode studied, the vulva is a homologous structure formed from a set of 12 ventral epidermal cells, the Pn.p cells. Within this group of cells, nearly every aspect of the developmental process to generate this homologous structure appears to be flexible (reviewed in Sommer and Sternberg, 1996; Sternberg and Felix, 1997; Chamberlin, 2000; Sommer, 2000a, 2000b, 2001; Felix, 2005). Developmental changes can take place at the level of cell lineage, cellular interactions, pre-patterning, number of competent cells, cell migration patterns, timing of events, programmed cell death and the signalling systems and transcription factors used. One of the surprising conclusions from the studies on nematode vulva formation is that developmental processes can vary, whether or not the final morphology of the vulva is different between the different species.

In this review we describe a comparative study of two different ascidian species. The fate maps and

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Key words: ascidian, evolution, induction, muscle cell fate, tunicate.
Abbreviations used: BMP, bone morphogenetic protein; CNS, central nervous system; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor; MEK, mitogen-activated protein kinase/ERK kinase; MRF, myogenic regulatory factor; TGF, transforming growth factor.

cleavage patterns of these two species are highly similar. Nonetheless, examples of developmental changes have been identified during the specification of certain cell fates, notably the induced muscle lineages on which we will focus.

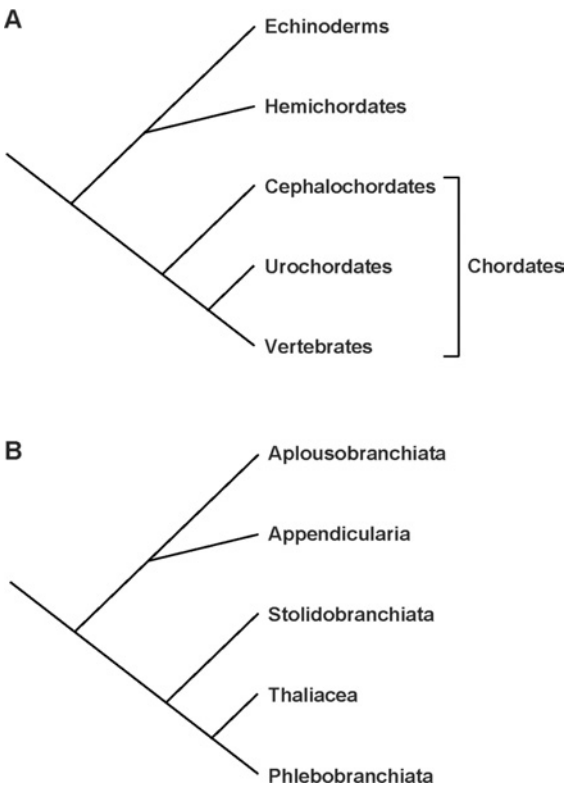
Ascidian early development and phylogeny

Ascidians, together with thaliaceans and larvaceans, form the subphylum Urochordata (also known as Tunicata). These invertebrate chordates are members of the phylum Chordata, which also includes the vertebrates and cephalochordates (Figure 1A). Chordates are characterized by possession of a notochord and a dorsal hollow CNS (central nervous system). In ascidians, these chordate characteristics are observed at the larval stage (Figure 2). Ascidian larvae subsequently metamorphose into sessile filter-feeding adults, which can be found in either solitary or colonial forms. Solitary ascidian species are favoured by developmental biologists due to the accessibility of their eggs and embryos. The larger embryos of colonial species are ovoviviparous, with development taking place inside the colony. Thus most of the information available is derived from solitary species. Among the solitary species studied, embryos develop with a small cell number, invariant cell lineage and produce extremely simple tadpole larvae (Figure 2). Very few changes are seen between the cleavage patterns, cell lineages and final body form of *Ciona* and *Halocynthia*, which are both used extensively in studies of developmental biology (Figures 2 and 3).

Many aspects of the early development of ascidians are initiated by maternal determinants that are inherited from different regions of the egg during cleavage and thus follow a 'mosaic' mode of development, so that isolated blastomeres develop autonomously into their predicted fates (Nishida, 1997). Despite this, there are an increasing number of examples of inductive interactions being required for cell-fate specification (Nishida, 1997; Kumano and Nishida, 2007). Ascidians employ both mosaic and inductive strategies in order to obtain the full complement of larval muscle cells.

Figure 1 | Phylogenetic position of ascidians

(**A**) Phylogenetic relationships of deuterostomes, which include echinoderms (starfish, sea urchin etc.), hemichordates (acorn worm), cephalochordates (amphioxus), urochordates and vertebrates. This relationship is based on studies which place urochordates as the sister group of vertebrates (Blair and Hedges, 2005; Delsuc et al., 2006; Bourlat et al., 2006), whereas the classic view places cephalochordates in this position. (**B**) Phylogenetic relationships of urochordates, which are based on a cladistic analysis by Stach and Turbeville (2002).



Traditionally, ascidians have been grouped into two orders, the enterogona and the pleurogona, depending upon the position of the gonads; *Ciona* sp. belong to the enterogona and *Halocynthia* sp. to the pleurogona (reviewed in Satoh, 1994). Another classification method divides ascidians into three

Notochord: The notochord is an embryonic midline structure common to all members of the phylum Chordata. It serves as a source of midline signals that pattern surrounding tissues and as the axial skeleton of the developing embryo. In ascidians, the notochord is present only during larval stages, whereas in cephalochordates it persists throughout life. In higher vertebrates, the notochord exists transiently before it becomes ossified in regions of forming vertebrae. Roles of the notochord in patterning surrounding tissues have been revealed only in vertebrates and are mediated by secreted molecules, such as Hedgehog proteins.

Figure 2 | Ascidian tadpole larvae of *H. roretzi* and *C. intestinalis*

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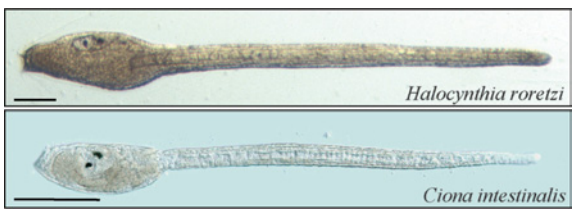


groups depending upon the structure of the brachial basket: the Aplousobranchiata and Phlebobranchiata (which are the enterogona) and the Stolidobranchiata (pleurogona). Within this classification, *Halocynthia* belongs to the Stolidobranchiata clade and *Ciona* to the Phlebobranchiata clade (Swalla et al., 2000; Stach and Turbeville, 2002). Other studies suggest that *Ciona* sp. may, alternatively, belong to the Aplousobranchiata clade (Turon and López-Legentil, 2004). Some phylogenetic studies suggest that ascidians are paraphyletic, with the Phlebobranchiata more closely related to other non-ascidian urochordates, the thaliaceans (pyrosomids, doliolids and salps), than to the Stolidobranchiata ascidians (Figure 1B) (Wada, 1998; Swalla et al., 2000; Stach and Turbeville, 2002; Yokobori et al., 2005). Although the precise phylogenetic relationships among the different groups of ascidians are not yet clear, particularly with regard to the Aplousobranchiata, it is clear that *Ciona* and *Halocynthia* belong to different clades of ascidians and are distantly related species.

The phylogenetic position of ascidians within the chordate lineage, together with their unique mode of development, has led to a recent surge of interest in ascidian development. The draft genome of *C. intestinalis* is available, as well as extensive EST (expressed sequence tag) collections, virtual three-dimensional embryos and a draft whole embryo gene-regulatory network (Dehal et al., 2002; Satou et al., 2005; Imai et al., 2006; Tassy et al., 2006). Although the molecular tools available are greater for *Ciona*, *Halocynthia* remains an excellent model for embryological studies. The eggs and embryos of *Halocynthia* are twice as large as those of *Ciona,* making embryo manipulation much easier. In this review, we will be comparing the cellular and molecular mechanisms of muscle-fate induction in two species: *Ciona* (*C. intestinalis* with some data from its close relative *C. savignyi*) and *H. roretzi*.

Larval muscle cells and their cell lineages of *Ciona* and *Halocynthia*

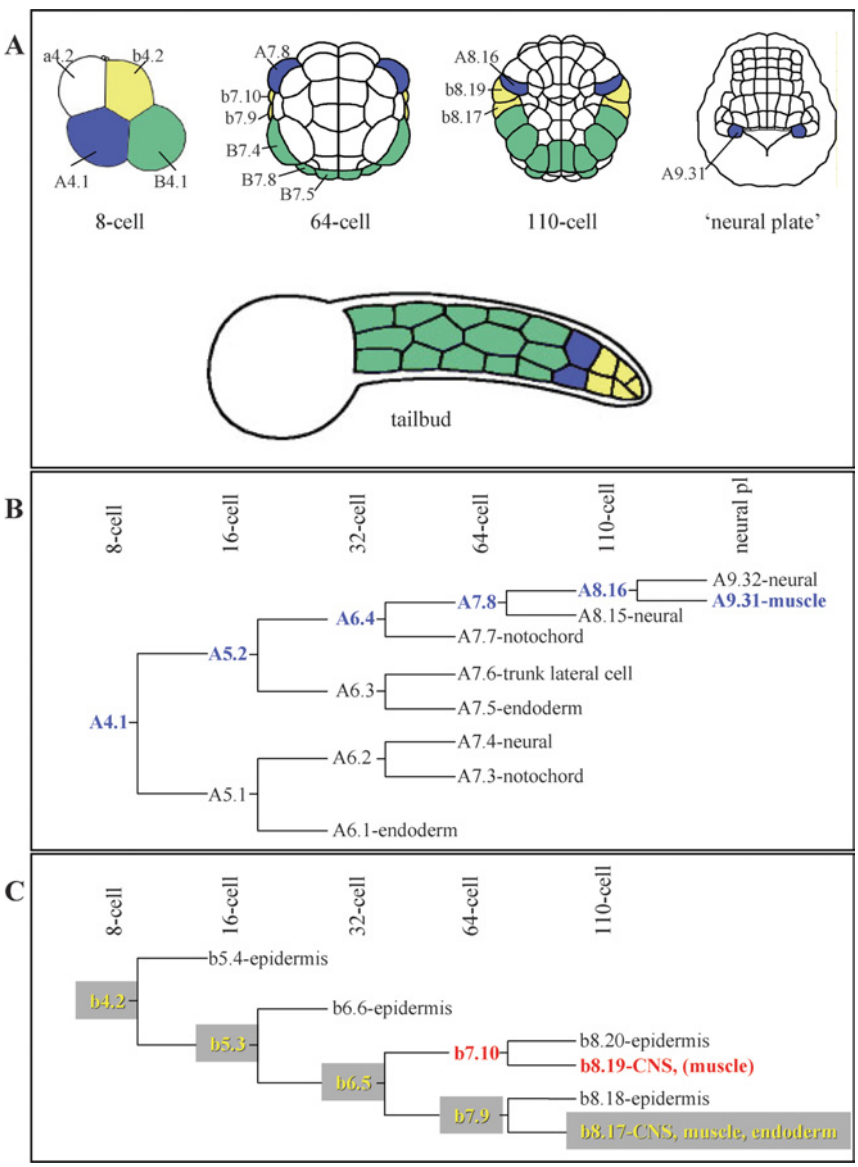
The swimming activity of the ascidian larva is generated by the tail muscle cells. These muscle cells are organized in two longitudinal bands, either side of the notochord. In *Ciona* and *Halocynthia*, each muscle band consists of 18 and 21 muscle cells respectively. Muscle-cell myofibrils exhibit a sarcomeric organization, running along the long axis of the larval tail. Muscle cells in a band are electrically coupled by gap junctions and neuromuscular transmission is cholinergic. Despite their many similarities to vertebrate skeletal muscle cells, ascidian larval muscle cells remain mononucleated, unlike vertebrate skeletal muscle cells which fuse to become multinucleated (reviewed in Meedel, 1998).

In both *Ciona* and *Halocynthia*, cell lineages leading to generation of muscle cells are well documented. By the 8-cell stage of ascidian development, stereotypical asymmetric divisions have already led to the creation of the founders of the a-, b-, A- and B- lineages (Figure 3A). The embryos are bilaterally symmetrical and, by convention, the equivalent pairs of cells are designated a4.2, b4.2, A4.1 and B4.1 respectively (Figure 3A) (Conklin, 1905). Muscle cells derive from three of the four cell types, the B-, A- and b-cells (Figure 3) (Nishida and Satoh, 1983, 1985; Nishida, 1987). These founder cells will also give rise to other cell types of the larvae, and the specification of muscle precursors from within these different lineages occurs following distinct mechanisms and at different developmental time points. B-lineage-derived muscles form the bulk of the larval muscle bands and are called 'primary' muscle cells, whereas those originating from A- and b-lineages are called 'secondary'

Clade: A clade is a taxonomic group comprising a single common ancestor and all the descendants of that ancestor. Any such group is considered to be a monophyletic group.

Figure 3 | Ascidian muscle lineages (Nishida, 1987)

(**A**) Primary and secondary muscle lineages, on the basis of a *Halocynthia* embryo, are shown. Adapted from Development, Genes and Evolution, vol. 217, 2007, pp. 1515–527, 'FGF9/16/20 and Wnt-5alpha signals are involved in specification of secondary muscle fate in embryos of the ascidian, *Halocynthia roretzi*' by M. Tokuoka, G. Kumano and H. Nishida, Figure 1 © Springer with kind permission of Springer Science and Business Media. The embryonic stage is indicated below the drawings. Muscle lineages: green, primary B-line muscle; blue, secondary A-line muscle; yellow, secondary b-line muscle. At the neural plate stage, only the A-line secondary muscle precursor is indicated and is formed in the posterior lateral part of the neural plate. The 'tailbud' drawing indicates the final number and position of muscle cells from tailbud-to-larval stages of development. The B- and A-line muscle lineages are invariant between *Ciona* and *Halocynthia*, whereas the b-lineages differ. In *Halocynthia*, five pairs of muscle cells in the tip of the tail originate from the b-lineages (yellow), whereas in *Ciona* the b-lineages generate only two pairs of muscle cells. In *Ciona,* the b7.10 lineage of the 64-cell stage embryo does not contribute to muscle. (**B**) The entire lineage of the A4.1 blastomere is shown until the fate restriction stage for each lineage. The secondary muscle lineage is indicated in blue. Developmental stages are indicated above the lineage. neural pl, neural plate stage of development. (**C**) The b4.2 lineage until the 110-cell stage of development. The muscle lineages are indicated in yellow, with a grey background for those conserved between *Ciona* and *Halocynthia* and red for those only observed in *Halocynthia*. Developmental stages are indicated above the lineages.



(Meedel et al., 1987). The secondary lineages form the posterior-most tail muscles, with b-line muscle cells found at the very tip of the tail (Figure 3A).

Invariance in the A- and B-line muscle lineages

In both *Ciona* and *Halocynthia*, both the B- and A-line muscle lineages are invariant, producing exactly the same number of muscle cells. The B-lineage generates 28 muscle cells and the A-lineage four muscle cells (Figure 3A). The B7.4 and B7.8 blastomeres of the 64-cell-stage embryo are restricted to muscle-cell fate, with B7.4 generating 16 muscle cells (eight cells on each side of the tail) and B7.8 generating eight muscle cells (four cells on each side of the tail) (Nishida, 1987). B7.5 goes on to generate four tail muscle cells and the heart precursors, known as trunk ventral cells at larval stages (Nishida, 1987; Hirano and Nishida, 1997). Interestingly, in both *Ciona* and *Halocynthia*, the fate restriction of these muscle precursors coincides with the activation of the single representative of vertebrate bHLH (basic helix–loop–helix) MRFs (myogenic regulatory factors), *Ci-MRF* in *Ciona* and *AMD1* in *Halocynthia* (Satoh et al., 1996; Satou et al., 2003; Meedel et al., 2007). This factor has been shown to be required for differentiation of muscle cells from both primary and secondary lineages in *Ciona* (Meedel et al., 2007).

The A-line secondary muscle precursors are closely associated with the A-line-derived part of the CNS (Figures 3A and 3B) (Nicol and Meinertzhagen, 1988a, 1988b; Nishida, 1990; Cole and Meinertzhagen, 2004). By the late gastrula stage, when the neural plate has formed, the secondary muscle precursor, A9.31, becomes restricted to a muscle fate, emerging in a lateral–posterior position of the neural plate (Figure 3A). As for the B-line-derived muscle lineage, the fate-restricted A-line muscle precursors start to express the *Ciona MRF* gene (Meedel et al., 2007). This pair of precursors will cleave once more to generate four larval muscle cells. The sister cell (A9.32) of the A-line secondary muscle cell will give rise to the lateral part of the tail nerve cord, part of the CNS (Figure 3B). Despite the common position and embryonic origin of the A-line muscle cells in *Halocynthia* and *Ciona*, it appears that remarkably different cell signalling strategies are used in order to generate this cell type, as described in more detail below.

Interspecies differences in the b-line muscle lineage

Unlike the A- and B-muscle lineages, the b-muscle lineage shows a number of differences between *Halocynthia* and *Ciona,* forming 10 and 4 muscle cells respectively. b-line muscle cells become restricted to a muscle fate relatively late in development. In *Halocynthia*, the b7.9 lineage of the 64-cell-stage embryo will generate, at the following cleavage, one epidermis precursor and one mixed fate precursor for the CNS, muscle and endoderm, eventually generating six muscle cells in the tail (Figures 3A and 3C). The b7.10 lineage will generate the four muscle cells at the very tip of the *Halocynthia* larvae (Figures 3A and 3C). On the other hand, in *Ciona*, the b7.9 lineage will produce four muscle cells (instead of six) at the tip of the *Ciona* larval tail, and the b7.10 lineage will contribute to the epidermis and CNS, but not to muscle (red lineage in Figure 3C) (Nishida, 1987). More muscle cells may be important for *Halocynthia* embryos, as they are larger than *Ciona* larvae (Figure 2). Interestingly, only those cells at the very tip of the tail are variable, suggesting they may be under less evolutionary constraint.

Caudalization of ascidian larvae

The presence of extra muscle cells in different ascidian species is relatively common and called caudalization (Jeffery and Swalla, 1992). *Halocynthia* is a modest example, with only a few additional secondary muscle cells in the tail tip. More extreme caudalization, whereby the larvae contain a much greater number of muscle cells, may occur by additional divisions of muscle cells before terminal differentiation. This has been described in ascidians with much larger eggs and larvae, which also often undergo adultation, the initiation of adult development, while still at

MRFs: Myogenic regulatory factor (*MRF*) genes are a family of conserved bHLH (basic helix–loop–helix) transcription factors that participate in muscle development in a variety of animals. In vertebrates, the generation of skeletal muscle fates depends on combinatorial roles of four *MRF* genes, including *MyoD*, *Myf5*, myogenin and *MRF4* (reviewed in Rudnicki and Jaenisch, 1995). In contrast, most invertebrates have a single MRF gene, which, in those that have been studied (*Caenorhabditis elegans* and *Drosophila*), plays a much less important role in myogenesis than in vertebrates. So far, the *Ciona* homologue of MRF gene (*Ci-MRF*) is the only invertebrate MRF that plays a critical role in myogenesis. Thus the crucial role of *MRF* genes in myogenesis is conserved between ascidians and vertebrates (Meedel et al., 2007).

the swimming larval stage (reviewed in Jeffery and Swalla, 1992). Extreme examples include *Ecteinascidia turbinata* and *Diplosoma macdonaldi*, colonial ascidians with giant larvae each containing more than 1000 larval tail muscles (reviewed in Satoh, 1994; Jeffery and Swalla, 1992). It is not yet known which lineages generate the additional muscle cells in cases of extreme caudalization. The increase in cell number associated with caudalization is not applied to all tail cell types, as these larvae still contain only 40 notochord cells, as do *Halocynthia* and *Ciona* (reviewed in Satoh, 1994). Others ascidian clades, notably the Molgulidae, contain species that bypass the larval tail altogether (reviewed in Jeffery and Swalla, 1992). Detailed analysis of muscle development in a greater variety of ascidian species would help to bring further insights into the mechanisms whereby the number of muscle cells alters between different ascidian species.

Autonomous and inductive mechanisms act in the different muscle lineages

Formation of the muscle cells in the primary (B-) lineage relies directly on the inheritance of maternal determinants within the B-line (Deno et al., 1984; Meedel et al., 1987; Nishida, 1990). In both *Halocynthia* and *Ciona*, this involves the inheritance of transcription factors of the Zic family, although details differ between the two species. In *Halocynthia*, the inheritance of a maternal transcription factor of the Zic family, *macho-1*, is sufficient to promote muscle fate (Nishida and Sawada, 2001). In *Ciona*, although loss of *macho-1* also results in the early loss of muscle specification, some muscle cells are eventually recovered (Satou et al., 2002). Loss of a second zygotic macho-like factor, *ZicL*, together with loss of *macho-1*, completely abolishes muscle development in *Ciona* (Imai et al., 2002a). In both *Ciona* and *Halocynthia*, a T-box transcription factor of the Tbx6-subfamily is zygotically expressed in muscle-lineage cells prior to fate restriction and is required for muscle specification (Mitani et al., 2001; Yagi et al., 2005). At least in *Ciona*, this *Tbx6* gene appears to mediate the function of Macho-1 during muscle specification (Yagi et al., 2005). The relationship between *Tbx6* and *MRF* and how they act to control activation of muscle structural genes remain to be elucidated.

In contrast with the primary muscle lineages, the secondary muscle lineages (A and b) require inductive cellular interactions in order to give rise to muscle fate (Meedel et al., 1987; Nishida, 1990). We will limit our comparison to the A-line secondary muscle lineages, since the mechanisms of muscle fate induction in b-lineages have not been studied in *Ciona*.

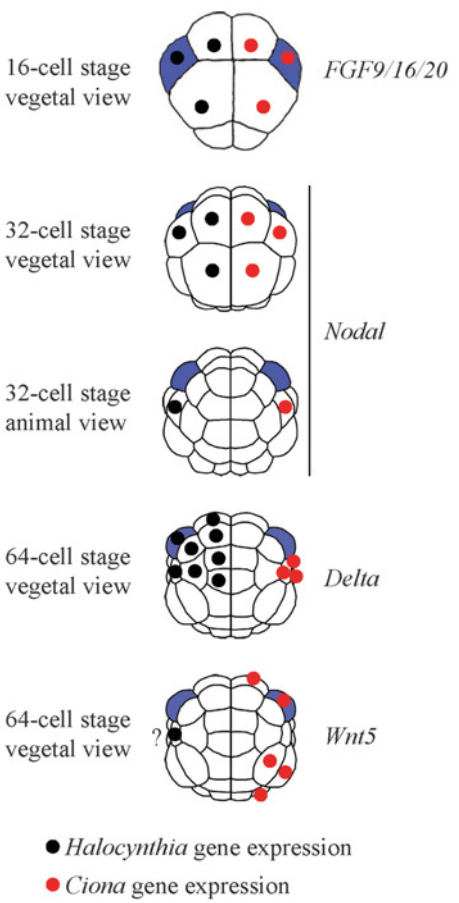
Induction of a-line muscle fates in *Ciona*: cellular interactions and signalling molecules

In *Ciona*, multiple steps of A-line muscle-fate induction have been uncovered and involve sequential inputs from the FGF (fibroblast growth factor), TGFβ (transforming growth factor β) (Nodal) and Delta/Notch signalling pathways. Expression patterns of the ligands involved in secondary muscle specification in *Ciona* and *Halocynthia* are summarized in Figure 4. The cellular and molecular interactions known to be involved in *Ciona* A-line secondary muscle formation are summarized in Figure 5(A).

Nodal induction in the b6.5 cell by FGF9/16/20 initiates A-line muscle cell induction

Ciona secondary muscle specification in the A-lineage begins with zygotic expression of *FGF9/16/20*, which is the single representative of the vertebrate FGF9 class of ligands (including FGF9, FGF16 and FGF20) (Dehal et al., 2002). *FGF9/16/20* is expressed in vegetal cells from the 16-cell stage and is required for the formation of A-line secondary muscle (Imai et al., 2002b). It is likely that FGF9/16/20 is acting indirectly during the early steps of secondary muscle specification by activating transcription of *Nodal*, which encodes a ligand of the TGFβ family. FGF9/16/20, acting via MEK [mitogen-activated protein kinase/ERK (extracellular-signal-regulated kinase) kinase]/ERK signalling during the 32-cell stage, induces *Nodal* expression in the b6.5 cell (Hudson and Yasuo, 2005). Nodal signals are required for the correct specification of all of the derivatives of the A7.8 lineage, which emerges at the 64-cell stage and will form the lateral part of the neural plate and the A-line secondary muscle (Figures 3 and 5A) (Hudson and Yasuo, 2005; Imai et al., 2006; Hudson et al., 2007). Inhibition of Nodal signalling leads to the loss of the entire lateral neural plate, including the A-line muscle precursor, and instead these cells adopt a medial neural plate fate (Hudson and Yasuo, 2005; Imai et al., 2006; Hudson et al., 2007). Furthermore, ablation of b5.3 (the precursor of b6.5) or b6.5 itself results in both

Figure 4 | Expression of key ligands involved in secondary muscle formation in *Halocynthia* and *Ciona*
Embryonic stage and orientation are indicated on the left-hand side. Dots indicate cells expressing the corresponding ligands indicated on the right-hand side, in *Halocynthia* (black dots; left-hand side of embryo) and *Ciona* (red dots; right-hand side of embryo). The A-line muscle lineage is indicated in blue. Expression patterns are shown for the following genes: *FGF9/16/20* (Imai et al., 2002b; Bertrand et al., 2003; Kumano et al., 2006), *Nodal* (Morokuma et al., 2002; Hudson and Yasuo, 2005), *Delta* (Akanuma et al., 2002; Hudson and Yasuo, 2006) and *Wnt5* (Imai et al., 2004). *Halocynthia Delta* is also expressed in a-line neural precursors at the 64-cell stage (not shown) (Akanuma et al., 2002). The expression of *Wnt5α* appears to be variable in *Halocynthia*. Maternal expression is detected in B7.6 (not visible in the Figure) and zygotic expression in A7.6 is seen in all cases, although expression is also variably reported in precursors of A-line neural, endoderm (A- and B-line) and epidermis (a- and b-line) (Sasakura et al., 1998; Nakamura et al., 2006; Tokuoka et al., 2007). For this reason, expression is shown only in the A7.6 cell, which was the strongest expression domain shown in Tokuoka et al. (2007) and is indicated by a question mark. In *Ciona*, *Wnt5* is also expressed in the B7.6 and B7.8 cells that are not visible in this Figure (Imai et al., 2004). Expression of *Ciona Wnt5* is also detected in B-line cells at the 32-cell stage (not shown; Imai et al., 2004).



loss of lateral neural gene marker expression and loss of secondary muscle fate (Hudson and Yasuo, 2005; C. Hudson and H. Yasuo, unpublished data). Thus A-line muscle specification relies on local Nodal signalling from the b6.5 cell, positioned on either side of the early embryo, and is intimately associated with the mechanisms that pattern the neural plate in *Ciona*.

Delta2/Notch signalling is required for A-line muscle fate

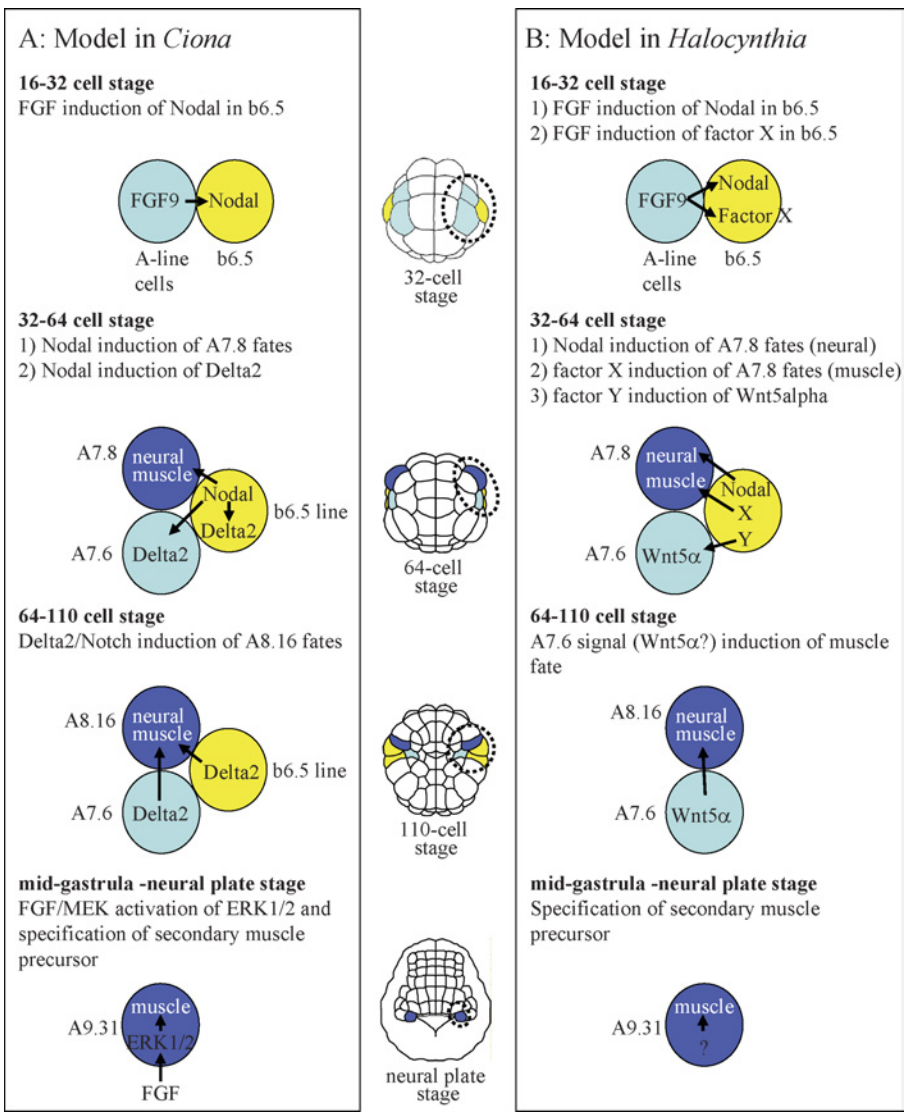
During the induction of A-line muscle, Nodal acts, in part, via the induction of another ligand, *Delta2*, in a small group of lateral cells (the A7.6, b7.9 and b7.10 cells) at the 64-cell stage (Figures 4 and 5A) (Hudson and Yasuo, 2006). These cells surround the secondary muscle-lineage cell, A7.8, at the 64-cell stage. Following cleavage of A7.8 into A8.15 (neural fate) and A8.16 (neural and muscle fates), *Delta2*-expressing cells remain in contact with only one daughter cell, A8.16, of muscle lineage, and not with A8.15, which has a neural fate (Hudson et al., 2007). Delta2 and the Notch signalling pathway are required for specification of all A8.16-cell-derived fates, including the lateral-most neural plate and the A-line muscle (Figures 3 and 5A) (Hudson et al., 2007). Lateralization of the neural plate cells by Nodal signalling appears to be a prerequisite for this role of the Delta2/Notch signals during muscle-fate induction, since ectopic expression of *Delta2* results in the specification of additional muscle fate within the neural plate, but only in descendents of the A7.8 cell, which received Nodal signals at the 64-cell stage (Hudson et al., 2007). Thus it is likely that Nodal acts both directly, as well as indirectly, via *Delta2* induction during A-line secondary muscle specification. In summary, Nodal and Delta2 form part of a regulatory relay that acts in a combinatorial and sequential manner during the induction of secondary muscle fate.

FGF/MEK/ERK signalling during the final step of A-line muscle induction

The final step in the specification of the A-line muscle precursors involves the cell division of A8.16, which

Figure 5 | Current models of A-line secondary muscle formation in *Ciona* and *Halocynthia* embryos

Models of secondary muscle formation are summarized for *Ciona* (**A**) and *Halocynthia* (**B**). The developmental stage and the cellular and molecular interactions are summarized above the drawings. Light blue circles, A-lineage cells; yellow circles, b-lineage cells; dark blue circles, A-line secondary muscle lineage. Positions of these cells at the relevant developmental stage are indicated by broken circles on the drawings of the embryos (between the two summaries). Arrows indicate interactions between the cells and signalling molecules. Cells expressing *FGF9/16/20* are labelled with 'FGF9" due to space constraints. For simplicity, the b-line cells (yellow) involved in these processes are represented as a single circle labelled 'b6.5-line' (b7.9 and b7.10 at the 64-cell stage, and b8.20, b8.19, b8.18 and b8.17 at the 110-cell stage).



separates neural and muscle lineages (Figure 3B). This cell division takes place along the anterior–posterior axis, generating an anteriorally positioned A9.32 and its posteriorally positioned sister cell A9.31. ERK1/2 activation is observed only in the A9.31 cell, which is specified as a muscle precursor, whereas A9.32, which is negative for ERK1/2 activation, adopts a neural fate. Although the ligand responsible for ERK1/2 activation is not identified, FGF9/16/20 is a good candidate. *FGF9/16/20* is expressed throughout the A-line neural plate precursors at this stage. Furthermore, inhibition of MEK (the kinase acting upstream

of ERK1/2) from the early gastrula stage, or expression of a dominant-negative form of the FGF receptor in the A5.2 lineage (Figure 3B), is sufficient to block muscle-fate specification in the A9.31 cell, which instead adopts the fate of its sister cell, neural fate, under these experimental conditions (Hudson et al., 2007). Thus it is likely that FGF/MEK/ERK signals are acting during the final step of muscle-fate specification in the A-lineage.

In summary, in *Ciona*, the secondary muscle precursors of the A-lineage are specified by combinatorial and sequential inputs from the Nodal, Delta2/Notch and FGF/MEK/ERK signalling pathways, and involve small groups of laterally positioned cells expressing the *Nodal* and *Delta2* ligands.

Similar cell interactions, but different signalling molecules, are involved in a-line muscle induction in *Halocynthia*

In *Halocynthia*, the cellular interactions that take place during A-line muscle induction appear to be similar to those in *Ciona*. However, there are some surprising differences in the identity of the ligands responsible for these cellular interactions.

b6.5 cell: FGF, Nodal and 'signal X'

The cellular and molecular interactions known to be involved in *Halocynthia* A-line secondary muscle formation are summarized in Figure 5(B). In *Halocynthia*, similar to *Ciona*, secondary muscle formation requires the FGF9/16/20–MEK signalling pathway (Kim and Nishida, 2001; Tokuoka et al., 2007). In addition, in *Halocynthia*, similar to *Ciona*, it has been shown that FGF9/16/20 is required for transcriptional activation of the *Nodal* gene in the b6.5 cell, and that Nodal signals are required for patterning of the A-line neural precursors along the medial–lateral axis (Tokuoka et al., 2007). However, strikingly, Nodal signals are not required for specification of the A-line muscle fates, which are generated in the lateral neural plate (A9.31 cell), as they are in *Ciona* (Tokuoka et al., 2007). Thus the mechanisms patterning the neural plate and specifying the secondary muscle precursor are separable processes in *Halocynthia* embryos. Despite this fundamental difference, formation of the secondary muscle does depend on the b6.5 blastomere, as ablation of this blastomere results in loss of A-line muscle (Tokuoka et al., 2007). Furthermore, FGF signals were shown to act indirectly via the b-line cells, as disruption of FGF signal transduction in b-line cells (via injection of a morpholino oligonucleotide against the ETS transcription factor) was sufficient to inhibit A-line muscle-fate specification (Tokuoka et al., 2007). Thus, in *Halocynthia*, an unidentified signal, 'signal X', which acts downstream of FGF9/16/20 in the b6.5 lineage, fulfils the role of *Ciona* Nodal during the induction of secondary muscle fate. Like Nodal, *Halocynthia* 'signal X' might also be involved in patterning of the neural plate.

A7.6 cell: Wnt5α and 'signal Y'

Another probable difference between A-line muscle specification in *Ciona* and *Halocynthia* is that the Notch signalling pathway does not appear to be required for secondary muscle formation in *Halocynthia* (Tokuoka et al., 2007). A *Halocynthia* homologue of the *Delta* gene is expressed more broadly than *Ciona Delta2*, although it is not yet known whether *Halocynthia Delta* is a target of Nodal signals (Figure 4) (Akanuma et al., 2002). Treatment of embryos with DAPT {*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester}, a pharmacological inhibitor of γ-secretase, an enzyme required for Notch-receptor processing, does not block secondary muscle formation in *Halocynthia*, whereas this same treatment blocks muscle-fate specification in the A-lineage in *Ciona* (Hudson et al., 2007; Tokuoka et al., 2007). It will be interesting to investigate whether Notch signalling is involved during patterning of the neural plate in *Halocynthia*, given its dual role in *Ciona*.

Despite the difference in requirement for Notch signalling, the A7.6 cell, which expresses *Delta2* in *Ciona* and is thus implicated in the induction of the A8.16 muscle lineage precursor (Figure 5A), is required for A-line muscle formation in *Halocynthia* (Tokuoka et al., 2007). The signal from the A7.6 cell may involve *Wnt5α*, which is strongly expressed in the A7.6 cell, as well as weakly in other cells at the 64-cell stage (Figure 4) (Tokuoka et al., 2007). Expression of *Wnt5α* in A7.6 depends upon the b5.3 lineage (Tokuoka et al., 2007). However, it is unlikely

Morpholino oligonucleotide: Morpholino antisense oligonucleotides are used in a variety of developmental systems in order to block translation or splicing processes of targeted mRNAs.

to be a target of either Nodal or 'factor X', as both of these factors depend on FGF9/16/20 signals, whereas *Wnt5α* expression does not (Tokuoka et al., 2007). Therefore, another FGF9/16/20-independent signal, 'factor Y', derived from b6.5 lineage, is required for *Wnt5α* expression in the A7.6 cell (Figure 5B). It is difficult to unravel the role of Wnt5α during A-line muscle specification, as this signalling molecule is required for many aspects of development. Inhibition of *Wnt5α* results in loss of notochord, mesenchyme and some B-line muscle, as well as secondary muscle (Nakamura et al., 2006). In addition, the potential requirement for Wnt5α in A-line neural cells, which share the same lineage as the secondary muscle has not been tested, so it is not yet clear how many cell types depend on Wnt5α. The developmental role of Wnt5 has not yet been tested in *Ciona* embryos. However, the expression pattern of *Wnt5* is different in *Ciona* and *Halocynthia* (Figure 4) (Imai et al., 2004).

In *Halocynthia*, there is no role for FGF/MEK signals during the final division generating the A-line muscle precursors

Finally, FGF/MEK signals do not play a later role during A-line muscle specification in *Halocynthia*, as they do in *Ciona*. Generation of muscle fate from the A-lineages in *Halocynthia* becomes independent of FGF/MEK signals at the 64-cell stage (Tokuoka et al., 2007). Furthermore, inhibition of FGF signal transduction in the entire A-lineage (via injection of a morpholino oligonucleotide against the ETS transcription factor) does not disrupt A-line muscle formation (Tokuoka et al., 2007). How the final step that segregates muscle and neural fates and generates the A-line muscle precursor is achieved in *Halocynthia* is thus unclear, although it appears that the precursor, the A8.16 cell, can cell autonomously generate muscle fate when isolated late in its cell cycle (Nishida, 1990).

Conclusions

Similar cellular interactions, but different signalling molecules, govern A-line muscle induction in *Ciona* and *Halocynthia*

It is clear that the cellular interactions required for secondary muscle induction, involving the b6.5 and A7.6 blastomeres, are similar in *Halocynthia* and *Ciona* (Figure 5). Surprisingly, however, the molecular nature of the signals utilized during these cellular interactions are not well conserved, with no apparent role for Nodal or Delta/Notch signals during secondary muscle formation in *Halocynthia*, despite well-defined roles in *Ciona* (Hudson and Yasuo, 2005; Imai et al., 2006; Hudson et al., 2007; Tokuoka et al., 2007). These observations suggest that the constraints upon cell lineage, cell position and cellular interaction during evolution in the ascidians have been higher than those for the cell signalling molecules used during these inductive processes.

Identification of the downstream signalling components and transcription factors involved in A-line muscle-fate specification in both species would help to explain how these different molecular processes converge to activate muscle precursor specific genes, such as *MRF*, and to promote muscle fate (Satoh et al., 1996; Meedel et al., 2007).

Ascidian secondary notochord induction may provide another example of evolutionary flexibility

The notochord of the ascidian larval tail consists of 40 cells, aligned along the anterior–posterior axis. In both *Ciona* and *Halocynthia*, these 40 notochord cells originate from two lineages, with the primary lineage generating the anterior 32 cells and the secondary lineage producing the eight cells at the caudal tip of the tail. The molecular and cellular mechanisms underlying primary notochord specification appear to be conserved between these two ascidian species (Kim et al., 2007; Picco et al., 2007). However, as is the case for secondary muscle, the specification of the secondary notochord, which like the secondary muscle is found at the tip of the tail, may provide an example of flexibility in the upstream regulatory mechanisms used to make the same cell type in the same temporal and spatial position. The B8.6 pair of secondary notochord precursors arises at the 110-cell stage and they divide twice to generate the eight caudal notochord cells in both species. In *Ciona*, a signalling relay of FGF → Nodal → Delta2/Notch is required, whereby FGF is required both to suppress muscle fate in the B-line notochord lineage precursor, as well as to induce *Nodal* expression; Nodal then acts both within the B-line for notochord formation, as well as in the A-line for induction of a second ligand, *Delta2*, which then induces notochord fate in B8.6 at the 110-cell stage (Hudson and Yasuo, 2006). In *Halocynthia* it is not known whether Nodal or Delta play

any role in secondary notochord formation. Rather, BMP2/4 (bone morphogenetic protein 2/4), together with FGF-like signals, have been implicated in secondary notochord formation in *Halocynthia*, whereby BMP2/4 signals from the anterior endoderm are required during the 64-cell stage for the induction of secondary notochord fate in B8.6 cells (Darras and Nishida, 2001). This is unlikely to be the case in *Ciona*, as *BMP2/4* is not expressed in the anterior endoderm (Imai et al., 2004). Although more work is clearly needed to confirm if there is a difference, induction of secondary notochord fates may provide another example where the position and lineage of a cell is under high constraint, but the molecular mechanisms and, perhaps, in this case, the cellular interactions involved are variable.

Future directions

b-line muscle cell lineages

The cell lineages and cell division patterns in ascidian embryos appear to be highly constrained, being largely invariant between the distantly related ascidian species discussed in this review, as well as many other ascidian species. There are a few minor differences within the b-line muscle lineages, whereby the b7.9 cell gives rise to different numbers of muscle cells in *Ciona* and *Halocynthia*, and its neighbour, the b7.10 cell, gives rise to muscle only in *Halocynthia* (Figure 3). The most likely scenario is that the mechanisms used to induce muscle fate in the b7.9 lineage are also used to induce muscle fate in the b7.10 lineage in *Halocynthia*. Indeed, Tokuoka et al. (2007) found no differences in the mechanisms used in the b7.10 and b7.9 lineages during the induction of muscle fate; both rely on FGF9/16/20 and Wnt5α, and both become insensitive to inhibition of MEK signalling by the 130-cell stage.

The embryological basis for the differences in the number of b-line muscle cells in *Halocynthia* compared with *Ciona* will be interesting to address. One possibility is that the cell position of the b7.10 cell lineage relative to muscle-inducing cells is slightly different between *Ciona* and *Halocynthia*, in such a way that muscle-inducing cells are in contact with both cells of the b7.9 and b7.10 lineage in *Halocynthia*, whereas in *Ciona* the muscle-inducing cells contact only the b7.9 lineage (or make relatively less contact with the b7.10 lineage). It has recently been shown in ascidian embryos that a differential response between competent cells to inductive cellular interactions can be achieved via a threshold in responsiveness to the signal, depending on the area of cell-surface contact between inducing and responding cells (Tassy et al., 2006). If one could increase the area of cell contact between the *Ciona* b7.10 lineage and the inducing cells (for example, by cell manipulation or by ectopic expression of the inductive ligand), would this lineage also produce muscle cells similar to *Halocynthia*? Finally, it should be noted that the b7.9 lineage produces different numbers of muscle cells in *Ciona* and *Halocynthia*, four and six cells respectively, suggesting that the cell-cycle control of muscle precursors may be differently regulated between these two species. Further work is clearly needed in order to understand the precise embryological and molecular basis of these differences in the muscle lineages of *Ciona* and *Halocynthia*.

Evolutionary implications of more muscle

The number of larval muscle cells is likely to have important consequences for survival and thus for evolution. Increased tail size and swimming power are likely to result in a wider dispersion of the larvae. It will be very interesting to address the embryological basis for the range of muscle cell number in a wider number of ascidian species, particularly those with giant larvae and a greater number of muscle cells. Where exactly do these extra muscle cells come from? Do all larval tail muscles simply undergo additional rounds of cell division or are additional muscle cells specified from different cell lineages? Unfortunately, the larger embryos and larvae tend to belong to colonial species of ascidian, which develop in an ovoviviparous manner. Thus their early eggs and embryos are inaccessible and have so far been subject to very few investigations.

Constraints on cell positions and interactions may be greater than those on the underlying molecular mechanisms

In contrast with the b-line muscle, the A-line muscle lineages are invariant between the two species addressed in this review. The cellular interactions taking place between the b6.5 cell and the A-line muscle precursor at the late 32-cell stage, and between A7.6 and the A-line muscle precursor at the 64-cell stage, appear to be highly constrained (Figure 5). However, the intracellular signalling molecules involved

in these inductive events appear to be highly flexible (Figure 5). Flexibility in the use of signalling pathways may be a common theme in evolution (True and Haag, 2001). In nematodes, Wnt signalling promotes vulval fate in *Caenorhabditis elegans*, but inhibits vulva formation in *Pristionchus pacificus*, which develops with a similar organization of vulval fate pattern (reviewed in Felix, 2005; Zheng et al., 2005). Thus, in the minimalistic mode of development adopted by many ascidians and nematodes, in which cell lineage, cell position, cell fate and cellular interactions are high constrained, the underlying molecular mechanisms can exhibit striking flexibilities.

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