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RESEARCH ARTICLE

Regulators specifying cell fate activate cell cycle regulator genes to determine cell numbers in ascidian larval tissues

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ABSTRACT

In animal development, most cell types stop dividing before terminal differentiation; thus, cell cycle control is tightly linked to cell differentiation programmes. In ascidian embryos, cell lineages do not vary among individuals, and rounds of the cell cycle are determined according to cell lineages. Notochord and muscle cells stop dividing after eight or nine rounds of cell division depending on their lineages. In the present study, we showed that a Cdk inhibitor, *Cdkn1.b*, is responsible for stopping cell cycle progression in these lineages. *Cdkn1.b* is also necessary for epidermal cells to stop dividing. In contrast, mesenchymal and endodermal cells continue to divide even after hatching, and *Myc* is responsible for maintaining cell cycle progression in these tissues. Expression of *Cdkn1.b* in notochord and muscle is controlled by transcription factors that specify the developmental fate of notochord and muscle. Likewise, expression of *Myc* in mesenchyme and endoderm is under control of transcription factors that specify the developmental fate of mesenchyme and endoderm. Thus, cell fate specification and cell cycle control are linked by these transcription factors.

KEY WORDS: Ascidian, *Ciona*, Cell cycle control, Cdkn1, Myc

INTRODUCTION

In animal development, cell cycle control is important for proper differentiation of tissues and organs. In many tissues, terminal differentiation is coupled with withdrawal from the cell cycle (G0 arrest). If cell cycle withdrawal is interrupted, it may affect normal development and could lead to cancer or other diseases (Buttitta and Edgar, 2007; Sun and Buttitta, 2017). For example, a myogenic basic helix-loop-helix transcription factor, Myog, induces cell cycle exit in differentiated myocytes in mammalian embryos (Liu et al., 2012). In nematode embryos, mutations in genes encoding cyclin-dependent kinase inhibitors induce hyperplasia in multiple somatic tissues, suggesting that these proteins normally stop cells from dividing (Fukuyama et al., 2003). Similarly, in *Drosophila* embryos, *dacapo*, encoding a cyclin-dependent kinase inhibitor, is necessary to stop epidermal cell proliferation (de Nooij et al., 1996; Lane et al., 1996). The retinoblastoma protein (Rb) is another important regulator of cell cycle withdrawal (Wikenheiser-Brokamp, 2006). In mice, deficiency of Rb in epidermis leads to hyperplasia (Ruiz et al., 2004). It is also important to maintain cell cycle progression for different cell populations. In mice, Mycn is required to maintain proliferating neural cell progenitors, and a mutation of this gene results in precocious differentiation of neural cells (Knoepfler et al., 2002).

In ascidian embryos, cell lineages are invariant among individuals, and numbers of cell divisions are strictly controlled (Conklin, 1905; Nishida, 1987). Notochord cells become post-mitotic after nine rounds of cell division, and every larva contains exactly 40 notochord cells. Similarly, in *Ciona*, 36 muscle cells are differentiated in the larval tail, and the anterior 28 cells are derived from the posterior vegetal quadrant of each embryo. Among these cells, 16 cells become post-mitotic after nine rounds of cell division, and 12 cells become postmitotic after eight rounds of cell division. Epidermal cells stop dividing after 11 rounds of cell division (Ogura et al., 2011; Pasini et al., 2006; Yamada and Nishida, 1999), although these cells resume cycling around the time when a larva develops competence for metamorphosis (Nakayama et al., 2005). By contrast, mesenchymal cells and endodermal cells, which produce adult tissues after metamorphosis, may continue to divide after hatching (Nakayama et al., 2005; Yamada and Nishida, 1999). In this way, these tissues in ascidian larvae regulate the cell cycle differently, which suggests that developmental programmes for these tissues are involved in determining when cells stop dividing.

In the notochord lineage of embryos of *Halocynthia roretzi*, an ascidian belonging to a different order than that of *Ciona*, the number of cell divisions is controlled by a mechanism that is under the control of Brachyury (T) (Fujikawa et al., 2011), a key transcription factor for notochord differentiation (Chiba et al., 2009; Satou et al., 2001a; Yasuo and Satoh, 1994). Similarly, the number of cell divisions in muscle cells is controlled by Tbx6-r (Kuwajima et al., 2014), a key transcription factor for muscle differentiation (Yagi et al., 2005; Yu et al., 2019). Because *Cdkn1.b* (also known as *CKI-b*), a gene encoding a Cdk inhibitor, is expressed in notochord and muscle cells, and because its expression in the notochord is under control of Brachyury, *Cdkn1.b* may be involved in regulation of cell cycle in these tissues (Kuwajima et al., 2014). Indeed, injection of *Cdkn1.b* mRNA into fertilized eggs halts the cell cycle around the 8- or 16-cell stage (Kuwajima et al., 2014), although its function in differentiation of notochord and muscle has not been evaluated.

By contrast, mesenchymal and endodermal cells likely continue to divide even after hatching (Nakayama et al., 2005), and produce a large number of small cells; however, it is not known how these

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tissues maintain cell cycle progression. It is also uncertain whether *Twist-r.a/b* and *Lhx3/4*, which encode key transcription factor genes for fate specification of mesenchyme and endoderm (Imai et al., 2003; Satou et al., 2001b), control cell numbers in these tissues. In the present study, we addressed these issues and found that *Cdkn1.b* and *Myc* serve essential functions in cell cycle control in ascidian embryos.

RESULTS

Zygotic gene expression is necessary for notochord, muscle and epidermal cells to stop dividing

Notochord cells stop dividing after nine rounds of cell division, and epidermal cells stop after 11 rounds of cell division (Nishida, 1987; Ogura et al., 2011; Pasini et al., 2006; Yamada and Nishida, 1999). The primary lineage of muscle cells (the anterior 28 muscle cells) stops dividing after eight or nine rounds of cell division depending on their lineages (Nishida, 1987). To examine whether zygotic gene expression is involved in regulation of cell cycle counts, we isolated cells of presumptive notochord and muscle at the 64-cell stage, and presumptive epidermal cells at the 32-cell stage using a fine glass needle, because developmental fate of these lineages is restricted to notochord, muscle and epidermis at these stages (Nishida, 1987; Nishida and Satoh, 1985). Because development of *Ciona savignyi* embryos is stable, especially in case of isolation of blastomeres, we used *C. savignyi* embryos in the present study unless otherwise noted.

First, we isolated one presumptive notochord cell (A7.3/A7.7) from each of 11 normal 64-cell embryos. When isolated cells were incubated in normal sea water until unperturbed control embryos hatched, they divided three times to become eight cells in most cases (Fig. 1A). Because each of these cells produces eight notochord cells in normal embryos, this observation indicated that the number of cell cycle rounds in these isolated cells was regulated tissue-autonomously, as previously indicated in *Halocynthia* embryos (Fujikawa et al., 2011). When we isolated presumptive notochord cells from unperturbed 64-cell embryos and isolated cells were incubated in sea water containing actinomycin D, which is an inhibitor of transcription (Crowther and Whittaker, 1984; Davidson and Swalla, 2001; Green et al., 2002; Krasovec et al., 2021; Meedel, 1983; Meedel and Whittaker, 1979; Miyaoku et al., 2018; Nishida and Kumano, 1997; Nishikata et al., 1987; Satoh and Ikegami, 1981; Shirae-Kurabayashi et al., 2006; Terakado, 1973; Whittaker, 1973, 1977), each of these cells became 13.4 cells on average

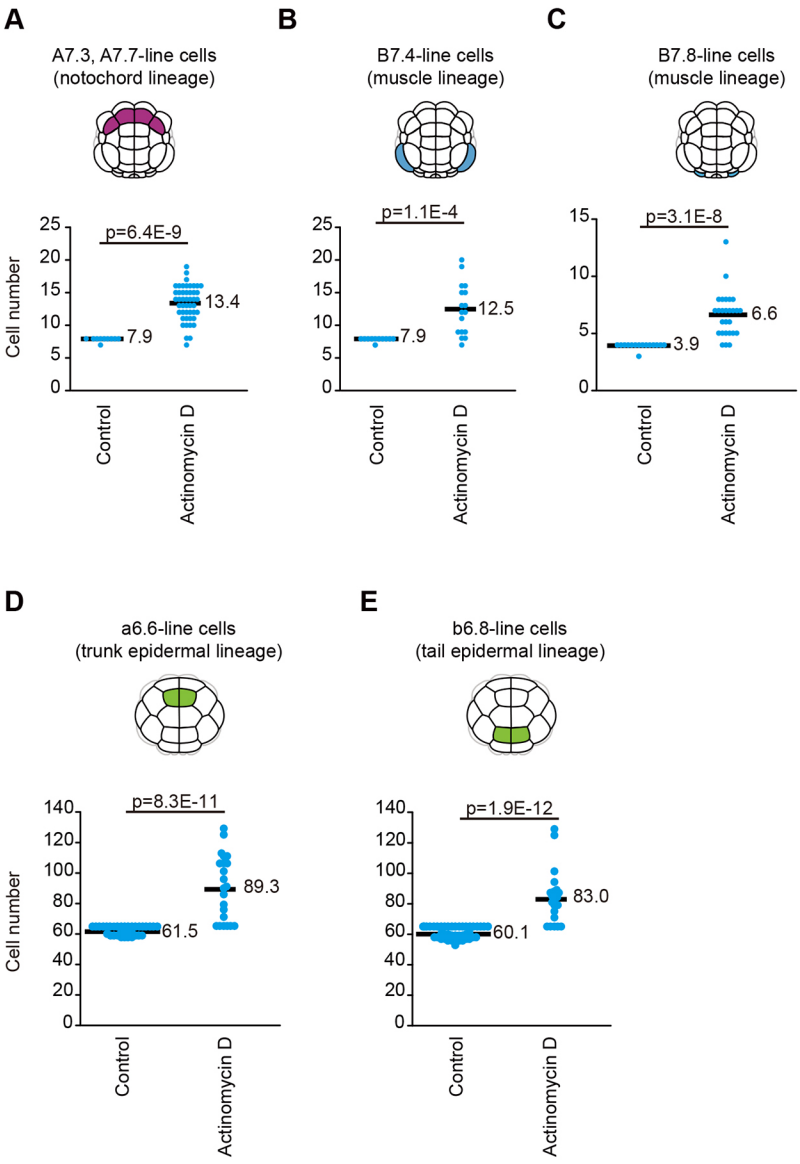

Fig. 1. Numbers of cells produced from isolated presumptive notochord, muscle or epidermal cells. (A-C) At the 64-cell stage, one presumptive notochord cell (A7.3 or A7.7; A) or presumptive muscle cell (B7.4 in B; B7.8 in C) was isolated from each normal embryo (isolated blastomeres are coloured in illustrations above the graphs). Isolated cells were incubated in normal sea water or sea water containing actinomycin D until control embryos hatched. The numbers of cells in the resulting partial embryos were counted. Differences in cell number were examined with the two-sided Wilcoxon rank sum test, and *P*-values are indicated. Each circle represents one partial embryo and indicates the number of cells in a single partial embryo. Averages are indicated by black bars. (D,E) Presumptive epidermal cells (a6.6 in D; b6.8 in E) were similarly isolated at the 32-cell stage, and counted.

Development (2022) 149, dev201218. doi:10.1242/dev.201218

(Fig. 1A). Thus, inhibitors of transcription increased the number of rounds of the cell cycle of isolated presumptive notochord cells, indicating that genes zygotically expressed after the 64-cell stage were required to stop cell division in the notochord lineage.

Similarly, we isolated two different lineages of presumptive muscle cells from normal 64-cell embryos. First, when B7.4 cells were isolated and incubated in normal sea water, isolated cells divided three times to become 7.9 cells on average (Fig. 1B). This number was close to the expected number ($8=2^3$), because B7.4 cells divide three times before stopping division in normal embryos (Nishida, 1987). Therefore, as in notochord and muscle cells in *Halocynthia* embryos (Fujikawa et al., 2011), this experiment showed that the number of cell cycle rounds of isolated blastomeres was tissue- or lineage-autonomously determined without requiring interactions with other cell lineages. By contrast, when we isolated this lineage of cells from unperturbed 64-cell embryos and incubated isolated cells in sea water containing actinomycin D, each of them became 12.5 cells on average (Fig. 1B). Thus, genes zygotically expressed after the 64-cell stage are also required to stop cell division in the muscle lineage.

Similarly, a previous study reported that B7.8 presumptive muscle cells divide two additional times before stopping division in normal embryos (Nishida, 1987), and we found that isolated B7.8 cells usually divide twice in normal sea water (Fig. 1C). However, isolated B7.8 cells incubated in sea water containing actinomycin D became 6.6 cells on average (Fig. 1C). Therefore, zygotic gene expression after the 64-cell stage is also necessary to stop the cell cycle in this muscle lineage.

Finally, we isolated presumptive epidermal cells (a6.6 and b6.8) from unperturbed 32-cell embryos. These cells divide six more times (11 times in total from 1-cell embryos) in normal embryos (Ogura et al., 2011; Pasini et al., 2006; Yamada and Nishida, 1999). When isolated a6.6 and b6.8 cells were incubated in normal sea water, they became 61.5 and 60.1 cells on average. These numbers were close to the expected value ($64=2^6$) (Fig. 1D,E). By contrast, a6.6 and b6.8 cells incubated in sea water containing actinomycin D became 89.3 and 83.0 cells (Fig. 1D,E). Therefore, cell cycle rounds of epidermal cells were also under control of zygotic gene expression after the 32-cell stage. Note that cell numbers did not always increase with actinomycin D treatment, because numbers of mesenchymal and endodermal cells were reduced with this treatment (see Fig. 6A,B).

A Cdk inhibitor controls the number of cell divisions of notochord, muscle and epidermal cells

The above experiments indicated that a gene or genes zygotically activated after fate specification are required to stop cell division properly in notochord, muscle and epidermis. A gene encoding a Cdk inhibitor was a strong candidate, because involvement of a Cdk inhibitor in regulation of cell division of notochord and muscle cells has been indicated in another ascidian (Kuwajima et al., 2014). Indeed, *in situ* hybridization showed that an orthologue (*Cdkn1.b*) was expressed zygotically, as shown in Fig. 2. Although no clear signal for zygotic expression was observed before the gastrula stage, the first evident *in situ* hybridization signal was detected in primary muscle lineage cells at the middle-gastrula stage. Soon after, presumptive notochord cells expressed *Cdkn1.b*. Signals were also detected in a few neural cells at the early neurula stage, and many neural cells expressed *Cdkn1.b* at the middle- and late-neurula stages. At the late-neurula stage, signals in epidermal cells also became evident transiently. At early and middle tailbud stages, signals in notochord, muscle and neural cells were evident, whereas signals in epidermal cells became undetectable.

Next, we injected a morpholino antisense-oligonucleotide (MO) into unfertilized eggs, which was designed to inhibit translation of *Cdkn1.b* mRNA. After insemination, injected eggs were incubated until the 32- or 64-cell stage. Then, to count accurately how many times cells divided, we isolated presumptive notochord cells (A7.3 or A7.7), presumptive muscle cells (B7.4 and B7.8) and presumptive epidermal cells (a6.6 and b6.8), and incubated them until uninjected control embryos hatched. As expected, isolated cells from *Cdkn1.b* MO-injected embryos produced a larger number of cells than isolated cells from embryos injected with a control MO against *Escherichia coli lacZ*, and the differences were statistically significant in all cases (Fig. 3A-E). These numbers were scarcely changed when we incubated for an additional 6 h. These results strongly supported the previously presented hypothesis that *Cdkn1.b* is responsible for stopping cell division in notochord and muscle (Kuwajima et al., 2014). In addition, the present study showed that *Cdkn1.b* is also transiently expressed in epidermal cells in order for epidermal cells to stop dividing.

To confirm the specificity of the MO, we designed another MO against *Cdkn1.b*. We isolated presumptive notochord (A7.3/A7.7) and muscle (B7.4) cells from embryos injected with the second *Cdkn1.b* MO, and found that injection of this MO similarly produced a larger number of cells than the control MO (Fig. S1).

Next, to confirm that knockdown of *Cdkn1.b* promoted extra rounds of cell cycle, not only in isolated cells but also in intact embryos, we injected the *Cdkn1.b* MO into eggs, and labelled presumptive notochord (A7.3/A7.7), muscle (B7.4 and B7.8) and epidermal (a6.6 and b6.8) cells with DiI, and incubated them until uninjected control embryos hatched. As shown in Fig. S2, the numbers of labelled cells were significantly increased in *Cdkn1.b* morphant embryos.

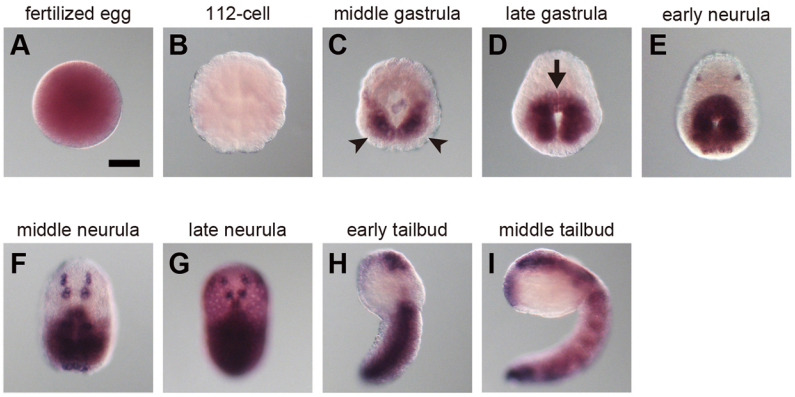

Fig. 2. Expression pattern of *Cdkn1.b* in *C. savignyi* embryos. (A-I) Representative images of whole-mount *in situ* hybridization of a fertilized egg (A) and embryos at the 112-cell (B), middle gastrula (C), late gastrula (D), early neurula (E), middle neurula (F), late neurula (G), early tailbud (H) and middle tailbud (I) stages. Expression in the muscle lineage was first evident at the middle gastrula stage (arrowheads). Expression in the notochord lineage was first evident at the late gastrula stage (arrow). Expression in epidermal cells was transiently evident at the late neurula stage. Scale bar: 50 µm.

Development (2022) 149, dev201218. doi:10.1242/dev.201218

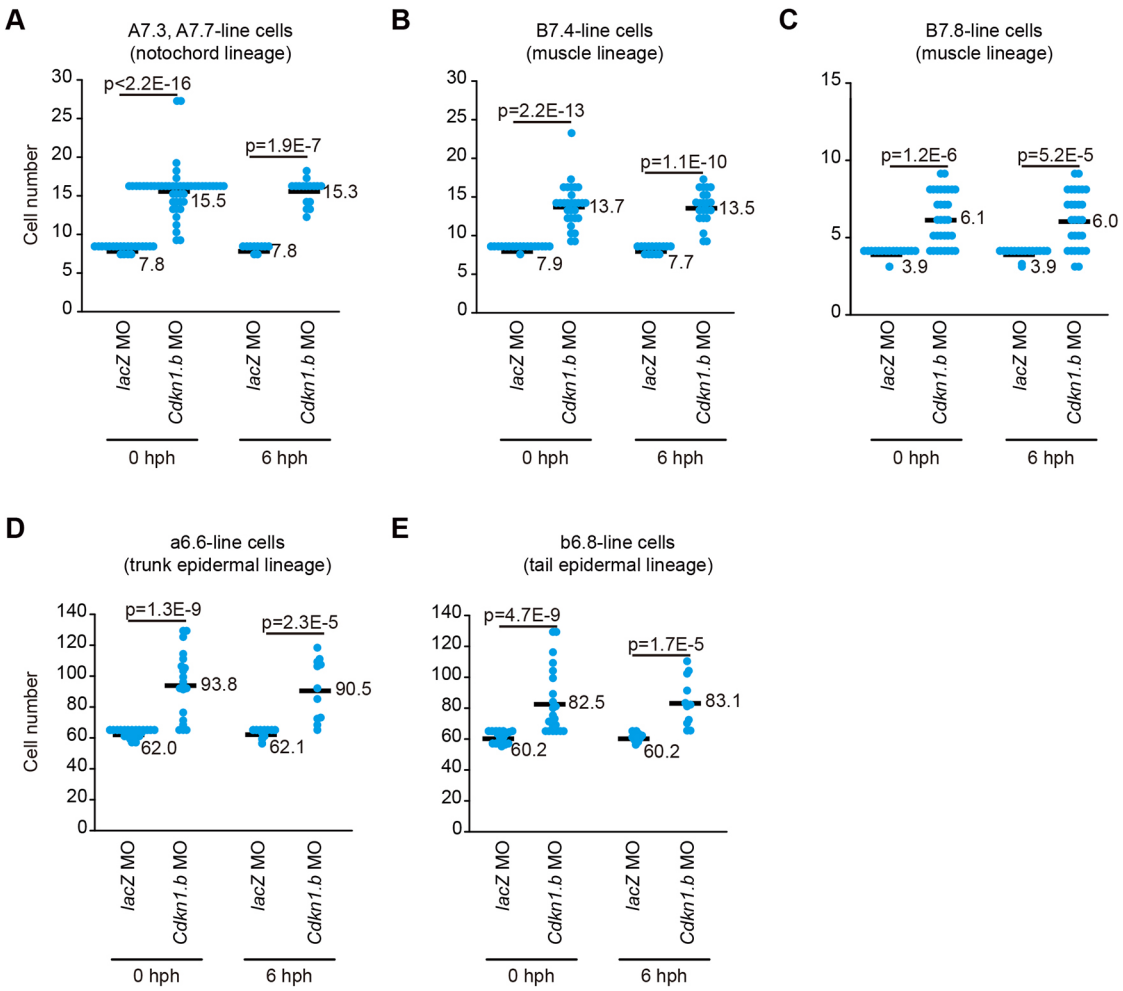

Fig. 3. Presumptive notochord, muscle or epidermal cells isolated from *Cdkn1.b* morphant embryos produced more cells than those isolated from control embryos. (A-E) The control *lacZ* or *Cdkn1.b* MO was injected into eggs. At the 32- or 64-cell stage, a presumptive notochord (A7.3 or A7.7; A), muscle (B7.4, B; B7.8, C) or epidermal cell (a6.6, D; b6.8, E) was isolated and incubated until uninjected sibling embryos hatched (0 hph) or 6 h later (6 hph). Embryos were fixed and cell numbers were counted. Differences in cell number were examined with the two-sided Wilcoxon rank sum test, and *P*-values are indicated. Each circle represents one partial embryo and indicates the number of cells in a single partial embryo. Averages are indicated by black bars.

Finally, we injected *Cdkn1.b* mRNA into eggs. A previous study reported that cell division stopped at the 8-cell or 16-cell stage in *Halocynthia* embryos in response to overexpression of *Cdkn1.b* (Kuwajima et al., 2014). However, in *C. savignyi* embryos, cells of embryos injected with *Cdkn1.b* mRNA divided normally until the 64-cell stage. Therefore, we isolated the presumptive notochord (A7.3/A7.7) and muscle (B7.4 and B7.8) cells at the 64-cell stage, and epidermal cells (a6.6 and b6.8) at the 32-cell stage, and incubated them until uninjected control embryos hatched. As expected, isolated cells from *Cdkn1.b* mRNA-injected embryos produced a smaller number of cells than isolated cells from embryos injected with a control *lacZ* mRNA (Fig. S3A-E). Therefore, overexpression of *Cdkn1.b* delayed or stopped cell cycle in the notochord, muscle and epidermal lineages.

Cdkn1.b is under the control of *Brachyury*, *Zic-r.b* and *Zic-r.a* in notochord and muscle cells

Because *Brachyury* directly or indirectly controls many notochord-specific genes (Takahashi et al., 1999), we examined whether expression of *Cdkn1.b* is under control of *Brachyury* in notochord. *Cdkn1.b* expression was indeed lost in presumptive notochord cells of embryos injected with a *Brachyury* MO, whereas it was unchanged in embryos injected with the control MO (Fig. 4A,B). This observation is consistent with what has been shown in *Halocynthia* embryos (Kuwajima et al., 2014). Next, we injected an MO against *Zic-r.b* (also known as *ZicL*). Because knockdown of *Zic-r.b* abolishes *Brachyury* expression (Imai et al., 2002), it was expected that knockdown of *Zic-r.b* would also suppress *Cdkn1.b*. Indeed, *Cdkn1.b* expression was lost in presumptive notochord cells of these embryos (Fig. 4C). These data indicated that this gene is under control of *Zic-r.b* and *Brachyury*. However, the data included in a previous study indicate that overexpression of *Brachyury* does not induce *Cdkn1.b* (Reeves et al., 2017), which suggests that *Brachyury* alone may not be sufficient to activate *Cdkn1.b* in the notochord lineage.

Zic-r.b is also involved in specification of muscle fate by activating *Tbx6-r.b* and *Mrf* in cooperation with a paralogous gene, *Zic-r.a* (also known as *Macho-1*) (Nishida and Sawada, 2001; Yu et al., 2019). Although knockdown of *Zic-r.a* or *Zic-r.b* does not completely abolish expression of muscle-specific genes, simultaneous knockdown of *Zic-r.a* and *Zic-r.b* abolishes it completely (Imai et al., 2002; Satou et al., 2002). Similarly, knockdown of *Zic-r.b* alone did not have an apparent effect on *Cdkn1.b* expression in muscle cells (Fig. 4C), but double

Development (2022) 149, dev201218. doi:10.1242/dev.201218

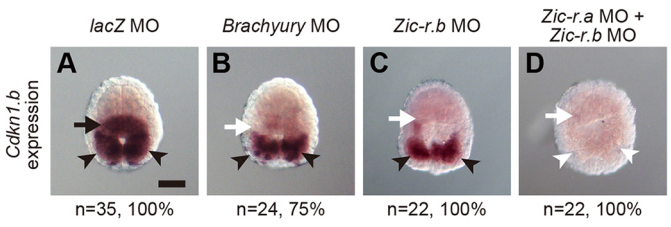

Fig. 4. *Cdkn1.b* is controlled by *Brachyury*, *Zic-r.b* and *Zic-r.a*. (A-D) *In situ* hybridization of *Cdkn1.b* at the late gastrula stage in embryos injected with the control *lacZ* MO (A), *Brachyury* MO (B) or *Zic-r.b* MO (C). The embryo in D was developed from an egg simultaneously injected with two MOs for *Zic-r.a* and *Zic-r.b*. Presumptive notochord and muscle cells are indicated by arrows and arrowheads, respectively. Black arrows and arrowheads indicate expression of *Cdkn1.b*, and white arrows and arrowheads indicate that *Cdkn1.b* expression was lost or decreased. Numbers of embryos examined and the proportion of embryos that each image represents are shown below the panels. Scale bar: 50 µm.

knockdown of *Zic-r.a* and *Zic-r.b* abolished *Cdkn1.b* expression in muscle cells (Fig. 4D). Thus, *Cdkn1.b* is controlled by the same developmental programmes that direct fate specification in notochord and muscle.

Finally, to demonstrate the importance of cell cycle control in proper formation of notochord, we injected the *Cdkn1.b* MO into eggs. The resulting larvae had short and thick tails, compared with normal larvae (Fig. 5A,B). Because major morphogenetic events of notochord formation start after notochord cells become post-mitotic (Jiang and Smith, 2007), it is likely that inhibition of *Cdkn1.b* prevented notochord morphogenesis. Indeed, at 0 h post-hatch (hph), there were many small notochord cells, and normal morphogenesis was apparently disturbed (Fig. 5C,D). At 6 hph, whereas a cell-free tubular lumen was conspicuous in the notochord of normal larvae (Fig. 5C′), such a lumen was not observed in *Cdkn1.b* morphants (Fig. 5D′).

Such morphogenetic defects were observed even before lumen formation. In early tailbud embryos injected with the control MO, 40 notochord cells were intercalated and aligned in a line (Fig. S4A). By contrast, in *Cdkn1.b* MO-injected embryos notochord cells were not aligned, although the number of notochord cells was the same as in normal embryos at this stage (Fig. S4B). In this experiment, we injected the *Cdkn1.b* MO into a pair of anterior vegetal blastomeres (A4.1 cell pair) of 8-cell embryos. Because this cell pair produces 32 notochord cells, but does not produce primary lineage muscle cells or epidermal cells, we can assess the effects of *Cdkn1.b* MO injection on notochord more clearly.

We also examined expression of two marker genes, *Talin* (Satou et al., 2001c; Yamada et al., 2003) and *Tropomyosin-like 1* (Di Gregorio and Levine, 1999), by *in situ* hybridization (Fig. 5E-H). As shown in Fig. 5F,H, these markers were expressed in *Cdkn1.b* morphant embryos, indicating that knockdown of *Cdkn1.b* did not affect expression of *Talin* or *Tropomyosin-like 1* in notochord cells. Similarly, we confirmed that muscle markers and epidermal markers were expressed in *Cdkn1.b* morphant embryos (Fig. S5).

Myc is required for continued division of mesenchymal and endodermal cells

A previous study showed that mesenchymal and endodermal cells in *Ciona robusta* (also called *Ciona intestinalis* type A) continue to divide after hatching (Nakayama et al., 2005). We confirmed that these cells continue to divide after hatching in *C. savignyi* by labelling one of three presumptive mesenchyme cells (B7.7, B8.5 and A7.6) and five presumptive endoderm cells (A7.1, A7.2, A7.5, B7.1 and B7.2) with DiI. Numbers of labelled cells in larvae at 6 hph were greater than those at hatching (0 hph) (Fig. S6A,B).

For further confirmation, we transferred normal larvae into sea water containing 5-bromo-2'-deoxyuridine (BrdU) for 30 min before fixation. At 0 and 6 hph, incorporation of BrdU in mesenchymal and endodermal cells was confirmed with a specific antibody (Fig. S6C), as in the case of *C. robusta* (*C. intestinalis* type A) (Nakayama et al., 2005).

Next, we examined whether zygotic transcription was necessary for mesenchymal and endodermal cells to continue to divide. For this purpose, we similarly labelled B7.7 (presumptive mesenchyme) and A7.2 (presumptive endoderm) of unperturbed embryos with DiI between the 68- and 112-cell stages, and then incubated them in normal sea water (control) or sea water containing actinomycin D. When control embryos hatched, we counted the labelled cells. We observed 109.0 labelled mesenchymal cells and 62.4 labelled endodermal cells in control embryos on average (Fig. 6A,B), and 22.6 and 24.3 labelled cells, respectively, in embryos incubated in sea water containing actinomycin D (Fig. 6A,B). Thus, inhibitors of transcription decreased the number of rounds of cell division or delayed the cell cycle of the mesenchymal and endodermal lineages, indicating that zygotically expressed genes are required to promote cell cycle progression in these cell lineages.

A previous study showed that a proto-oncogene, *Myc*, is zygotically expressed in mesenchyme and endoderm in *C. robusta* (*C. intestinalis* type A) (Imai et al., 2004), and we confirmed that *Myc* was zygotically expressed the same way in *C. savignyi* (Fig. S7). On the basis of this expression pattern, we tested the possibility that *Myc* is involved in cell cycle regulation of mesenchymal and endodermal cells. For this purpose, we injected an MO against *Myc* into eggs, and labelled a presumptive mesenchymal or endodermal cell with DiI between the 68- and 112-cell stages. When the control *lacZ* MO was injected and B7.7 (presumptive mesenchymal cell) was labelled with DiI, 107.4 and 117.4 labelled cells were detected at 0 hph and 6 hph, respectively (Fig. 6C). By contrast, when the *Myc* MO was injected, 27.1 and 28.4 labelled cells were observed at 0 hph and 6 hph (Fig. 6C). This suggested that control larvae divided approximately two additional times compared with *Myc*-morphant embryos. We obtained similar results in the two remaining mesenchymal lineages (B8.5 and A7.6 lineages; Fig. S8A). Therefore, the above observation indicated that *Myc* is necessary for mesenchyme cells to continue to divide.

Similarly, when the control MO was injected and A7.2 (presumptive endodermal cell) was labelled with DiI, 60.5 and 102.5 cells were detected at 0 hph and 6 hph (Fig. 6D). By contrast, when the *Myc* MO was injected, 26.4 and 30.0 cells were detected at 0 hph and 6 hph (Fig. 6D). We obtained similar results in the other four endodermal lineages (A7.1, A7.5, B7.1 and B7.2 lineages; Fig. S8B). Therefore, as in the case of mesenchymal cells, our data indicated that *Myc* is necessary for endodermal cells to continue to divide.

We could not obtain another *Myc* MO that could sufficiently knock down *Myc*; therefore, we made a construct that drove a dominant-negative version of *Myc* (*dnMyc*) under the upstream regulatory region of *Twist-r.a*. Because *C. robusta* (*C. intestinalis* type A) eggs are more suitable for electroporation, we used *C. robusta* eggs for this experiment. When we introduced a construct that contained a *lacZ* reporter and the same *Twist-r.a* upstream region into fertilized eggs, resultant larvae expressed the reporter in mesenchymal cells. In larvae co-introduced with constructs encoding *dnMyc* and *lacZ*, we found larger cells than those in normal larvae (Fig. S9A,B). To quantify larvae with large cells, we

Development (2022) 149, dev201218. doi:10.1242/dev.201218

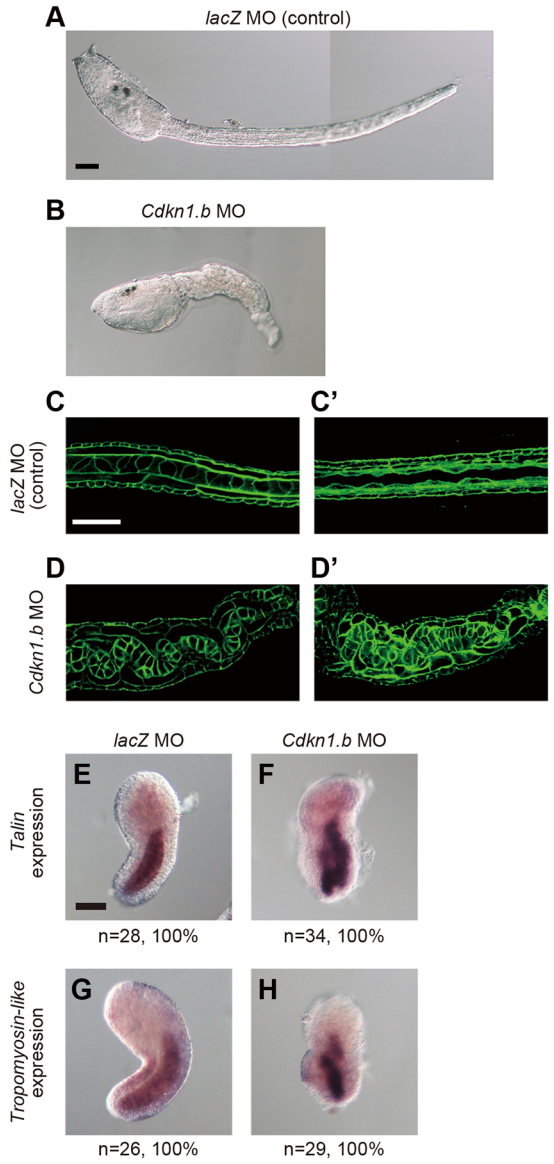

Fig. 5. Notochord cells in embryos injected with the *Cdkn1.b* MO. (A,B) The control *lacZ* MO (A) and *Cdkn1.b* MO (B) were injected into eggs, and resultant larvae are shown. Two images for the same embryo are combined in A. (C-D′) Resultant larvae were fixed at 0 hph (C,D) and 6 hph (C′,D′), and stained with phalloidin. Tail regions are shown. (E-H) Expression of *Talin* (E,F) and *Tropomyosin-like 1* (G,H) in early tailbud embryos injected with the control *lacZ* MO (E,G) or *Cdkn1.b* MO (F,H). Although morphology was severely disrupted, these notochord marker genes were expressed. Scale bars: 50 µm.

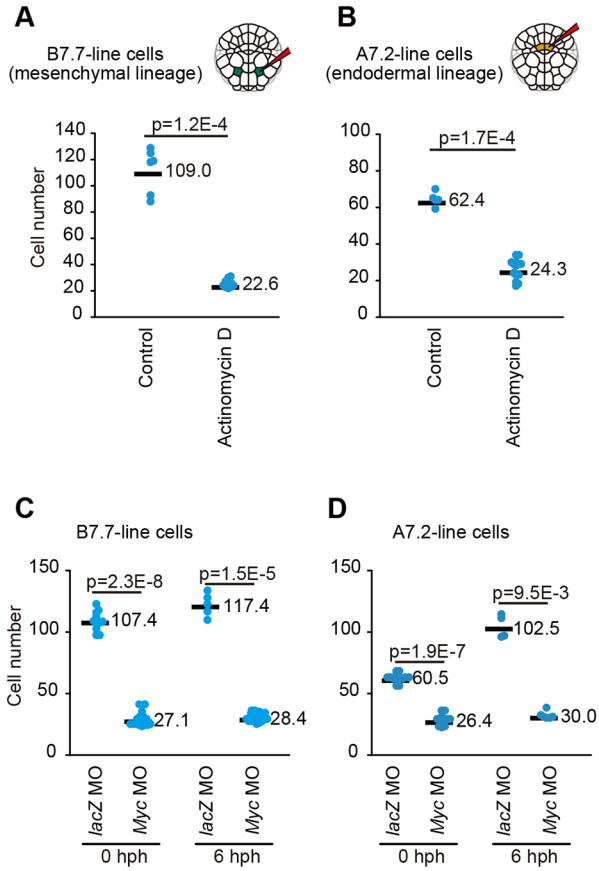

Fig. 6. *Myc* knockdown decreases cell numbers of mesenchymal and endodermal cells. (A,B) One presumptive mesenchyme (B7.7; A) or presumptive endoderm cell (A7.2; B) of unperturbed embryos was labelled with DiI between the 68- and 112-cell stages, and then incubated in normal sea water (control) or sea water containing actinomycin D. Numbers of labelled cells were counted when control embryos hatched. (C,D) The control *lacZ* or *Myc* MO was injected into eggs. A presumptive mesenchymal cell (B7.7; C) and an endodermal cell (A7.2; D) were labelled with DiI between the 68- and 112-cell stages, and incubated until hatching (0 hph) or 6 h after hatching (6 hph). Larvae were fixed and the number of DiI-labelled cells was counted. Each circle indicates the DiI-labelled cell number of a single larva. Differences in cell number were examined with the two-sided Wilcoxon rank sum test, and *P*-values are indicated. Each circle indicates the number of labelled cells in a single larva, and averages are indicated by black bars.

picked several cells that appeared largest in each of five control and eight experimental larvae into which only *dnMyc* was introduced, and measured areas of confocal images of these cells. As shown in Fig. S9C, embryos that expressed *dnMyc* contained larger cells than control embryos. We also counted the number of cells expressing β-galactosidase in embryos into which *lacZ* and *dnMyc* were co-introduced. The number of β-galactosidase-positive cells was markedly smaller in larvae with *lacZ* and *dnMyc* constructs than in larvae with the *lacZ* construct only (Fig. S9D). These results indicated that the dominant-negative form of Myc suppressed cell division, and were consistent with results obtained using the *Myc* MO in *C. savignyi* larvae; therefore, it is likely that the *Myc* MO successfully inhibited translation of *Myc* mRNA.

Myc is under the control of *Twist-r.a/b* and *Lhx3/4* in mesenchymal and endodermal cells

Twist-r.a/b and *Lhx3/4* are key transcription factor genes for fate specification of mesenchyme and endoderm, respectively (Imai et al., 2003; Satou et al., 2001b). *Myc* expression was lost in mesenchymal cells of embryos injected with a *Twist-r.a/b* MO and in endodermal cells of embryos injected with an *Lhx3/4* MO (Fig. 7). By contrast, expression of two mesenchymal marker genes [*Hm13* (previously called *Psl3*) and *Tram1/2*] (Tokuoka et al., 2004) and two endodermal marker genes (*Thr* and *Gata.a*) (Imai et al., 2004, 2006) were not changed in *Myc* MO-injected embryos (Fig. S10). These data indicated that *Myc* is under control of *Twist-r.a/b* and *Lhx3/4* in mesenchymal cells and endodermal cells, respectively, and that *Myc* is not involved in specification of mesenchymal fate or endodermal fate.

Development (2022) 149, dev201218. doi:10.1242/dev.201218

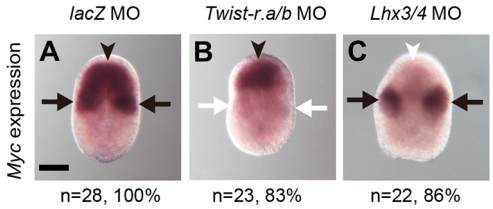

Fig. 7. *Myc* is controlled by *Twist-r.a/b* and *Lhx3/4*. *In situ* hybridization of *Myc* at the middle neurula stage in embryos injected with the control *lacZ* MO (A), *Twist-r.a/b* MO (B) or *Lhx3/4* MO (C). Presumptive mesenchymal and endodermal cells are indicated by arrows and arrowheads, respectively. Black arrows and arrowheads indicate expression of *Myc*, and white arrows and arrowheads indicate loss of expression. The number of embryos examined and the proportion of embryos that each image represents are shown below the panels. Scale bar: 50 µm.

Myc regulates transcription of cell cycle regulators

Because Myc is a transcription factor, it may regulate cell cycle regulator genes. To test this hypothesis, we quantified levels of mRNAs encoding Ccna (Cyclin A), Ccnb (Cyclin B), Ccnd (Cyclin D), Ccne (Cyclin E), Cdk1, Cdk2/3 and Cdk4/6 by reverse-transcription followed by quantitative PCR (RT-qPCR). As shown in Fig. 8, expression levels of *Ccnb*, *Ccne*, *Cdk1*, *Cdk2/3* and *Cdk4/6* were obviously reduced in *Myc* MO-injected embryos, whereas *Ccna* and *Ccnd* expression levels were not significantly altered.

DISCUSSION

Previous studies have shown that zygotic factors are required to control cell cycling in ascidian early and late embryos (Dumollard et al., 2013; Kuwajima et al., 2014; McDougall et al., 2012). The present study showed that numbers of cell cycle rounds in normal embryos are controlled by zygotically expressed *Cdkn1.b* and *Myc*, depending on cell lineages. In the A-line notochord lineage, cells stopped dividing after precisely nine rounds of cell division under control of *Cdkn1.b*. Similarly, in the primary muscle lineage, cells stopped dividing after eight (B7.8 lineage) or nine (B7.4 lineage) rounds of cell division, under control of *Cdkn1.b*.

As in the case of *Halocynthia Cdkn1.b*, *Ciona Cdkn1.b* begins to be expressed in notochord and muscle lineages within the cell cycle once or twice before the final cycle. Kuwajima et al. suggested the possibility that a certain duration, but not a certain number of cell divisions, is necessary for a sufficient amount of Cdkn1.b to accumulate (Kuwajima et al., 2014). Our data are consistent with this hypothesis. Expression of *Cdkn1.b* in epidermal cells is restricted to a short time during the neurula stage. This might be related to the fact that epidermal cells resume cell cycling several hours after hatching (Nakayama et al., 2005).

Numbers of notochord, muscle and epidermal cells in embryos injected with the *Cdkn1.b* MO were slightly greater in intact embryos than in isolated partial embryos (compare Fig. 3 with Fig. S2). Although this may be due to damage unexpectedly caused by isolation, it is possible that these cells may receive unknown signalling molecules that promote cell division through cell–cell interactions.

Mesenchymal and endodermal cells do not have apparent functions in larvae, and they produce adult mesodermal and endodermal tissues after metamorphosis (Hirano and Nishida, 1997, 2000; Tokuoka et al., 2005). The number of mesenchymal cells is estimated to be around 900 at 0 hph (Monroy, 1979). As we showed in the present study, numbers of the three lines of mesenchymal cells became 1.1-1.6 times (1.3 times on average) larger at 6 hph than at 0 hph (Fig. S6); therefore, there may be ∼1200 cells at 6 hph. Because mesenchymal cells are derived from five pairs of cells (ten cells) in the eighth generation (Nishida, 1987), these presumptive cells are estimated to divide six or seven more times (13-14 times in total from 1-cell embryos).

Similarly, the number of endodermal cells is estimated to be around 500 at 0 hph (Monroy, 1979). We observed that numbers of the five lineages of endodermal cells became 1.1-1.9 times (1.6 times on average) greater at 6 hph than at 0 hph; therefore, there may be ∼800 cells at 6 hph. Endodermal cells are derived from five pairs of cells in the seventh generation. Hence, it is likely that the five pairs of presumptive endodermal cells (ten cells) divide six or seven additional times (12-13 times in total). Thus, the number of cell cycles of mesenchymal and endodermal cells greatly exceed the number of cell cycles of the notochord and muscle lineages of cells. The present study indicated that *Myc* expression is required for mesenchymal and endodermal cells to divide so many times.

Myc is a well-known oncogene that controls cell cycling. One function of *Myc* is to regulate cell cycle-related genes

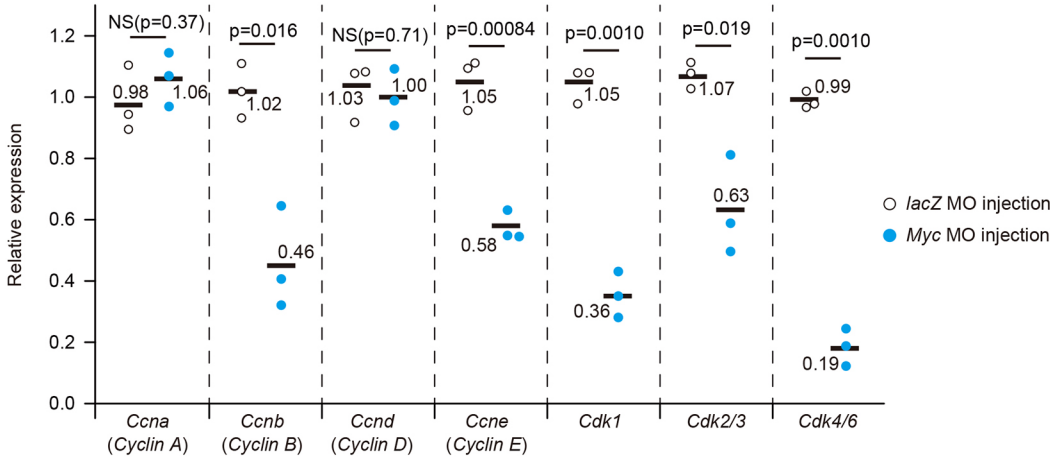

Fig. 8. Expression levels of mRNAs encoding cell cycle regulators. Relative mRNA expression levels were measured by RT-qPCR at the larva stage (0 hph). Expression levels in control *lacZ* MO-injected larvae (white circles) and *Myc* MO-injected larvae (blue circles) are represented as values relative to those of uninjected control larvae. Each circle shows a result for each of three independent experiments. *Ywhaz* was used as an internal control for normalization. Differences in relative expression were analysed with unpaired, two-tailed Student's *t*-tests using ΔΔCt values. NS, not statistically significant (P>0.05).

Development (2022) 149, dev201218. doi:10.1242/dev.201218

(Bretones et al., 2015). We examined this possibility in the present study. In our experiment, we used whole embryos that included tissues in which *Myc* was not expressed. Because these tissues might mask effects of *Myc* knockdown, it is possible that *Ccna* and *Ccnd*, levels of which were not changed in the present study, are also controlled by *Myc* in mesenchymal and endodermal cells. By contrast, despite the possible masking effect, *Ccnb*, *Ccne*, *Cdk1*, *Cdk2/3* and *Cdk4/6* were clearly downregulated by injection of the *Myc* MO. Thus, *Myc* controls cell cycle-related genes directly or indirectly, and ascidians use this conserved mechanism to continue the cell cycle in embryonic/larval mesenchyme and endoderm. Myc can also regulate the cell cycle by hyperactivating Cyclin/Cdk complexes, inhibiting transcription of *CDKN1A*, encoding a Cdk inhibitor, and activating genes that encode proteins involved in DNA replication (Bretones et al., 2015). It is possible that these mechanisms also act in ascidian mesenchyme and endoderm. We injected the *Myc* MO into eggs to evaluate its effects in whole embryos. Therefore, we cannot rule out the possibility that mesenchyme and endoderm interact with each other to control cell cycle rounds under control of *Myc*. However, because cell cycle rounds of these two tissues are determined without interaction among tissues in *Halocynthia* embryos (Fujikawa et al., 2011), it is likely that cell cycle rounds of mesenchyme and endoderm are also controlled tissue-autonomously in *Ciona* embryos.

In the *in situ* hybridization experiment, we detected maternal *Cdkn1.b* mRNA, but this maternal mRNA was hardly seen in 112-cell embryos. Injection of the *Cdkn1.b* MO did not apparently change the morphology of embryos before the 112-cell stage. Therefore, it is highly likely that the phenotypes observed in the present study were due to knockdown of zygotic *Cdkn1.b* mRNA. Likewise, we detected maternal *Myc* mRNA, and this maternal mRNA was hardly detected at the gastrula stage. Injection of the *Myc* MO did not apparently change the morphology of embryos before the gastrula stage. Therefore, it is highly likely that the observed phenotypes are due to knockdown of zygotic *Myc* mRNA.

Inhibition of progression of the cell cycle by Cdk inhibitors and maintenance of cell proliferation by Myc are well-known mechanisms for cell cycle control in animal development (de Nooij et al., 1996; Fukuyama et al., 2003; Knoepfler et al., 2002; Lane et al., 1996; Liu et al., 2012). The present study showed that both of these mechanisms are used in different tissues of ascidian larvae, and identified their upstream regulators. *Cdkn1.b* is under control of *Brachyury* (and its upstream regulator *Zic-r.b*) in the notochord lineage, and it is under control of *Zic-r.a* and *Zic-r.b* in the muscle lineage, whereas *Myc* is controlled by *Twist-r.a/b* in the mesenchymal lineage and by *Lhx3/4* in the endodermal lineage. These upstream regulators are transcription factors important for specifying cell fate (Corbo et al., 1997; Imai et al., 2002, 2003; Nishida and Sawada, 2001; Reeves et al., 2017; Satou et al., 2001b; Takahashi et al., 1999; Yasuo and Satoh, 1993, 1994; Yu et al., 2019). In muscle cells, *Tbx6-r.b* is activated under control of *Zic-r.a* and *Zic-r.b* (Yagi et al., 2004, 2005; Yu et al., 2019), and involvement of *Tbx6-r.b* in cell cycle regulation in muscle cells has been suggested in *Halocynthia* embryos (Kuwajima et al., 2014). Therefore, it is possible that *Cdkn1.b* is under the control of *Tbx6-r.b* in *Ciona* embryos. In this way, the present study showed that the same transcription factors that specify cell fate also regulate cell cycle regulator genes, directly or indirectly. However, our data do not necessarily indicate that tissue specification mechanisms and cell cycle control mechanisms are decoupled. In a related species, *Ciona robusta* (*Ciona intestinalis* type A), *Dlx.b* and *Tfap2-r.b* contribute to epidermal fate specification (Imai et al., 2017). Although currently we cannot efficiently knock down or knock out these genes in *C. savignyi*, it is possible that *Cdkn1.b* is regulated by either or both of these factors. Thus, cell fate specification and cell cycle control are tightly linked at the point of these key transcription factors in ascidian embryos.

MATERIALS AND METHODS

Animals and gene identifiers

Adult specimens of *Ciona savignyi* were obtained from the International Coastal Research Center (the University of Tokyo), the Onagawa Field Center (Tohoku University), the Research Center for Marine Biology (Tohoku University), the Maizuru Fisheries Research Station (Kyoto University), Honmoku fishing harbour (Kanagawa, Japan), and the University of California, Santa Barbara (CA, USA). Identifiers in Ensembl (Howe et al., 2021) for genes examined in this study are ENSCSAVG00000005700 for *Cdkn1.b*, ENSCSAVG00000000363 for *Myc*, ENSCSAVG00000002247 for *Brachyury*, ENSCSAVG00000001907 for *Zic-r.a*, ENSCSAVG00000002442/2450/2454/2459/2465 for *Zic-r.b* (multiple copies exist), ENSCSAVG00000011698 for *Talin*, ENSCSAVG00000009518 for *Tropomyosin-like 1*, ENSCSAVG00000008180 for *Ma1*, ENSCSAVG00000003085 for *Epi1*, ENSCSAVG00000009525 for *Cesa*, ENSCSAVG00000008611/8613 for *Twist-r.a/b*, ENSCSAVG00000000590 for *Lhx3/4*, ENSCSAVG00000007855 for *Hm13* (*Psl3*), ENSCSAVG00000009046 for *Tram1/2*, ENSCSAVG00000006056 for *Thr*, ENSCSAVG00000008350 for *Gata.a*, ENSCSAVG00000010339 for *Ccna* (*Cyclin A*), ENSCSAVG00000003185 for *Ccnb* (*Cyclin B*), ENSCSAVG00000003746 for *Ccnd* (*Cyclin D*), ENSCSAVG00000005373 for *Ccne* (*Cyclin E*), ENSCSAVG00000001660 for *Cdk1*, ENSCSAVG00000009914 for *Cdk2/3* and ENSCSAVG00000005088 for *Cdk4/6*.

Adult specimens of *Ciona robusta* (also called *Ciona intestinalis* type A) were obtained from the National Bio-Resource Project for *Ciona*. Identifiers for genes examined in this study are KY21.Chr1.384 for *Myc* and KY21.Chr5.361 for *Twist-r.a* (gene models were based on annotation of the latest genome assembly of this species; Satou et al., 2019, 2022).

Gene knockdown and overexpression/ectopic expression

Nucleotide sequences of MOs (custom-made by Gene Tools) were as follows: *Cdkn1.b*, 5′-CATTGTACGAAGGCGGTGGAACCAT-3′ and 5′-AAGGCGGTGGAACCATTTTGATTAC-3′; *Myc*, 5′-TGTTTATCGGTGTGCTGAGCTTCAT-3′; *Brachyury*, 5′-AACACGATTCTAAAGTGCTGGTCAT-3′; *Twist-r.a/b*, 5′-CTTGATTGTACTCTAGTGATGTCAT-3′; *Lhx3/4*, 5′-AAAGCGGGCTTGACTCGATACACAT-3′; *Zic-r.a*, 5′-AACCAAGCGTGCCAACGAAAGCCAT-3′; *Zic-r.b*, 5′-GTACATGATATTGATTGCTGTCTAA-3′. These MOs were designed to block translation. MOs for *Brachyury*, *Twist-r.a/b*, *Zic-r.a* and *Zic-r.b* have been described previously (Imai et al., 2002, 2003; Satou et al., 2001a, 2002). Specificity of MOs for *Cdkn1.b* and *Myc* was examined and is described in the Results section. In larvae injected with the *Lhx3/4* MO, alkaline phosphatase activity was rarely detected by histochemical staining (Fig. S11). This phenotype was similar to that obtained in a previous study in which a different MO against *Lhx3/4* was used (Satou et al., 2001b), suggesting that the *Lhx3/4* MO used in the present study acted specifically. We also used an MO against *E. coli lacZ* as a negative control (5′-TACGCTTCTTCTTTGGAGCAGTCAT-3′). These MOs were microinjected into unfertilized eggs unless otherwise specified. Concentrations of the MOs were 1.5 mM for *Cdkn1.b*, 1.0 mM for *Brachyury* and *Zic-r.b*, and 0.5 mM for all others.

Synthetic capped mRNAs for *Cdkn1.b* and *lacZ* were synthesized from *Cdkn1.b* or *lacZ* cDNA cloned into the pBluescript RN3 vector (Lemaire et al., 1995) using the mMESSAGE mMACHINE T3 Transcription Kit (Thermo Fisher Scientific). The concentration of the mRNA was 1.5 µg/µl.

A mutant *C. robusta Myc* encoding a dominant-negative form of Myc lacking Myc box II (Haque et al., 2016) was fused to the upstream region of *Twist-r.a*, and *E. coli lacZ* was similarly fused to the upstream region of *Twist-r.a*. These constructs were introduced into *C. robusta* fertilized eggs by electroporation. To measure the area of a cell, we used a confocal slice in which a given cell looked largest among all slices, and approximated each

Development (2022) 149, dev201218. doi:10.1242/dev.201218

cell using polygons. All image analysis was performed in ImageJ (https://imagej.nih.gov/ij/).

All experiments were performed at least twice using different batches of embryos. They gave essentially the same results. Total numbers of embryos examined are shown with images in figures.

Isolation of blastomeres, DiI labelling, cell counts and inhibition of transcription

Blastomeres were isolated with a fine glass needle under a binocular microscope, and isolated blastomeres were incubated using agar-coated dishes without further isolation or dissociation. DiI (CellTracker CM-DiI, Thermo Fisher Scientific) was dissolved in soybean oil at a concentration of 1 mg/ml. We labelled blastomeres with the DiI solution under a binocular microscope. To count cell numbers, isolated partial embryos, embryos and larvae were fixed for 30 min in 4% paraformaldehyde solution and mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, H-1200). Numbers of nuclei were counted under a fluorescence microscope or a confocal laser-scanning microscope. Actinomycin D was dissolved in DMSO and added to sea water at a final concentration of 200 µg/ml.

Whole-mount *in situ* hybridization, phalloidin staining, histochemical staining for alkaline phosphatase and acetylcholine esterase, and immunostaining of β-galactosidase

Whole-mount *in situ* hybridization was performed as described previously (Satou et al., 1995). To delineate cell shape, embryos were stained with Alexa Fluor 488 Phalloidin (1:200; Thermo Fisher Scientific, A12379) after fixation with 4% paraformaldehyde in MOPS buffer (0.5 mM NaCl, 0.1 M MOPS, pH 7.5) or 3.7% formaldehyde.

For histochemical detection of alkaline phosphatase activity, embryos were fixed with 4% paraformaldehyde in MOPS buffer for 10 min at room temperature. Embryos were washed in AP staining buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) twice, and then incubated with staining buffer (2.25 mg/ml Nitro Blue Tetrazolium, 1.75 mg/ml 5-bromo-4-chloro-3-indolyl phosphate).

For histochemical detection of acetylcholinesterase (AChE), embryos were fixed with 4% paraformaldehyde in MOPS buffer for 10 min at room temperature. Fixed embryos were washed in 100 mM sodium phosphate buffer three times, and incubated in AChE staining buffer (0.5 mg/ml acetylthiocholine, 65 mM sodium phosphate, 3 mM copper sulphate, 0.5 mM potassium ferricyanide, 5 mM sodium citrate, pH 6.0).

To detect β-galactosidase, embryos were fixed with 3.7% formaldehyde, and then incubated overnight with an anti-β-galactosidase monoclonal antibody (1:1000; Promega, Z3781) in Can-Get-Signal-Immunostain Solution B (TOYOBO). The signal was visualized using Alexa Fluor 555 anti-mouse secondary antibody (1:1000; abcam, ab150106) in Can-Get-Signal-Immunostain Solution B.

BrdU incorporation and immunodetection

To monitor DNA synthesis, we added BrdU (Nacalai Tesque, 05650) to sea water at a concentration of 100 µM. Incorporation of BrdU was monitored with a specific antibody (1:50; Merck, 11170376001) following a protocol described previously (Nakayama et al., 2005). Mesenchymal cells and endodermal cells were identified by cell size and cell location.

RT-qPCR

Total RNA was extracted from 30 larvae (0 hph) using NucleoSpin RNA XS (Macherey-Nagel, U0902A). cDNA was synthesized with an iScript cDNA synthesis kit (Bio-Rad, 1708891). Quantitative PCR was performed using the MiniOpticon Real-Time PCR Detection System (Bio-Rad) with SsoFast EvaGreen Supermix (Bio-Rad, 1725200). A one-embryo-equivalent quantity of cDNA was used for each reaction. Cycling conditions were preheating at 95°C for 30 s, and 40 cycles of 95°C for 5 s and 60°C for 10 s. We took three biological replicates. Relative expression values were calculated using the ΔΔCT method, and expression values were normalized to a housekeeping gene, *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide). Primers used were: *Ccna*, 5′-GCAGCAAGACATCACAGTTGG-3′ and 5′-TGGTTTCGGTGTGGAGTTTG-3′; *Ccnb*, 5′-CAACATACGCTCGCAAAATACC-3′ and 5′-AAGGCACAAGGAACCAGCA-3′; *Ccnd*, 5′-AAGGTGTGAAGATGATGTGTTTCC-3′ and 5′-GTTGAGTTCGTTGGATTGGTTG-3′; *Ccne*, 5′-GGAGGTGTGCGAGGTTTATTC-3′ and 5′-GGGTTTTATGGATGTCGGTTG-3′; *Cdk1*, 5′-CGGTGTGACGCAGTTGAAAG-3′ and 5′-ATACGAGGCATTTAGCGAGCA-3′; *Cdk2/3*, 5′-TCGCACAGAGTCCTACACAGAGA-3′ and 5′-ATACATCCGCACTGGCACAC-3′; *Cdk4/6*, 5′-AGCGTGAAACCCAACTAATGC-3′ and 5′-AGTCCCATCAACATCTGCCTC-3′; *Ywhaz*, 5′-TTTCTGGACTTGGACGCAAAC-3′ and 5′-CGGCTTTCCCTTGTCCTTATC-3′.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.K., M.T.; Methodology: K.K., M.T., S.F.; Validation: K.K., M.T., S.F., Y.S.; Formal analysis: S.F., Y.S.; Investigation: K.K., M.T., H.S., M.A., S.K., S.F.; Data curation: K.K., M.T., S.F., Y.S.; Writing - original draft: K.K., M.T., Y.S.; Writing - review & editing: K.K., M.T., S.F., Y.S.; Visualization: K.K., M.T., S.F., Y.S.; Supervision: S.F., T.K., Y.S.; Funding acquisition: M.T., T.K., Y.S.

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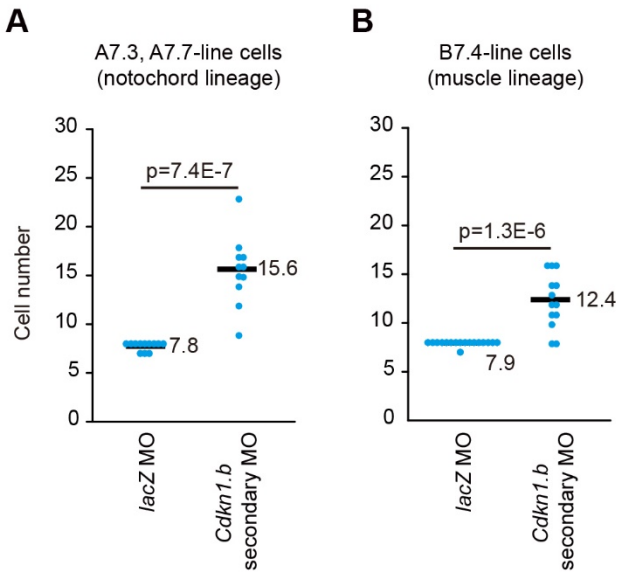


Fig. S1. A second MO against *Cdkn1.b* gave the same phenotype as the first MO. Presumptive notochord, or muscle cells isolated from embryos injected with the second *Cdkn1.b* MO produced larger numbers of cells than those isolated from control embryos. The control *lacZ* or *Cdkn1.b* MO was injected into eggs. At the 64-cell stage, a presumptive notochord (A) or muscle (B7.4; B) cell was isolated and incubated until uninjected sibling embryos hatched (0 hph). Embryos were fixed and cell numbers were counted. Differences in cell number were examined with the two-sided Wilcoxon rank sum test, and p-values are indicated. Each dot indicates the number of cells in a single patrial embryo, and averages are indicated by black bars.

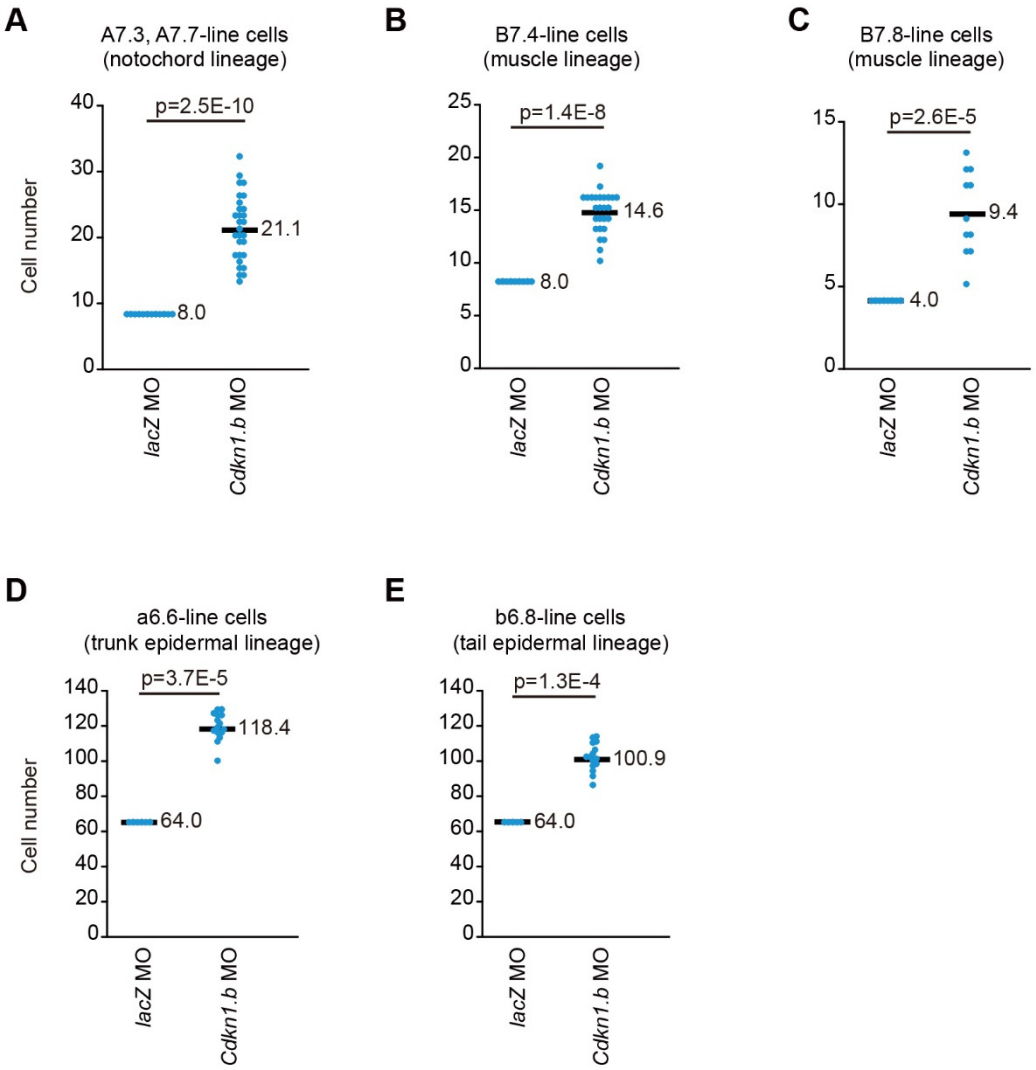


Fig. S2. Presumptive notochord, muscle, or epidermal cells in *Cdkn1.b* morphants produced larger numbers of cells than those in control larvae. The control *lacZ* or *Cdkn1.b* MO was injected into eggs. At the 32- or 64-cell stage, a presumptive notochord (A7.3 or A7.7; A), muscle (B7.4, B; B7.8, C), or epidermal cell (a6.6, D; b6.8, E) was labelled with DiI and incubated until uninjected sibling embryos hatched (0 hph). Embryos were fixed and labelled cell numbers were counted. Differences in cell number were examined with the two-sided Wilcoxon rank sum test, and p-values are indicated. Each dot indicates the number of labelled cells in a single embryo, and averages are indicated by black bars.

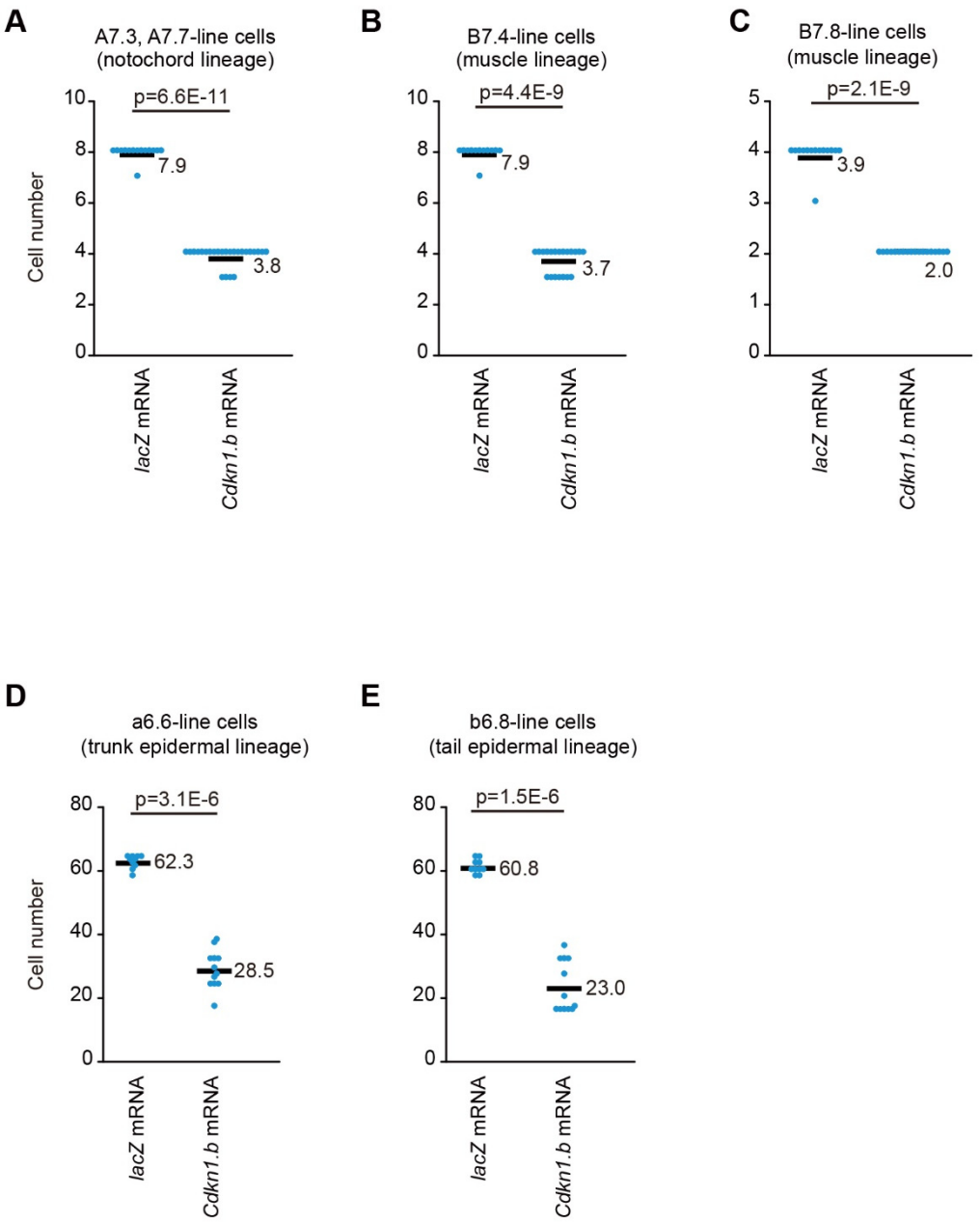


Fig. S3. Presumptive notochord, muscle, or epidermal cells isolated from *Cdkn1.b* mRNA-injected embryos produced smaller numbers of cells than those isolated from control embryos. The control *lacZ* mRNA or *Cdkn1.b* mRNA was injected into eggs. At the 32- or 64-cell stage, a presumptive notochord (A7.3 or A7.7; A), muscle (B7.4, B; B7.8, C), or epidermal cell (a6.6, D; b6.8, E) was isolated and incubated until uninjected sibling embryos hatched (0 hph). Embryos were fixed and cell numbers were counted. Differences in cell number were examined with the two-sided Wilcoxon rank sum test, and p-values are indicated. Each dot indicates the number of cells in a single patrial embryo, and averages are indicated by black bars.

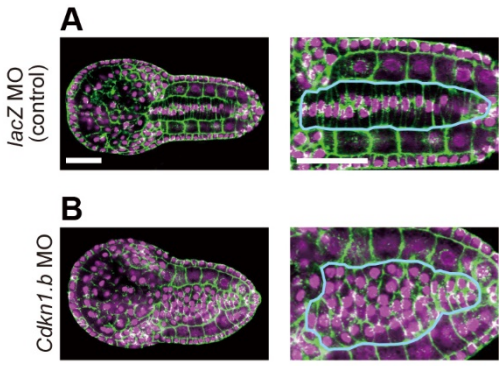


Fig. S4. Notochord cells in embryos injected with the *Cdkn1.b* MO. (A,B) We injected the (A) control *lacZ* MO and (B) *Cdkn1.b* MO into a pair of anterior vegetal blastomeres (A4.1 cell pair) of 8-cell embryos. The resultant tailbud embryos were fixed and stained with phalloidin (green). The tail region is magnified in the right panels. In contrast to control embryos, notochord cells are not properly aligned in a line in *Cdkn1.b* MO-injected embryos. Nuclei were stained with DAPI (magenta), and notochord is enclosed by cyan lines in the right panels. Scale bars, 50 μm.

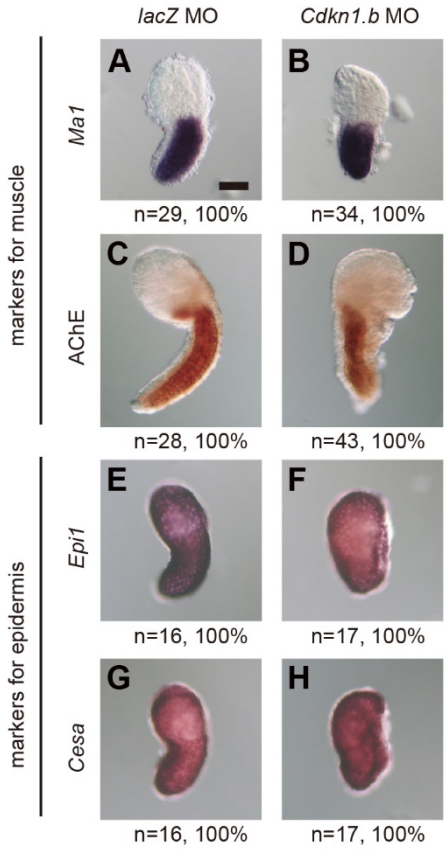


Fig. S5. Differentiation markers of muscle and epidermis. Differentiation markers for muscle (A-D) and epidermis (E-H) were tested in embryos injected with the control *lacZ* MO (A,C,E,G) or *Cdkn1.b* MO (B,D,F,H). Expression of (A,B) muscle actin gene (*Ma1*), (E,F) *Epi1*, and (G,H) *Cesa* was examined by *in situ* hybridization. (C,D) Expression of acetylcholine esterase (AChE) was examined by histochemical staining. Numbers of embryos examined and the proportion of embryos that each photograph represents are shown below the panels. Scale bar, 50 μm.

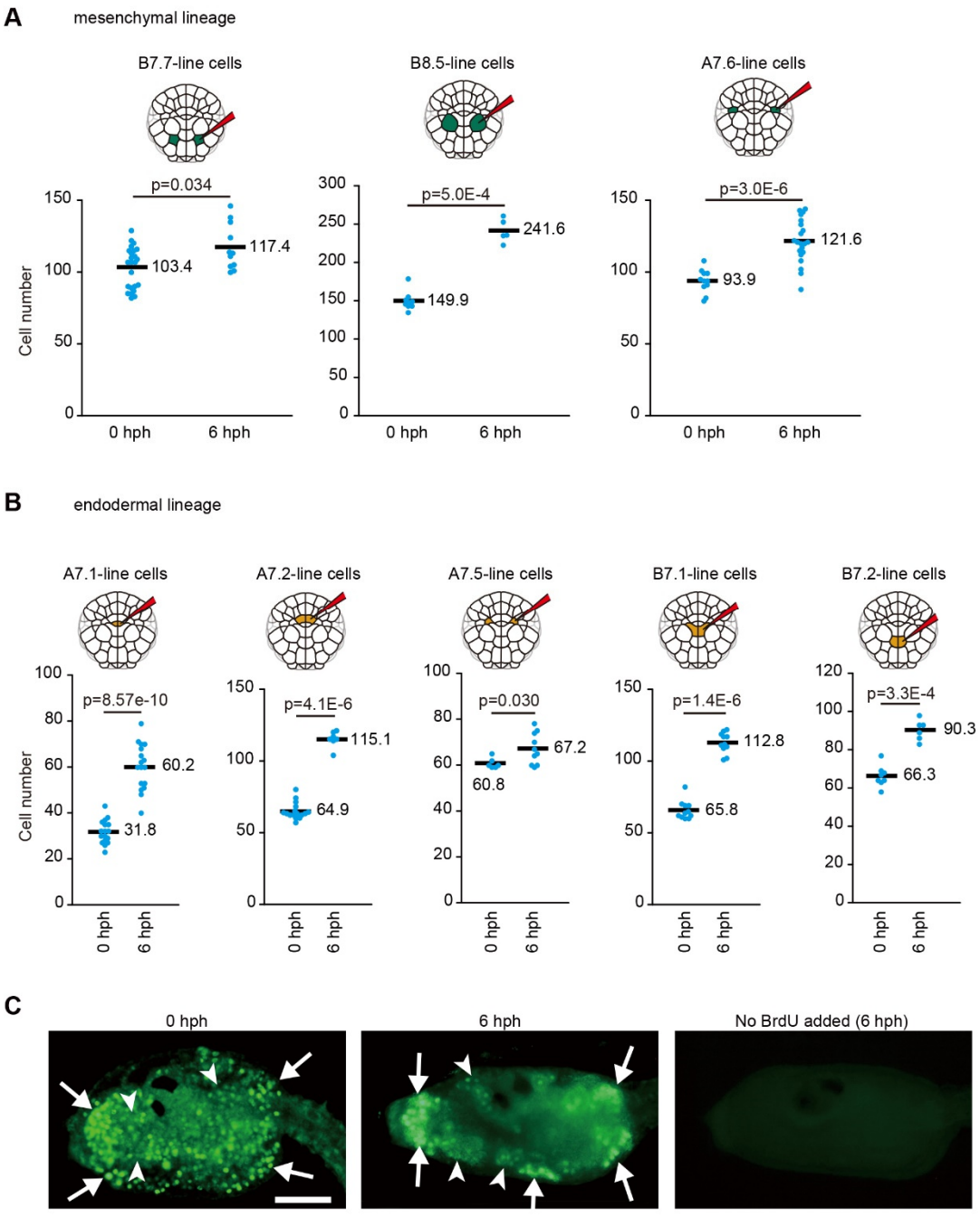


Fig. S6. Mesenchymal and endodermal cells divide even after hatching. (A,B) One of three presumptive mesenchymal cells (A) or one of five presumptive endodermal cells (B) was labelled with DiI between the 68- and 112-cell stages. Numbers of labelled cells were counted immediately after hatching (0 hph) or 6 h later (6 hph). Differences in cell number between 0 hph and 6 hph were examined with the one-sided Wilcoxon rank sum test, and p-values are indicated. Each dot indicates the number of labelled cells in a single larva, and averages are shown by black bars. (C) Larval trunk

regions stained with an anti-BrdU antibody. This larva was incubated in sea water containing BrdU for 30 min from 0 hph (left) and 6 hph (center). Signals were evident in mesenchymal and endodermal cells, while no signals were observed in larvae incubated in sea water that did not contain BrdU (right). Some mesenchymal cells and endodermal cells are indicated by arrows and arrowheads, respectively. Scale bar, 50 μm.

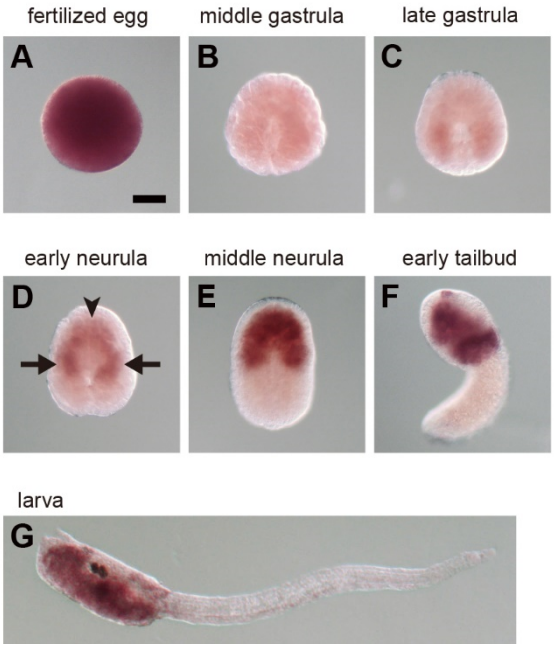


Fig. S7. Expression pattern of *Myc* in *C. savignyi* embryos. Photographs of whole mount *in situ* hybridization of (A) a fertilized egg, and embryos at the (B) middle gastrula, (C) late gastrula, (D) early neurula, (E) middle neurula, (F) early tailbud, and (G) larval stages. Expression in the mesenchyme lineage was first evident at the early neurula stage (arrows). Expression in the endodermal lineage was also first evident at the early neurula stage (arrowhead). Scale bar, 50 μm.

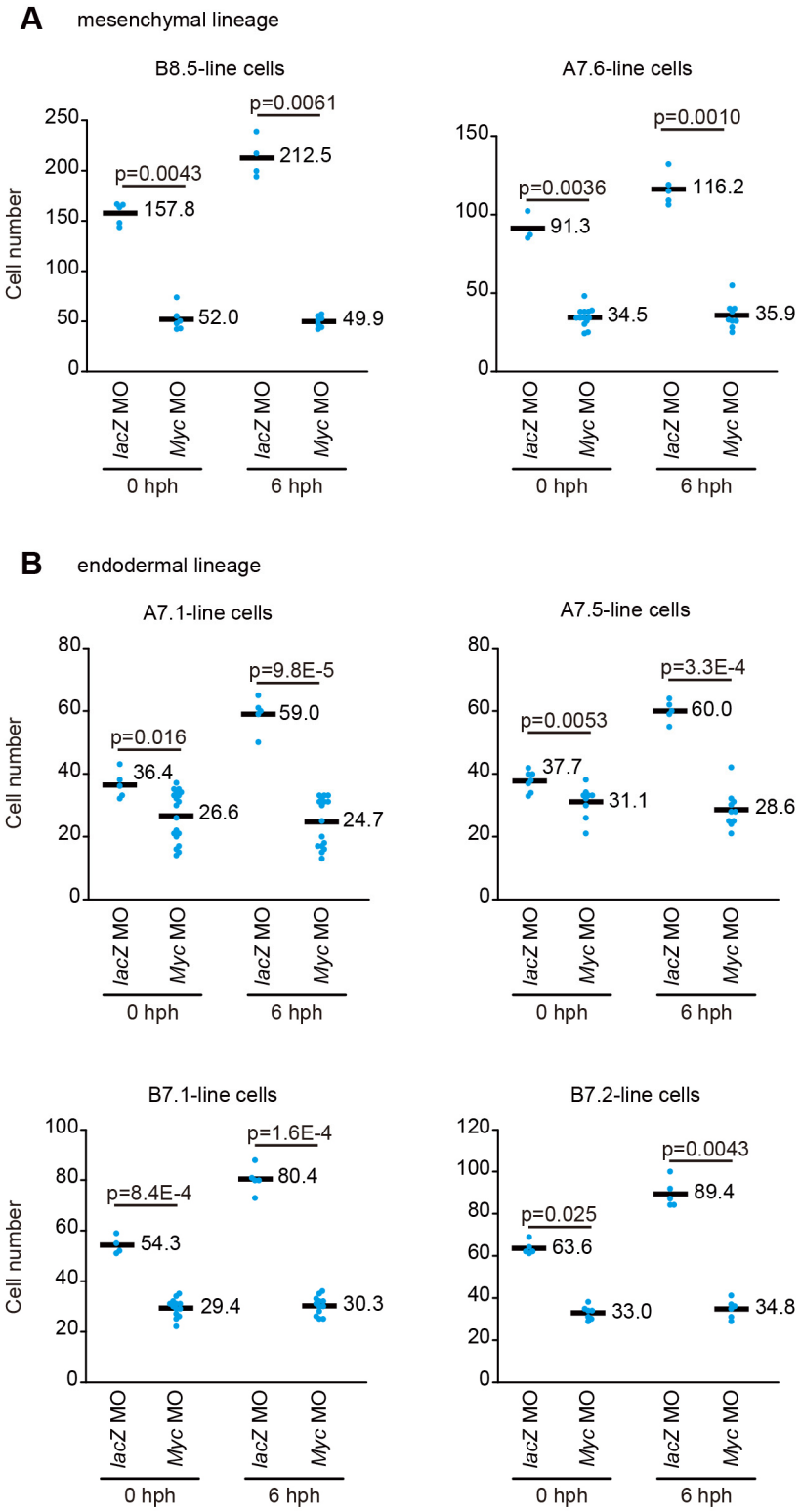
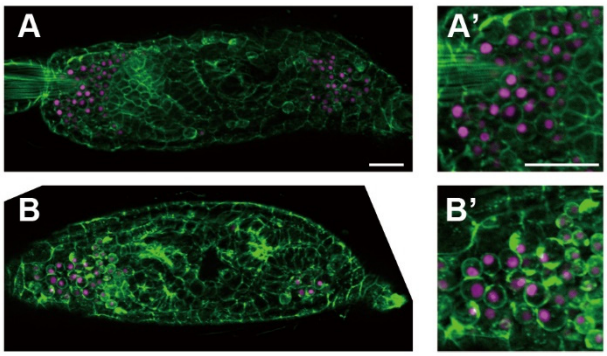
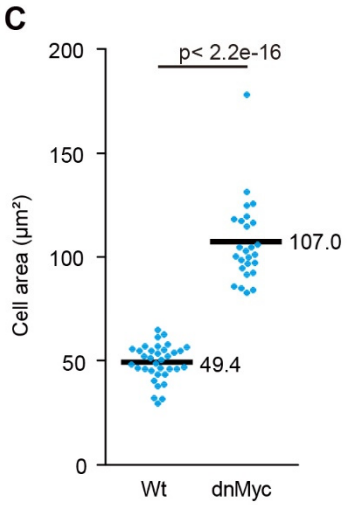


Fig. S8. Knockdown of *Myc* decreases numbers of mesenchymal and endodermal cells. (A,B) The control *lacZ* or *Myc* MO was injected into eggs. A presumptive mesenchymal cell (B8.5 or A7.6; A) and endodermal cell (A7.1, A7.5, B7.1 or B7.2; B) was labelled with DiI between the 68- and 112-cell stages, and incubated until hatching (0 hph) or 6 h after hatching (6 hph). Larvae were fixed and numbers of DiI-labelled cells were counted. Differences in cell number between larvae injected with the control *lacZ* MO and *Myc* MO were examined with the two-sided Wilcoxon rank sum test, and p-values are indicated. Each dot indicates the number of labelled cells in a single larva, and averages are indicated by black bars.





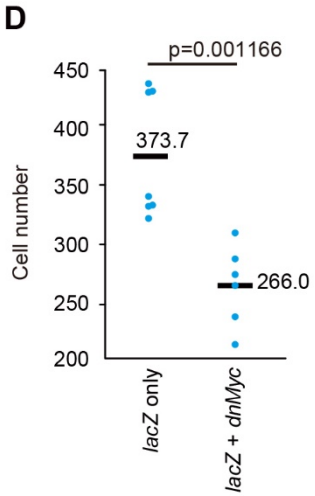


Fig. S9. A dominant negative form of *Myc* suppresses cell division in the mesenchymal and endodermal lineages. (A,B) A DNA construct that expressed *lacZ* under control of the upstream regulatory sequence of *Twist-r.a* was introduced into fertilized eggs of *C. robusta* (*C. intestinalis* type A) by electroporation. In B, a DNA construct that expressed a dominant negative form of Myc under the same upstream sequence was co-introduced. Larvae were fixed at 6 hph. An anti-β-galactosidase antibody was used to detect β-galactosidase protein (magenta). Larvae were also stained with phalloidin (green). Higher magnification views of trunk regions close to the tail are shown (A',B'). Scale bars, 25 μm. (C) We measured cell areas in confocal slices of 35 cells from five control embryos, and 26 cells from eight embryos introduced with *dnMyc*. Embryos were fixed at the late tailbud stage. We picked several cells that looked largest in individual embryos. Each dot represents a cell, and mean values are indicated by black bars. Differences in cell area were examined with the two-sided Wilcoxon rank sum test, and p-values are indicated. (D) Numbers of β-galactosidase-positive cells were smaller in larvae that expressed β-galactosidase and a dominant negative from of Myc than in larvae that expressed β-galactosidase only. Larvae were fixed at 6 hph. The difference in cell number was examined with the two-sided Wilcoxon rank sum test, and p-values are indicated. Each dot indicates the number of labelled cells in a single larva, and mean values are indicated by black bars.

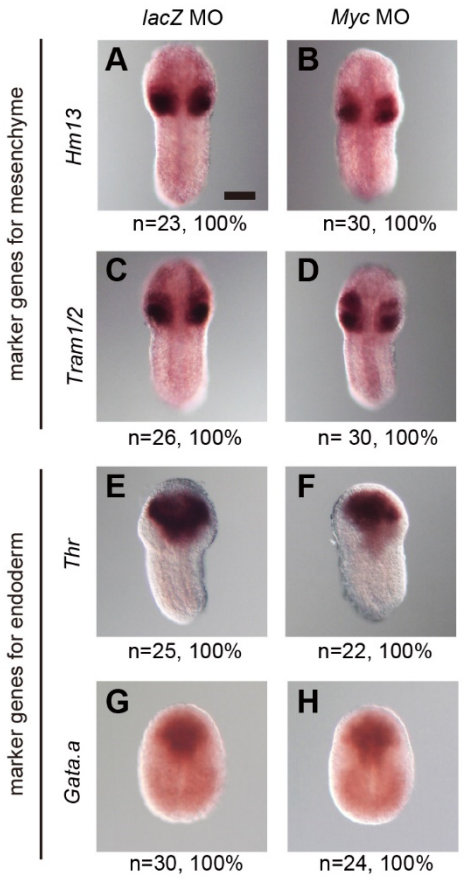


Fig. S10. Expression of marker genes for mesenchymal and endodermal cells. Expression of two mesenchymal marker genes, (A,B) *Hm13* and (C,D) *Tram1/2*, and two endodermal marker genes, (E,F) *Thr* and (G,H) *Gata.a*, was examined by *in situ* hybridization at (A-F) the early tailbud stage and (G,H) the middle neurula stage in (A,C,E,G) control *lacZ* MO-injected embryos and (B,D,F,H) *Myc* MO-injected embryos. Numbers of embryos examined and proportions of embryos that each photograph represents are shown below the panels. Scale bar, 50 μm.

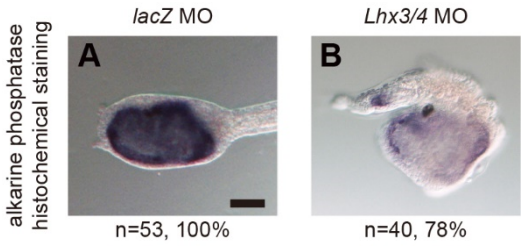


Fig. S11. Detection of alkaline phosphatase activity by histochemical staining. Larvae developed from eggs injected with (A) the control *lacZ* MO and (B) the *Lhx3/4* MO were histochemically stained to detect alkaline phosphatase activity. Numbers of larvae examined and proportions of larvae that each photograph represents are shown below the panels. In A, only the trunk region was shown. Loss of alkaline phosphatase activity has been reported in a previous study that used a different MO against *Lhx3/4* (Satou et al., 2001b). Scale bar, 50 μm.