

Review Article

Control of the number of cell division rounds in distinct tissues during ascidian embryogenesis

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An important question in developmental biology is how the number of cells constituting the body is regulated during development. After termination of cell divisions, cells enter the postmitotic phase, but the mechanisms determining the timing of final cell division remain unclear. In ascidians, the egg develops into a tadpole larva consisting of a relatively small number of cells (approximately 2800), indicating that embryonic cells divide 11.5 times on average after fertilization, although the number of cell division rounds differs among tissues. This article discusses the possible mechanisms that control the timing of the final cell division: these include (i) the analog and digital clock models, in which the elapsed time and number of cell division cycles are measured after fertilization, respectively, and (ii) the analog and digital timer models, in which the elapsed time and number of cell divisions are measured after cell fate specification, respectively. In notochord and muscle cells of ascidian embryos, cell division termination involves cell fate specification by inductive FGF signaling, followed by activation of the tissue-specific key transcription factors, *Brachyury* and *Tbx6*, which then induce gene expression of an effector, Cdk inhibitor. The present evidence suggests that these timing mechanisms comprise two functional phases: (i) an analog timer, which is mediated by accumulation of Cdk inhibitor protein after cell fate specification, and (ii) a timekeeper, which confines the timing, when the above timer is triggered by *Brachyury* and *Tbx6*, to the appropriate stage.

Key words: ascidian, Cdk inhibitor, cell division number, developmental clock, developmental timer.

Background

During animal development, a single cell, the fertilized egg, divides a fixed number of times to develop into an organism with a variety of differentiated cell types. Previous extensive investigations have clarified the mechanisms underlying the processes of cell fate specification and cell differentiation, and it is also now clear that each cell cycle is mediated by cell cycle regulators such as cyclins. However, the mechanisms that regulate the numbers of cell division rounds during development remain elusive. Since cells have to proliferate to generate a complete organism with an appropriate size and shape, the numbers of cell divisions are strictly regulated and linked to differentiation

(Myster & Duronio 2000; Zhu & Skoultschi 2001). So far, most studies relevant to this issue have been limited to those involving tissue culture cells and stem cells (Olson 1992; Le Grand & Rudnicki 2007; Doe 2008; Zuchero & Barres 2013). In this article, we discuss how the number of cell division rounds is controlled during ascidian embryogenesis, focusing on control of the timing of terminal cell divisions in the context of cell fate specification.

Ascidians are chordates, and classified into the subphylum Tunicata (Urochordata), which is a sister group to the vertebrates (Putnam *et al.* 2008). The ascidian tadpole larva possesses the basic body plan of a chordate (Satoh 2003), but comprises a relatively small number of cells (approximately 2800; Monroy 1979; Yamada & Nishida 1999) relative to vertebrate larvae (Fig. 1A). The cell lineages arising during embryogenesis are invariant and well documented. Fate restriction of blastomeres to a single cell type is almost complete as early as the 110-cell stage (Conklin 1905; Nishida 1987). Blastomeres that are isolated from the 110-cell embryo develop as a partial embryo, and they can differentiate as in normal embryos in a cell-autonomous

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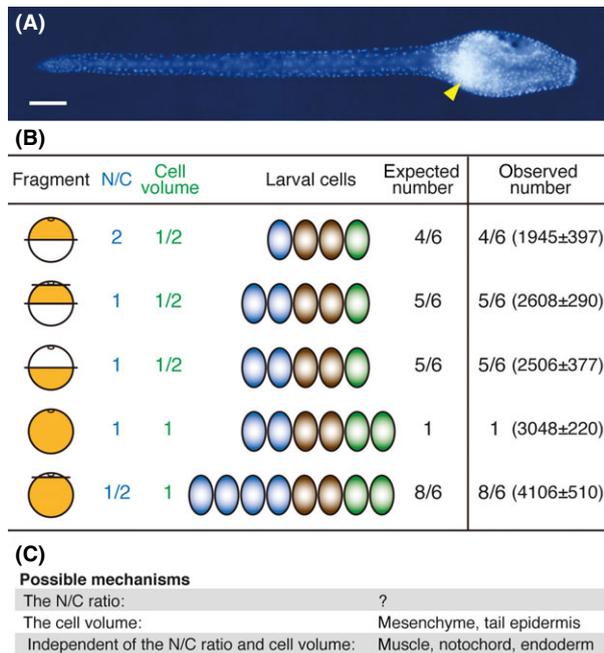


Fig. 1. Possible mechanisms involved in regulation of cell number in the tadpole larva. (A) A newly hatched ascidian tadpole larva, whose nuclei are visualized with DAPI (4',6'-diamidino-2-phenylindole dihydrochloride) staining. Anterior to the right. The bright region in the trunk (*arrowhead*) includes many small mesenchyme cells. Scale bar, 100 μm . (B) Various egg fragments that were prepared by cutting unfertilized eggs are shown in the left column. The small half circle at the top of the eggs depicts the female nucleus. The amount of DNA after fertilization and the volume of cytoplasm in an intact egg are supposed as '2n' and 1, respectively. The amount of DNA is 'n' when the female nucleus was removed. The volume of cytoplasm is 1/2 in half fragments. The N/C ratio and volume of egg fragments are indicated relative to those for a normal egg (fourth lane) as 1. The simulation was performed assuming that the number of cell divisions was controlled by three kinds of mechanism. Blue, green, and brown ovals represent cell groups regulated by the N/C ratio, cell volume, and a factor independent of both N/C ratio and cell volume, respectively. Each of the ovals represents about 500 cells. When the volume of cytoplasm was half that of a normal egg, the total cell number decreased, and when the N/C ratio was half it increased. The expected and observed cell numbers are shown on the right. Modified from Yamada & Nishida (1999) with permission from John Wiley & Sons. (C) Possible mechanisms controlling the number of cell divisions in each tissue.

manner (Nishida 1992). The mechanisms of cell fate specification in ascidian embryos are now well understood at the cellular and molecular levels for most of the cell types (Nishida 2005; Kumano & Nishida 2007). These characteristics of ascidian embryos make them well suited to studies aimed at clarifying the mechanisms controlling cell division rounds in connection with cell fate determination and differentiation.

Generally, cell proliferation and cell differentiation are thought to be incompatible, but in early embryogenesis they might be able to progress simultaneously in terms of gene expression. Various cell-type-specific transcription factors are already expressed by cells before they enter the postmitotic phase. Furthermore, in ascidians, expression of muscle-specific structural genes such as *actin*, *myosin*, and *sarcoplasmic Ca-transporter* is initiated at the 32-cell stage in muscle-lineage cells (Satou *et al.* 1995; Sawada *et al.* 2005). Although it is unclear whether these transcripts are translated into proteins in those cells, the available data indicate that, in terms of gene expression, cell differentiation has already started prior to the final cell division. On the other hand, cells never divide after they have started the process of functional (or morphological) differentiation, such as muscle contraction and axon formation, even in ascidian embryos, supporting the idea that cell proliferation and functional differentiation are incompatible.

Possible nature of mechanisms measuring time during embryogenesis

The timing of terminal cell division, as well as other events during embryogenesis, is regulated in a temporal manner. Here, we hypothetically use the terms developmental 'clock' and 'timer' to specify mechanisms that measure embryonic time. The 'clock' is defined as a mechanism that can measure the period elapsed after fertilization, to which cells refer for the decision to cease dividing. When the clock measures the time elapsed after fertilization, we refer to it as the analog clock. In contrast, the clock that counts the actual number of cell division rounds after fertilization is called the digital clock. Alternatively, the start button of the 'timer' for determining the point at which cell division ceases would be activated when cell fates are specified. In this case, embryonic cells start to measure time after their cell fates have been specified. In the 'timer' model, the analog and digital timer models are also applicable, as in the clock models. Thus, four possible models, (i) the analog clock, (ii) the digital clock, (iii) the analog timer, and (iv) the digital timer, can be postulated as the mechanisms capable of measuring the time during development until termination of cell division. Several specific mechanisms involved in temporal control of cell division have been proposed so far.

N/C ratio

The embryos of most animal species do not feed during embryogenesis. Therefore, the volume of the embryo is constant, and the cytoplasm of each cell is

reduced by approximately half at every cell division. The nucleo-cytoplasmic ratio (N/C ratio; ratio of the number of nucleus relative to the volume of cytoplasm within each cell, or the total number of nuclei in an embryo divided by the total amount of embryonic cytoplasm) has been proposed as a factor controlling the timing of the mid blastula transition (MBT) in the frog (Newport & Kirschner 1982a,b). During the cleavage stage, the transition from synchronous to asynchronous cell divisions, which is one of the phenomena observed at the MBT, occurs one division earlier in embryos that have twice the N/C ratio of normal embryos, and one division later in embryos with half the N/C ratio. MBT control based on the N/C ratio appears to be a widespread mechanism, since a similar effect of the N/C ratio has been reported in newt, zebrafish, and starfish embryos (Kobayakawa & Kubota 1981; Mita 1983; Mita & Obata 1984; Kane & Kimmel 1993). A similar role of the N/C ratio has also been observed during the blastoderm cell divisions in *Drosophila* (Edgar *et al.* 1986). Newport & Kirschner (1982b) have further demonstrated that injection of DNA, equal to the amount of nuclear DNA present at MBT, causes premature MBT in *Xenopus*, and on that basis proposed the nuclear DNA titration model. In fact, it was recently reported that at least four factors involved in DNA replication are listed as titration factors in the MBT of *Xenopus* (Collart *et al.* 2013). Although the N/C ratio has been implicated in the MBT, if factors required for cell cycle progression were titrated by nuclear DNA, cells would stop dividing when they exceeded a critical threshold value of the N/C ratio. As the N/C ratio doubles at every cell division during embryogenesis, it could conceivably function as a digital clock or timer. Although similar to, but different from the N/C ratio, some factors could simply become exhausted at every cell division, and are not titrated by DNA. This would be too speculative at the moment because no candidate factor/molecule that works in such a manner has been reported so far. In this case, the clock or timer would proceed independently of the amount of DNA.

Cell volume

Cultured mammalian cells as well as yeast cells can gauge their own cell size or amount of cytoplasm (Edgar & Kim 2009). Cells may cease proliferating when they reach a definite final size during embryogenesis. However, it still remains unclear how cells gauge their own size. One possibility is that they might sense the amount of cell cycle activators such as Cdk. Maternally stored cell cycle activator is partitioned into

each embryonic cell with every cell division. When the amount falls below the threshold level required for cell cycle progression, cell division will be terminated. In this case, the factors are not titrated or exhausted. The stepwise reduction of the activator might act as a digital clock or timer.

In the abovementioned cases, cells sense their conditions (N/C ratio, cell size, and amounts of specific factors) at each stage. The timing of final mitosis depends only on threshold levels and terminal cell conditions, and does not depend on previous developmental history. It is also possible that cells might count and “remember” how many times they have divided, but no definite evidence for such a mechanism has emerged so far.

Cascade of gene activation

Termination of cell divisions may be the consequence of a cascade that sequentially activates genes to eventually promote the expression of effector genes, such as *Cdk inhibitor*. Cdk inhibitor negatively regulates cell cycle progression by inhibiting the initiation of S-phase (Sherr & Roberts 1999; Besson *et al.* 2008; Lim & Kaldis 2013). The *Drosophila* Cdk inhibitor, Dacapo, is required for withdrawal of embryonic cells from the cell cycle (Lane *et al.* 1996; de Nooij *et al.* 1996), and the Cdk inhibitor of *C. elegans*, cki-1, facilitates a transient arrest of cell divisions in vulval precursor cells (Clayton *et al.* 2008). Since the expression of each gene in the cascade is probably controlled independently of cell division number that the cells have experienced, this fits the analog clock or analog timer models.

Gradual reduction or degradation of factors

Gradual and continuous reduction of absolute amounts of certain maternal factors through degradation might also serve as an analog time-measuring mechanism.

The abovementioned possibilities of reduction of maternal factors by titration, exhaustion, partition, and degradation could work as a clock model starting at fertilization and during early stages of embryogenesis. However, it seems unlikely that they would work as a timer starting after cell fate specification since zygotic gene expression starts. It is also difficult to explain tissue-specific control of cell division through reduction of maternal factors. Here, we distinguish the developmental ‘clock’ and ‘timer’ as mechanisms measuring the time after fertilization and after cell fate specification, respectively.

Cell numbers during ascidian embryogenesis

Prior to discussing whether the above timing mechanisms are involved in ascidian embryogenesis, it may be pertinent to give an outline of cell proliferation in ascidian embryos. The first report to indicate cell numbers in **an ascidian larva** was presented by Monroy (1979), who estimated that the epidermis, nervous system, mesenchyme, **muscle**, notochord, and endoderm consist of 800, 250, 900, **40**, 40, and 500 cells, respectively, giving a total of 2530 cells. The larva of the Japanese ascidian, *Halocynthia roretzi*, comprises approximately 2800 cells just after hatching, which was estimated by counting all nuclei of compressed and squashed larvae stained with a fluorescent dye specific for DNA (Yamada & Nishida 1999), similarly to the estimation by Monroy (1979). The estimated '2800 cells' in a larva would indicate that embryonic cells divide 11.45 times on average after fertilization. However, the numbers of cell division rounds differ significantly among tissues, the differences being conserved in *Halocynthia* and *Ciona*. The two are distantly related solitary ascidian species, as they belong to distinct Orders, and the diameters of their eggs also differ two-fold. Table 1 summarizes the number of cell division rounds in various tissues known so far during embryogenesis of these two species, along with the pertinent references.

Epidermis. Lineage tracing by labeling precursor blastomeres of each tissue in the *Halocynthia* larva

showed that epidermal lineage cells divide 11 times from the egg to the middle tailbud stage. Although it is unclear whether epidermal cells do not divide after the middle tailbud stage in *Halocynthia*, this number corresponds well to that in *Ciona* epidermis throughout embryogenesis, as revealed by a similar tracing study and by a study using a fluorescent ubiquitination-based cell cycle indicator (Fucci).

Nerve cord. The nerve cord is the neural tube posterior to the brain vesicle (see Fig. 2B). The average number of cell division rounds is 11.8 in the nerve cord of *Halocynthia*, shown by labeling a single precursor blastomere of the nerve cord, A7.4. In *Ciona*, observations of whole-mount embryos by confocal microscopy have shown that the nerve cord precursor in *Ciona* undergoes 9–13 cell divisions depending on the descendent lineages. Cells constituting the brain vesicle undergo 9–12 cell divisions (Cole & Meinertzhagen 2004).

Mesenchyme. There are many small mesenchyme cells in the anterior trunk region (Fig. 1A, *arrowhead* and Fig. 2E). These mesenchyme cells undergo 13.5 and 12.8 cell divisions in two lineages, B8.5 and B7.7, respectively, in *Halocynthia*.

Muscle. Muscle in the tail comprises 42 and 36 post-mitotic cells in *Halocynthia* and *Ciona*, respectively, which are the result of eight or nine divisions, according to muscle lineages, as will be mentioned in detail

Table 1. Total number of cell divisions from egg to larval hatching in each tissue

Tissue	<i>Halocynthia roretzi</i>				<i>Ciona intestinalis</i>		
	Lineage	Number of cell divisions	Stage of the final division	References	Lineage	Number of cell divisions	References
Epidermis	a-line	~11	~mTB	Nishida (1987)	a-line	11	Ogura <i>et al.</i> (2011)
	b-line	~11	~mTB	Nishida (1987)	b-line	11	Ogura <i>et al.</i> (2011); Pasini <i>et al.</i> (2006)
Nerve cord	A7.4	11.8	n.d.	Fujikawa <i>et al.</i> (2011)	A7.4	10–12	Cole & Meinertzhagen (2004)
	A7.8	n.d.	n.d.		A7.8	9–13	Cole & Meinertzhagen (2004)
Mesenchyme	B8.5	13.5	Not terminated	Fujikawa <i>et al.</i> (2011)	B8.5	n.d.	
	B7.7	12.8	Not terminated	Kuwajima <i>et al.</i> (2014)	B7.7	n.d.	
Muscle	B7.4	9	IG-eN	Nishida (1987); Kuwajima <i>et al.</i> (2014)	B7.4	9	Nishida (1987); Passamaneck <i>et al.</i> (2007)
	B7.8	8	IG-eN	Nishida (1987); Kuwajima <i>et al.</i> (2014)	B7.8	8	Nishida (1987); Passamaneck <i>et al.</i> (2007)
	B7.5	8	n.d.	Nishida (1987)	B7.5	8	Nishida (1987); Passamaneck <i>et al.</i> (2007)
	A8.16	9?	n.d.	Nishida (1987, 1990)	A8.16	n.d.	
	b8.17	9?	n.d.	Nishida (1987)	b8.17	n.d.	
	b8.19	n.d.	n.d.	Nishida (1987)			b8.19 does not develop into muscle.
Notochord	A7.3	9	eN	Fujikawa <i>et al.</i> (2011)	A7.3	9	Nakamura <i>et al.</i> (2012)
	A7.7	9	eN	Nishida (1987)	A7.7	9	Nakamura <i>et al.</i> (2012)
	B8.6	9	n.d.	Nishida (1987)	B8.6	9	Nakamura <i>et al.</i> (2012)
Endoderm	A7.2	10.8	Not terminated	Fujikawa <i>et al.</i> (2011)	A7.2	n.d.	

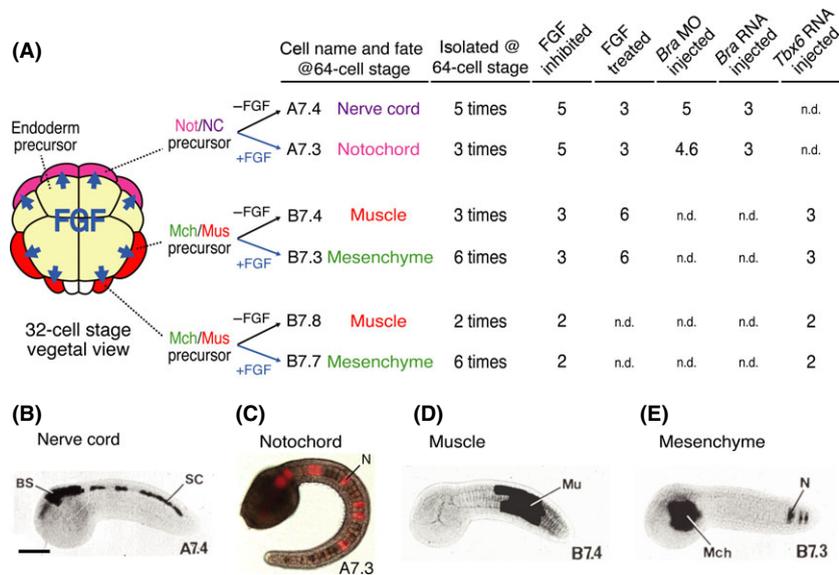


Fig. 2. Numbers of cell division rounds in isolated blastomeres at the 64-cell stage. (A) Vegetal view of the 32-cell embryo, color-coded for each tissue precursor. Anterior is up. In the anterior marginal zone of the vegetal hemisphere, notochord/nerve cord mother cells (in pink) divide asymmetrically into A7.3 notochord and A7.4 nerve cord of the 64-cell embryo. The FGF signal (blue arrows) at the 32- and 64-cell stages is required for induction of notochord fate while the default fate is nerve cord. In the posterior region, two kinds of mesenchyme/muscle mother cells (in red) divide asymmetrically into B7.3 mesenchyme and B7.4 muscle, and B7.7 mesenchyme and B7.8 muscle, respectively. FGF induces a mesenchyme fate, and the default fate is muscle. The number of cell division rounds after blastomere isolation at the 64-cell stage is shown for intact blastomeres and blastomeres derived from embryos in which the FGF signaling and transcription factors, Bra and Tbx6, are manipulated. n.d., not determined. (B–E) The fates of the A7.4, A7.3, B7.4, and B7.3 blastomeres in tailbud embryos. Anterior is to the left. Note that the A7.3 cell gives rise to eight notochord cells, while the B7.4 cell develops into eight muscle cells. They do not divide further, and are postmitotic. BS, brain stem (nerve cord); Mu, muscle; N, notochord; SC, spinal cord (nerve cord). Scale bar, 100 μ m. Images are reproduced from Nishida (1987) and Niwano *et al.* (2009) with permission from Elsevier and John Wiley & Sons, respectively.

later. The final cell division takes place by the early neurula stage. The difference in the total numbers of muscle cells between the two species is due to the number of muscle precursor cells in the 64-cell embryos, and not to the number of cell divisions (Nishida 1987; Hudson & Yasuo 2008).

Notochord. In both species, the notochord comprises exactly 40 postmitotic cells, which result from nine divisions. Notochord cells finish dividing by the early neurula stage.

Endoderm. The average number of cell division rounds is 10.8 in *Halocynthia* endoderm.

Thus, the number of cell division rounds varies from tissue to tissue, implying the presence of distinct mechanisms that regulate the number of cell divisions. It is worth noting that cells in the mesenchyme and endoderm do not stop dividing at the hatching stage and continue to divide after hatching until, and beyond, metamorphosis (Nakayama *et al.* 2005), giving rise to tunic cells and endodermal structures such

as the digestive organs and branchial sac, of juveniles, respectively (Hirano & Nishida 1997, 2000). Thus, the numbers of mesenchyme and endoderm cells at the hatching stage merely reflect the numbers of cell divisions until the hatching stage, and are not regulated by mechanisms terminating cell division.

Deducing the mechanisms that control the number of cell division rounds using egg fragments

Involvement of the N/C ratio and cell volume in control of the number of cell division rounds has been investigated by counting the numbers of cells in hatched larvae that have been derived from various egg fragments (Yamada & Nishida 1999). Unfertilized eggs were cut into halves, or the female pronucleus was removed, to alter the amount of egg cytoplasm and the N/C ratio (Fig. 1B). Such treated eggs divide with a normal cleavage pattern after fertilization and develop into tadpole larvae with a normal morphology (Reverberi & Ortolani 1962; Yamada & Nishida 1999). The total numbers of cells in the resulting larvae derived from

fragments of the animal-half, non-nucleate animal-half, vegetal-half, and non-nucleate egg fragments were approximately 4/6, 5/6, 5/6, and 8/6, respectively, relative to those of normal larvae (see right-hand column of Fig. 1B). The difference in cell number between larvae derived from the animal- and vegetal-half fragments is not due to differences in egg cytoplasm along the animal-vegetal axis because enucleated animal-half fragments develop into larvae with 5/6 cells, which is the same as that of vegetal-half fragments. The total number of cells in the larvae varies depending on the egg fragments. In particular, the larger the N/C ratio in egg fragments, the lower the number of larval cells (Fig. 1B, second column from the left). On the other hand, the non-nucleate animal-half, vegetal-half fragments and intact eggs have the same N/C ratio, but the numbers of larval cells of half fragments are lower, suggesting the involvement a cell volume factor (Fig. 1B, third column from the left).

In contrast to total cell number, the notochord in every kind of resulting larvae consists of about 40 cells, as in the normal larva (Yamada & Nishida 1999), suggesting the presence of a mechanism that is unaffected by either N/C ratio or cell volume. The difference in cell number between the larvae derived from various kinds of egg fragments can be explained by assuming that 1/3 each of the cells in an intact larva are controlled by the three types of mechanism: first by the N/C ratio, second by cell volume, and third by neither of the first two (Yamada & Nishida 1999). Accordingly, the expected numbers of cells in larvae developing from the animal-half, non-nucleate animal-half, vegetal-half, and non-nucleate egg fragments would be 4/6, 5/6, 5/6, and 8/6 relative to the normal larva, respectively (see columns of larval cells and expected numbers in Fig. 1B). This simulation corresponds well to the cell numbers observed, although the simulation is not necessarily reliable if mechanisms other than these three are involved.

Mechanisms controlling the number of cell divisions in various tissues

The above analysis suggests that distinct mechanisms are involved in the control of total cell number. The formation of each tissue has also been analyzed individually by counting the descendants of tissue precursor blastomeres isolated from embryos derived from various egg fragments (Fig. 1C; Yamada & Nishida 1999). The numbers of cells in partial embryos originating from isolated muscle precursors, notochord precursors, and endoderm precursors are constant, and do not depend on the type of egg fragments, suggesting that these tissues are unaffected by changes in N/C

ratio and cell volume. In contrast, mesenchyme precursors and tail epidermis precursors in partial embryos derived from animal-half and vegetal-half fragments divide into half the number of descendants in normal embryos, indicating that they are controlled by the cell volume factor. Mesenchyme cells especially become very small after many cell divisions (Fig. 1A, *arrowhead*), and therefore might divide down to a definitive minimal cell size limit. Division of tail epidermal cells may also be possibly regulated by the cell volume factor. Blastomere-isolation experiments have not revealed any example of a division mechanism that is dependent on the N/C ratio. However, N/C ratio control may operate in some tissues that have not yet been investigated, for example, the nervous system.

Most ascidian embryonic blastomeres isolated at the 64- and 110-cell stage show tissue-autonomous cell division control even in isolation, thus providing a useful system for analysis of tissue-specific cell division control. However, isolated blastomeres of some tissues do not divide as many times as intact embryos. According to Fujikawa *et al.* (2011), partial embryos of nerve cord, mesenchyme, and endoderm consist of slightly fewer cells in comparison with whole embryos, although they still show distinct tissue-specific cell division rounds. Therefore, it is still significant to compare cell numbers in partial embryos.

Cell fate conversion is accompanied by alteration of the number of cell division rounds

Analysis using egg fragments has shown that the numbers of cell divisions during muscle and notochord development are not affected by changes in the N/C ratio and cell volume. This suggests the presence of a developmental clock or timer mechanism that is independent of these two factors. Notochord and muscle consist of large postmitotic cells and stop dividing at an early stage in developing embryos. By showing that the number of cell division rounds can be altered upon conversion of cell fate, Fujikawa *et al.* (2011) and Kuwajima *et al.* (2014) have demonstrated that the number of cell divisions in notochord and muscle is determined at the fate specification stage. Fate restriction of notochord and muscle occurs at the 64-cell stage, when binary cell fate decisions between notochord and nerve cord in the anterior-vegetal region, and between muscle and mesenchyme in the posterior-vegetal region occur, being mediated by fibroblast growth factor (FGF) signaling. The FGF signal emanates from endoderm precursors in the 32-cell embryo (Fig. 2A). The fates induced by FGF are notochord and mesenchyme, while the default fates are nerve cord

and muscle (Nakatani *et al.* 1996; Minokawa *et al.* 2001; Kim *et al.* 2007). FGF promotes the induced fate in one of the daughter cells of the notochord/nerve cord mother cell and in one of the daughter cells of the muscle/mesenchyme mother cell, and the other daughter cells assume the default fates without FGF signaling. Thereafter, the cells divide a specific number of times, according to their fates. For example, the A7.4 nerve cord, A7.3 notochord, B7.4 muscle, and B7.3 mesenchyme precursors divide 5, 3, 3, and 6 times, respectively, between isolation at the 64-cell stage and the larval hatching stage (Fig. 2B–E).

The number of cell divisions undergone by these tissue precursor cells can be altered by manipulation of cell fates (Fig. 2A; Fujikawa *et al.* 2011; Kuwajima *et al.* 2014). Inhibition of inductive FGF signaling, for example, by a signaling inhibitor not only converts notochord and mesenchyme precursor cells to their default nerve cord and muscle fates, respectively, but also results in conversion of the number of cell divisions according to the altered fate. Conversely, ectopic activation of FGF signaling by treatment with FGF yields the opposite results, nerve cord and muscle fates being converted to induced notochord and mesenchyme fates, respectively, and the corresponding numbers of cell division rounds being altered accordingly. Thus, the number of cell divisions is determined at the fate specification stage, and thereafter the cells can autonomously accomplish the determined number of cell division rounds.

Cell-type-specific key transcription factors control both cell differentiation and the number of cell division rounds

After cell fates have been determined by an extracellular inductive signal, it is not possible to exogenously manipulate cell fates any further. However, it is still possible to interfere with cell differentiation processes by manipulation of transcription factors within the cells. The target of FGF signaling in notochord is the *Brachyury (Bra)* gene, which is essential for notochord differentiation (Yasuo & Satoh 1994, 1998; Nakatani *et al.* 1996; Chiba *et al.* 2009). Expression of *Bra* is initiated at the 64-cell stage soon after inductive events. Knockdown of *Bra* by injecting a *Bra* morpholino antisense oligo (*Bra* MO) results in a significant increase in the number of cells in notochord partial embryos (Fig. 2A; Fujikawa *et al.* 2011). After the normal three divisions after the 64-cell stage, cells continue to divide another 1.6 times on average, indicating that *Bra* regulates both cell differentiation and the number of cell divisions in notochord. This idea has also been supported by a *Bra* mis-expression experi-

ment. When *Bra* mRNA is injected, nerve cord and endoderm precursors divide three times, like notochord cells (Fig. 2A; Fujikawa *et al.* 2011).

Expression of the *Tbx6* gene in muscle lineage starts at the 64-cell stage (Yasuo *et al.* 1996; Mitani *et al.* 1999, 2001; Yagi *et al.* 2005). Similarly, mis-expression of this muscle-specific key transcription factor changes the number of cell divisions of mesenchyme precursors to that of muscle cells (Fig. 2A; Kuwajima *et al.* 2014). Thus, *Tbx6* likely activates both of the mechanisms that regulate muscle differentiation and muscle-specific cell division rounds.

However, precocious expression of *Bra* or *Tbx6* induced by injection of *Bra* or *Tbx6* mRNA into eggs and early cleavage-stage embryos does not affect the number of cell divisions undergone by the original notochord and muscle precursors, respectively, indicating that the stage of initiation of *Bra* and *Tbx6* expression does not appear to determine when cell division ceases. Precocious *Bra* and *Tbx6* expression from the 1-cell stage does not put the developmental clock or timer forward, but makes cells exit from the cell cycle at an appropriate time point, probably defined by the *Bra*- and *Tbx6*-independent clock or timekeeper mechanism, about which almost nothing is known. It would also be interesting to examine whether extra divisions occur in embryos showing delayed expression of *Bra/Tbx6*.

Numbers of cell division rounds differ between two muscle lineages

Although not mentioned particularly in the above sections, in fact, the number of cell division rounds differs between muscle lineages. This also offers an interesting opportunity for analysis of cell division number control. The cell fates of the B7.4 and B7.8 blastomeres of the 64-cell embryo are restricted to muscle, and thereafter divide three times and twice, respectively, to become postmitotic muscle cells, even in isolation (Nishida 1987, 1992; Fig. 2A and 3A–C). The B7.4 muscle cell is a sister cell of the B7.3 mesenchyme cell, and the B7.8 muscle cell is a sister cell of the B7.7 mesenchyme cell (Fig. 2A). These mesenchyme cells also differ from each other in the number of cell divisions they undergo: B7.3 divides about 7.5 times and B7.8 about 6.8 times after the 64-cell stage in intact embryos, whereas they divide 6.4 and 6.2 times, respectively, in partial embryos isolated at the 64-cell stage (Fig. 2A; Kuwajima *et al.* 2014).

When these two mesenchyme lineage cells are converted to give rise to muscle cells by suppression of FGF signaling and mis-expression of *Tbx6*, they divide a similar number of times to their respective counterpart

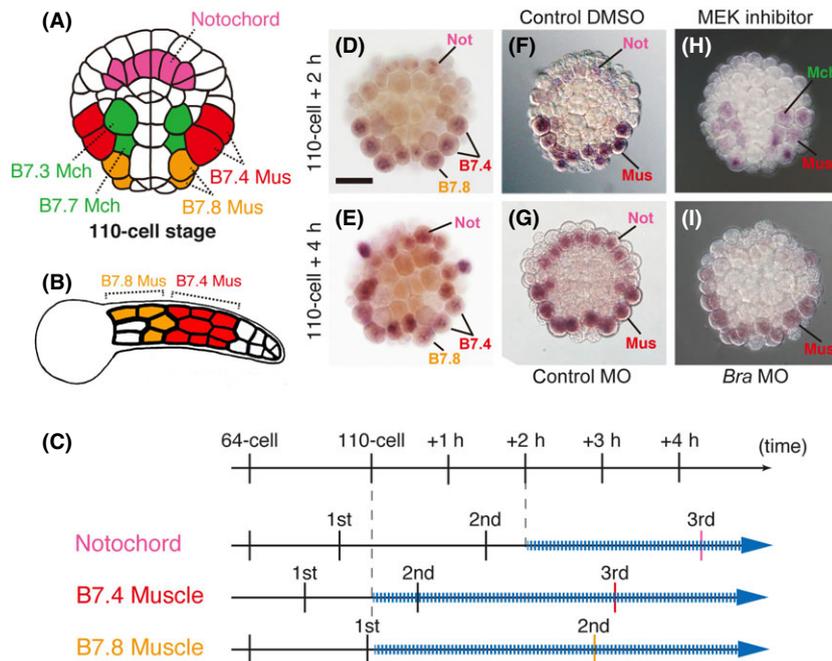


Fig. 3. Spatial and temporal expression of *CKI-b*. (A) Schematic representation of 110-cell stage embryos, showing the position of each tissue precursor cell in the vegetal hemisphere. Each tissue precursor is color-coded. The B7.4 and B7.8 muscle precursor cells of the 64-cell embryo have divided once and are colored in red and orange, respectively. Anterior is up. (B) Descendants of muscle precursor cells at the middle tailbud stage. The B7.4 blastomere and the B7.8 blastomere generate eight (in red, see also Fig. 2D) and four (in orange) muscle cells, respectively. (C) Time table of *CKI-b* expression and cell division progression after the 64-cell stage. Cell divisions after the 64-cell stage in each lineage are shown, and blue arrows indicate *CKI-b* gene expression. While the expression of *CKI-b* is first detected in the muscle lineages, B7.4 and B7.8, at the 110-cell stage, which in the notochord lineage is delayed by 2 h. The 64-cell stage is attained at 8 h of development, and the larva hatches at 35 h. Modified from Kuwajima *et al.* (2014). (D–I) *In situ* hybridization of *Hr-CKI-b*. To precisely identify the *CKI-b*-expressing lineage cells, cell division has been arrested at the 110-cell stage using cytochalasin B. Vegetal view. (D, F, H) Two hours after the 110-cell stage. (E, G, I) Four hours after the 110-cell stage. (D, E) Untreated embryos. (F, H) Treatment with control DMSO (dimethylsulfoxide) and MEK inhibitor to suppress FGF signaling, respectively. (G, I) Injection of control MO and *Bra* MO, respectively. Mch, mesenchyme; Mus, muscle; Not, notochord. Scale bar, 100 μ m. Images are reproduced from Kuwajima *et al.* (2014).

sister muscle cells. The manipulated B7.3 divides three times like the sister B7.4 muscle cell, and B7.7 divides twice like the sister B7.8 muscle cell (Kuwajima *et al.* 2014; Fig. 2A). This suggests the presence of a lineage-specific control of cell divisions, even in muscle precursor cells, and indicates that the muscle precursors probably do not count cell division rounds but instead measure elapsed time before they stop dividing. This idea has been supported by observations of cell division timing in these two muscle lineages (Fig. 3C). The cell cycles of B7.8 are longer than those of the B7.4 lineage, and the final divisions of B7.4 and B7.8 occur at almost the same time. The difference in the number of cell divisions (three times and twice) seems to be achieved by differences in cell cycle length, rather than the timing of terminal cell division. It appears that muscle precursors measure time that has elapsed after the fate determination (64-cell) stage, so that they finish cell division at the appropriate time. This idea fits the analog timer model.

Role of the Cdk inhibitor, *CKI*, in termination of cell divisions in notochord and muscle cells

It seems that a certain common process (analog timer) terminates cell division in both the B7.4 and B7.8 muscle lineages. What finally terminates cell division in muscle as well as in notochord cells? Cdk inhibitors negatively regulate cell cycle progression by inhibiting the initiation of S-phase (Sherr & Roberts 1999; Besson *et al.* 2008; Lim & Kaldis 2013), and the kinase activity of Cdk/cyclin complexes is tightly regulated by binding to Cdk inhibitors. Similarly to *Drosophila* and *C. elegans* (Lane *et al.* 1996; de Nooij *et al.* 1996; Clayton *et al.* 2008), the ascidian Cdk inhibitor, *CKI-b*, is involved in the termination of cell divisions in muscle and notochord cells in ascidian embryos (Kuwajima *et al.* 2014). *CKI-b* gene expression is initiated at the 110-cell stage in muscle precursors of the B7.4 and B7.8 lineages at the same time (Fig. 3C) and approximately 2 h later in

notochord (Fig. 3C, D). The expression continues up to the neurula stage. It should be noted that cells still divide once or twice even after the initiation of *CKI-b* gene expression. Although the amount of CKI-b protein has not been reported, it has been proposed that a certain time is required to accumulate a sufficient amount of CKI protein for termination of cell division, as *CKI-b* mRNA-injected fertilized eggs stop dividing at the 8- or 16-cell stage, approximately 2 h after the injection.

In embryos in which FGF signaling has been inhibited, *CKI-b* expression is lost in the notochord lineage, and is ectopically activated in mesenchyme cells (Fig. 3F, H). In contrast, knockdown of *Bra* results in loss of *CKI-b* expression specifically in notochord precursors (Fig. 3G, I). Thus, *CKI-b* gene expression is initiated by FGF signaling, which is followed by expression of the key transcription factor *Bra* in notochord. Unfortunately, it is not known whether *Tbx6* also promotes *CKI-b* expression because of the lack of an effective *Tbx6* MO, but it seems likely. One interesting aspect is that the *CKI-b* gene is simultaneously activated in two distinct muscle lineages, B7.4 and B7.8 (Fig. 3C), implying that the accumulation of CKI-b mRNA and protein serves as the analog timer commonly running to terminate cell division in the two muscle lineages at a similar developmental stage (110-cell stage plus 3 h). The delayed expression of *CKI-b* in notochord cells well coincides with the delay in final cell division relative to muscle.

The available data suggest that cell-division control involves the determination of cell fates by inductive events. Expression of the tissue-specific key transcription factors, *Bra* and *Tbx6*, which are regulated by the presence and absence of FGF inductive signaling, respectively, is also involved in regulation of the specific number of cell division rounds in notochord and muscle. *CKI-b* is likely a direct or indirect downstream effector molecule of tissue-specific key transcription factors, leading to cell division arrest. Accumulation of CKI-b mRNA and protein would act as a kind of analog timer measuring elapsed time before cell division is actually terminated.

Two phases of the timing mechanism in control of the number of cell divisions

The timing mechanism of final cell division in notochord and muscle seems to consist of at least two functional phases: (i) A timer, which involves accumulation of a sufficient amount of Cdk inhibitor protein after cell fate specification in notochord and muscle. If *Bra* and *Tbx6* do not directly activate the Cdk inhibitor gene, the gene activation cascade from these transcription factors down to Cdk inhibitor gene would also be included in

this timer mechanism. It seems to be a globally applicable concept that expression of tissue-specific transcription factors after cell fate specification elicits specific timer mechanisms for controlling the number of cell divisions. (ii) A timekeeper, which activates the “start button” of the above timer at the cell fate specification stage. The clock or timer of the timekeeper progresses independently of *Bra/Tbx6* gene expression, as precocious initiation of *Bra* and *Tbx6* expression does not influence the number of total cell division rounds in notochord and muscle cells. Thus, the timekeeper mechanism determines the time point at which these transcription factors become ready to act. It has been shown that the *Bra* and *Tbx6* proteins are also precociously translated from mRNA injected into the egg and then transported into the nucleus before the initiation of endogenous gene expression (Fujikawa *et al.* 2011; Kuwajima *et al.* 2014). It seems unlikely that there is specific temporal regulation of translation or nuclear transportation. Nothing is known about the nature of the timekeeper mechanisms, or about whether it works in digital or analog mode.

A similar situation operates for MBT control in zebrafish. Maternal factors already present in the egg, *Nanog*, *Pou5f1*, and *SoxB1*, activate global zygotic gene expression at the MBT (Lee *et al.* 2013), but it is unclear why these maternal factors do not operate until the initiation of the MBT. The N/C ratio could be the timekeeper mechanism in this case. In addition, it has been proposed that chromatin modification might be a key process in determining the timing of MBT in zebrafish and *Xenopus* (Heasman 2006; Schier 2007; Andersen *et al.* 2013). Chromatin remodeling might act as a timekeeper mechanism in starting the timer for cessation of cell division.

It would be of great interest to clarify the true nature of the timekeeper. The timekeeper mechanism proposed here would also temporally regulate the processes of cell differentiation, as the *Bra* and *Tbx6* proteins are pivotal for notochord and muscle differentiation. Timing of the expression of tissue differentiation markers remains to be analyzed.

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