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Evolution of Developmental Control Mechanisms

Beta-catenin patterns the cell cycle during maternal-to-zygotic transition in urochordate embryos

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ABSTRACT

During the transition from maternal to zygotic control of development, cell cycle length varies in different lineages, and this is important for their fates and functions. The maternal to zygotic transition (MZT) in metazoan embryos involves a profound remodeling of the cell cycle: S phase length increases then G2 is introduced. Although β-catenin is the master regulator of endomesoderm patterning at MZT in all metazoans, the influence of maternal β-catenin on the cell cycle at MZT remains poorly understood. By studying urochordate embryogenesis we found that cell cycle remodeling during MZT begins with the formation of 3 mitotic domains at the 16-cell stage arising from differential S phase lengthening, when endomesoderm is specified. Then, at the 64-cell stage, a G2 phase is introduced in the endoderm lineage during its specification. Strikingly, these two phases of cell cycle remodeling are patterned by β-catenin-dependent transcription. Functional analysis revealed that, at the 16-cell stage, β-catenin speeds up S phase in the endomesoderm. In contrast, two cell cycles later at gastrulation, nuclear β-catenin induces endoderm fate and delays cell division. Such interphase lengthening in invaginating cells is known to be a requisite for gastrulation movements. Therefore, in basal chordates β-catenin has a dual role to specify germ layers and remodel the cell cycle.

Introduction

During development the speed of the cell cycle changes. Such cell cycle remodeling is important for many aspects of embryonic development including the morphogenetic cell movements of gastrulation, which can be prevented if cell division is not halted (Duncan and Su, 2004; Kurth, 2005; Murakami et al., 2004). Ascidian embryos develop with a fixed number of cells and display a stereotyped pattern of cell division (Hotta et al., 2007). During early embryonic development in non-vertebrate chordates such as the ascidians, the cell cycle is remodeled early, in a predictable manner, and in specific blastomeres, making ascidians an excellent organism to study how cell cycle length is controlled during embryonic development at the single cell level.

Different mechanisms are employed to remodel the cell cycle during early embryonic development. Early embryos display successive phases of DNA duplication (S phase) and mitosis (M phase) without the intervening gap phases (G1 and G2) common to somatic cells. S phase is generally the first phase of the cell cycle subject to cell cycle remodeling during embryonic development. In large frog and fly embryos depletion of maternal factors induces cell cycle lengthening. For example, in *Xenopus* the nucleocytoplasmic (N/C) ratio increases with each round of cell division causing the titration of an as yet unidentified factor leading to a lengthening of S phase at the midblastula transition or MBT (Newport and Kirschner, 1982a; Vastag et al., 2011). In *Drosophila* embryos it is thought that cell cycle lengthening during cycles 10–13 (prior to the MBT at cycle 14) is provoked by depletion of cyclins A and B which causes an initial slowing of DNA replication that in turn activates chk1/grapes to further slowdown S phase and prophase (Royou et al., 2008; Sibon et al., 1997; Yu et al., 2000). Then at cycle 14, S phase lengthening is caused by loss of the maternal Cdc25 phosphatase Twine (Farrell et al., 2012). Such loss of maternal Cdc25 is due to a dramatic change in Cdc25 stability induced by an increased N/C ratio triggering zygotic transcription (Di Talia et al., 2013; Farrell and O'Farrell, 2013). In *Caenorhabditis elegans* the cell cycle is also remodeled but much earlier at the two cell stage. This reflects the fact that zygotic transcription starts at the 4-cell stage and gastrulation begins at the 20-cell stage in *C. elegans* (Baugh et al., 2003). Interestingly, the mechanism employed in *C. elegans* may be independent of cell volume (Schierenberg and Wood, 1985) and is brought about by a dual mechanism involving Plk1 and the DNA checkpoint system depending on ATL-1/CHK-1. For example, during cell division in *C. elegans* zygotes when anterior AB and posterior P1 cells are formed, more PLK-1 protein is retained in AB while the activation

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of ATL-1/CHK-1 occurs in P1 (reviewed in Budirahardja and Gönczy, 2009). PLK-1 protein is actively retained in AB cells (Budirahardja and Gönczy, 2008; Rivers et al., 2008) causing an increased amount of CDC-25.1 to accumulate in the nucleus thereby driving AB into mitosis ahead of its sister P1 (Rivers et al., 2008), while ATL-1/CHK-1 delays mitotic entry in P1 (Brauchle et al., 2003).

A maternal gradient of β-catenin is one of the most ubiquitous factors responsible for germ layer specification from cnidarians to protostomes and non-vertebrate deuterostomes (reviewed in Petersen and Reddien (2009). β-catenin is also the endoderm determinant in embryos of cnidarians, protostomes and some invertebrate deuterostomes (urochordates) and is thereby directly responsible for the onset of gastrulation (Petersen and Reddien, 2009; Woodland and Zorn, 2008). Despite such a conserved role for β-catenin during MZT and gastrulation at moments of major cell cycle remodeling, the possibility that β-catenin controls cell cycle duration before gastrulation has never been explored.

Ascidian embryos develop with a small fixed cell number and display cell cycle asynchrony from the 16-cell stage. For example, at the 16-cell stage, 8 vegetal blastomeres divide before the 8 animal blastomeres creating a brief 24-cell stage embryo. Remarkably, this pattern of cell division has been conserved in very distantly-related ascidian species from different orders (e.g. *Halocynthia roretzi* and *Phallusia mammillata*). However, it is not known what causes the difference in cell cycle timing between animal and vegetal cells at the 16-cell stage. The earliest known zygotic transcription starts at the 8-cell stage (*Halocynthia*: Miya and Nishida, 2003; *Ciona*: Rothbächer et al., 2007), and analysis of transcription factors and signaling molecules in *C. intestinalis* indicates that gene expression increases gradually during embryogenesis from the 16-cell stage onwards (Imai et al., 2004). Furthermore at this stage β-catenin accumulates in the nuclei of the vegetal cells to specify the endomesoderm (reviewed in Kumano and Nishida, 2007). β-catenin has been shown to promote cell proliferation in somatic cells (Niehrs and Acebron, 2012) but not during endomesoderm patterning in early embryos. Although the majority of zygotic genes are expressed at the MBT in *Xenopus*, it was recently found that β-catenin patterns the dorsal mesoderm by priming early expression of nodal-related genes at the 32-cell stage, i.e. 5 cell cycles before MBT (Blythe et al., 2010; Skirkanich et al., 2011; Yang et al., 2002). It is interesting to note that, during cell cycle 5 to 10, dorsal cells divide first followed by a wave of cell division over the embryo (Boterenbrood et al., 1983; Satoh, 1977). These dorsal cells are the blastomeres which accumulate nuclear β-catenin, but it is still unknown whether β-catenin modulates cell cycle length in these blastomeres prior to the MBT in *Xenopus* embryos.

Here we have investigated the relationship between β-catenin and cell cycle duration in ascidian embryos and reveal for the first time that β-catenin causes the presumptive endomesoderm cells to divide ahead of the ectodermal cells leading to the appearance of cell cycle asynchrony. We show that β-catenin is both necessary and sufficient for the asynchrony in cell cycle duration at the 16-cell stage. Knockdown of β-catenin protein or blocking β-catenin transactivation by Tcf both cause cell cycle slowing of the vegetal cells indicating that β-catenin is necessary to maintain a rapid cell cycle. Conversely, β-catenin over expression or inhibition of GSK3b both cause the 8 animal blastomeres to shorten interphase and divide at the same time as the vegetal cells, indicating that β-catenin is sufficient to speed up the cell cycle at the 16-cell stage. In contrast β-catenin was found to slow down the cell cycle in endoderm cells just before gastrulation at the 112 cell stage. Therefore β-catenin has a dual impact on cell cycle duration depending on the germ layer and time of development.

Materials and methods

Biological material

Eggs from the ascidians *P. mammillata* were harvested from animals obtained in Sète and kept in the laboratory in a tank of natural sea water at 16 °C. Egg preparation and microinjection have been described previously (see detailed protocols in Sardet et al., 2011). All imaging experiments were performed at 19 °C.

Molecular tools

H2B::mRfp and MAP7::GFP were used previously to monitor DNA and mitotic spindles in *Phallusia* embryos (Dumollard et al., 2011; Prodon et al., 2010; McDougall et al., 2012). Ci-shugoshin (KH.C12.362) was amplified from a Gateway-compatible cDNA library. Ci-β-catenin (KH.C9.53) and DN-Tcf (Hudson et al., 2013) were kindly provided by Yasuo Hitoyoshi (UMR7009). The 27 × TCF::H2B-Ch reporter codes for H2B::Cherry driven by a promoter hosting 27 Tcf/Lef binding sites (CCTTTGAT) multimerized upstream of the basal promoter fog. The 27 × TCF::H2B-Ch reporter was kindly provided by Dr Eileen Wagner (University California Berkeley, USA). The ability of DN-Tcf to inhibit β-catenin/Tcf-dependant transcription was assessed in live *Phallusia* embryos by monitoring 27 × TCF::H2B-Ch red fluorescence in embryos expressing DN-Tcf (Fig. 5B). Pm-Pem-1 (Genbank Id: GQ418163) that was cloned and characterized previously (Paix et al., 2011). NT-β-Catenin was generated by targeted mutations of R459 and H460 into ala. These 2 mutations are homologous to the mutations R469A and H470A in human β-catenin which were found to impair binding to Tcf1 (Von Kries et al., 2000).

All constructs were made using pSPE3 and the Gateway cloning system (Invitrogen, Roure et al., 2007) unless otherwise stated (see Sardet et al., 2011 for a detailed protocol).

Morpholino oligonucleotides injections

Sequences of *P. mammillata* genes were taken from EST collections and *Phallusia* genome project led by Hitoyoshi Yasuo and Genoscope.

Pm-β-catenin (CTGGTTCATCATCATTTCTGCCATG) morpholino oligonucleotides (MO) (GeneTools) was prepared at a concentration of 3 mM in dH_2O and stored at −80 °C. MOs were then injected at a pipette concentration of 1 mM (~0.2% egg volume). Importantly, in order to obtain the phenotypes published in this study for β-catenin MO, MO injection was realized not more than 1 h before fertilization. If MO-injected eggs remained unfertilized for longer time, stronger phenotypes were observed and problems of cleavage were observed as early as the one cell stage. MO was injected alone or co-injected with synthetic mRNAs before fertilization or in one blastomere at the 2-cell stage.

Time-lapse and fluorescence microscopy

Time-lapse imaging of Venus, GFP, mRfp1 and Cherry constructs was performed on an Olympus IX70 inverted microscope set up for epifluorescence imaging. Sequential brightfield and fluorescence images were captured using a cooled CCD camera (Micromax, Sony Interline chip, Princeton Instruments, Trenton NJ) and data collected was analysed using MetaMorph software (Molecular Devices, Sunnyvale CA). 4D imaging was performed with 6 to 10 z-planes acquired every minutes (or every 2 min) in 3 colours (BF, GFP/Venus and mRfp1/Cherry channel). Time series were reconstructed and analysed by MetaMorph software (Molecular Devices) and Image J (NIH, USA). Timing of the cell cycle was measured by scoring the time from NEB to NEB (visualised either