Antagonism between β-catenin and Gata.a sequentially segregates the germ layers of ascidian embryos

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RESEARCH REPORT

Antagonism between β-catenin and Gata.a sequentially segregates the germ layers of ascidian embryos

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ABSTRACT

Many animal embryos use nuclear β-catenin (nβ-catenin) during the segregation of endomesoderm (or endoderm) from ectoderm. This mechanism is thus likely to be evolutionarily ancient. In the ascidian embryo, nβ-catenin reiteratively drives binary fate decisions between ectoderm and endomesoderm at the 16-cell stage, and then between endoderm and margin (mesoderm and caudal neural) at the 32-cell stage. At the 16-cell stage, nβ-catenin activates endomesoderm genes in the vegetal hemisphere. At the same time, nβ-catenin suppresses the DNA-binding activity of a maternal transcription factor, Gata.a, through a physical interaction, and Gata.a thereby activates its target genes only in the ectodermal lineage. In the present study, we found that this antagonism between nβ-catenin and Gata.a also operates during the binary fate switch at the 32-cell stage. Namely, in marginal cells where nβ-catenin is absent, Gata.a directly activates its target, *Zic-r.b* (*ZicL*), to specify the marginal cell lineages. Thus, the antagonistic action between nβ-catenin and Gata.a is involved in two consecutive stages of germ layer segregation in ascidian embryos.

KEY WORDS: β-Catenin, *Ciona intestinalis*, Gata, Zic, Ascidian, Germ layer formation

INTRODUCTION

In early embryos of many invertebrate animals, including echinoderms, hemichordates, ascidians, nematodes and cnidarians, nuclear localized β-catenin (nβ-catenin) is used for the specification of endomesoderm (or endoderm) (Darras et al., 2011; Logan et al., 1999; Maduro, 2009; McCauley et al., 2015; Momose and Houliston, 2007; Wikramanayake et al., 2003, 1998). This process is thus likely to be an evolutionarily ancient mechanism.

In embryos of the chordate *Ciona intestinalis*, nβ-catenin is not only involved in the segregation of endomesoderm from ectoderm but also in the subsequent segregation of endoderm from mesoderm (and some neural) lineages (Hudson et al., 2013; Imai et al., 2000). Thus, germ layer segregation in *Ciona* embryos begins with two temporally separable steps.

In the first step, which occurs at the 16-cell stage, nβ-catenin activity promotes endomesoderm over ectoderm lineage-specific gene expression. In vegetal cells, from which endomesoderm is derived, *Foxd* and *Fgf9/16/20* are directly activated by nβ-catenin and its partner transcription factor Tcf7 (Imai et al., 2002a; Oda-Ishii et al., 2016). Conversely, *Efna.d* [formerly called *EphrinA-d*, and renamed according to the nomenclature guidelines for this animal (Stolfi et al., 2015)] and *Tfap2-r.b* are activated by the Gata.a transcription factor in animal cells, from which ectoderm is derived (Bertrand et al., 2003; Horikawa et al., 2013; Oda-Ishii et al., 2016; Rothbächer et al., 2007). Although Gata.a is present ubiquitously in the embryo, its activity is inhibited in vegetal cells by a physical interaction with β-catenin and Tcf7 (Oda-Ishii et al., 2016). This interaction thus restricts Gata.a activity to the animal hemisphere. Therefore, in vegetal cells, β-catenin/Tcf7 directly promotes the transcriptional activation of endomesoderm genes, and indirectly inhibits the expression of ectoderm genes. In the animal hemisphere, Gata.a, free from β-catenin/Tcf7-mediated repression, initiates the ectoderm-specific genetic program.

The second nβ-catenin binary fate decision takes place at the 32-cell stage, following a cell division that segregates the endoderm lineages (E cells) from the marginal (mesoderm and some neural) lineages (the NN and MM cells) (Fig. 1A). During this step, nβ-catenin promotes endoderm over marginal lineage specification, such that *Zic-r.b* (formerly *ZicL*) is specifically activated in margin cells, and *Lhx3/4* (formerly *Lhx3*) is activated in E cells (Hudson et al., 2013; Imai et al., 2002b; Satou et al., 2001). *Zic-r.b* is a key specifier for the marginal lineages, required for the anterior marginal cells (NN cells, A6.2 and A6.4) to give rise to notochord and caudal neural tissue, and for each of the posterior marginal cells [anterior MM (aMM) cells, B6.2; posterior MM (pMM) cells, B6.4] to give rise to both muscle and mesenchyme (Imai et al., 2006, 2002b; Satou and Imai, 2015; Yagi et al., 2004). It appears that *Zic-r.b* is regulated differently between aMM and pMM; when nβ-catenin is ectopically activated, *Zic-r.b* expression is lost in NN and aMM cells but not in pMM (Hudson et al., 2013). In the present study, we address how *Zic-r.b* is activated differentially between NN/aMM cells and E cells, and examine the possibility that the physical association of Gata.a and β-catenin/Tcf7 might also be involved during the segregation of cell lineages at the 32-cell stage.

RESULTS AND DISCUSSION

Gata.a is required for *Zic-r.b* expression

In order to test whether Gata.a is required for *Zic-r.b* expression in marginal cells, we knocked down *Gata.a* using morpholino oligonucleotides (MOs). As shown in Fig. 1B, *Zic-r.b* was expressed normally in NN and MM cells in embryos injected with a control MO against *Escherichia coli lacZ*. By contrast, in embryos injected with *Gata.a*-MO, *Zic-r.b* expression was reduced in NN and aMM cells, but not in pMM cells (Fig. 1C,D; Fig. S1). Thus, Gata.a is required for *Zic-r.b* expression in NN and aMM cells, but is dispensable for expression in pMM cells.

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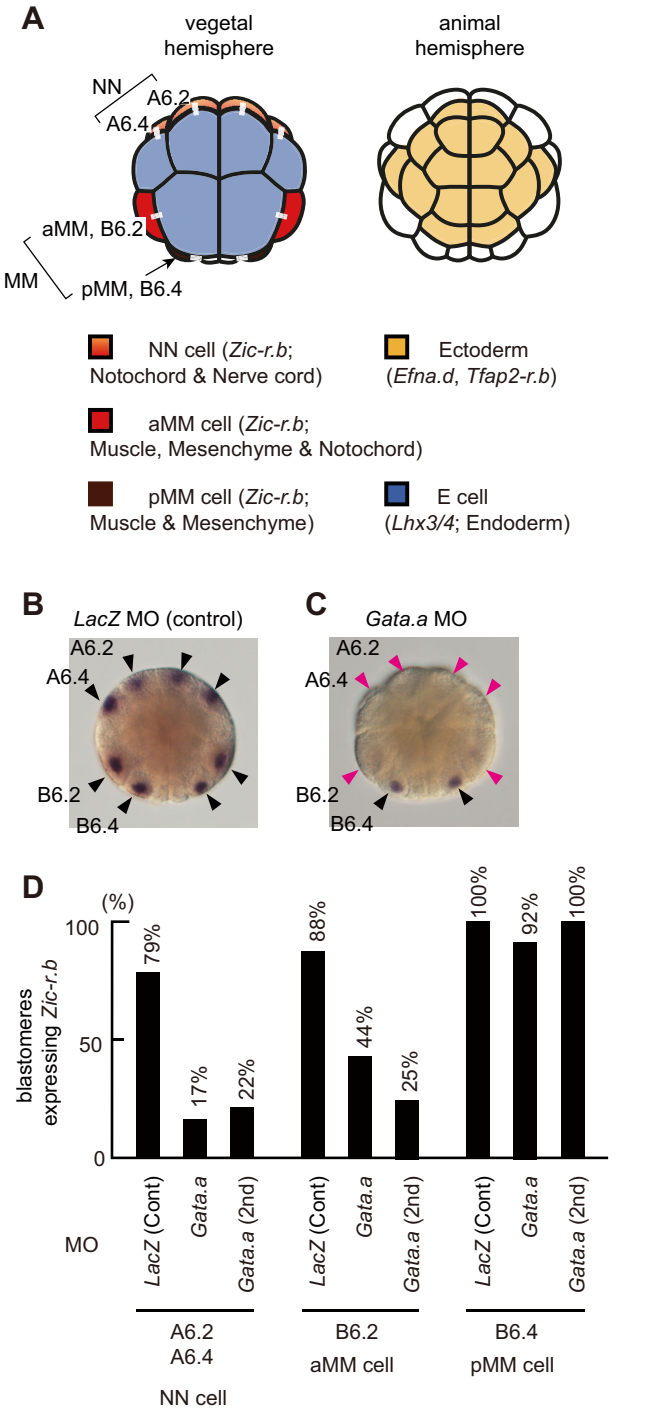

Fig. 1. *Zic-r.b* expression in the marginal cells is regulated by Gata.a. (A) An illustration of the 32-cell embryo. Because the embryo is bilaterally symmetrical, blastomere names are shown only in the left half. At this stage, the developmental fate of the animal hemisphere cells (yellow) is restricted to ectoderm, and the developmental fate of the vegetal-most cells (blue) is restricted to mostly endoderm. The marginal cells, NN and MM cells, with distinct lineage origins express *Zic-r.b* and give rise to mesodermal tissues and posterior neural tube. White bars connecting two cells indicate their sister cell relationship. (B-D) *Zic-r.b* expression in embryos injected with a *lacZ* control MO (B) and a *Gata.a* MO (C). Unfertilized eggs were injected with either of the MOs (1 mM), and inseminated 1 h after injection. Black arrowheads indicate *Zic-r.b* expression and magenta arrowheads indicate loss of *Zic-r.b* expression. We analyzed 40 *lacZ* morphants and 31 *Gata.a* morphants. We also analyzed 16 embryos injected with a second MO against *Gata.a*. The percentages of blastomeres that expressed *Zic-r.b* are shown in D.

Gata.a directly activates *Zic-r.b* expression

Zic-r.b is a multi-copy gene (Dehal et al., 2002; Yamada et al., 2003). By comparing the upstream regulatory regions of four copies, we found that the region approximately 230 bp upstream from the transcription start sites was highly conserved (Fig. S2A). We identified four putative Gata-binding sites in this highly conserved domain. Chromatin-immunoprecipitation (ChIP) mapping of Gata.a binding, generated in our previous study (Oda-Ishii et al., 2016), revealed a clear peak of Gata.a binding to the conserved upstream regions of each copy of *Zic-r.b* (Fig. 2A; Fig. S2B). A *lacZ* reporter construct containing this conserved upstream region was indeed activated in NN, aMM and pMM cells (Fig. 2B, C) (Anno et al., 2006).

In order to address whether the Gata sites were required for the expression driven by this conserved region, we introduced mutations in the Gata sites. We focused on the two proximal Gata sites for two reasons. First, the distal Gata sites are present in a region of the *Zic-r.b* upstream regulatory sequences specifically required for expression in MM cells (Anno et al., 2006). Second, the two proximal Gata sites were conserved in the upstream sequence of *Zic-r.b* of the closely related *Ciona savignyi*. We introduced three different mutations into both of the two proximal Gata sites. In each case, reporter gene expression driven by these mutant constructs was greatly reduced in both NN and MM lineages (Fig. 2B-E; Fig. S3). The second mutant construct (μ2; Fig. 2B,E) reveals that not only the core sequence, 'GATA', but also its flanking nucleotides are important, consistent with previous studies (Farley et al., 2015; Horikawa et al., 2013). Furthermore, gel-shift assays showed that the upstream region of *Zic-r.b* containing the proximal Gata sites (Fig. 2A; Fig. S2A) bound Gata.a protein *in vitro*, and that this binding was dependent upon intact Gata binding sites (Fig. 2F). Taken together, our results suggest that Gata.a directly activates *Zic-r.b* expression.

Curiously, although the *Gata.a*-MO analysis suggested that Gata.a activity is required for *Zic-r.b* expression in NN and aMM, but not pMM cells, our mutational analysis of the upstream regulatory sequences of *Zic-r.b* suggests that the proximal Gata sites are required for activation in all the marginal cells (NN, aMM and pMM) (Fig. 2B-E). This suggests that Gata.a contributes to *Zic-r.b* expression in pMM cells, but that transcription factors other than Gata.a are sufficient to control endogenous *Zic-r.b* expression in these cells. Regulatory elements that control this putative Gata.a-independent expression did not appear to be present even in a reporter construct containing longer upstream sequences (−682; Fig. S3).

β-catenin/Tcf7 reduces the DNA-binding activity of Gata.a

We next tested the possibility that β-catenin/Tcf7 prevents Gata.a from binding to the upstream regulatory sequences of *Zic-r.b*, as it does in the upstream regions of two ectoderm genes, *Efna.d* and *Tfap2-r.b* (Oda-Ishii et al., 2016). Consistent with this idea, gel-shift assays showed that Gata.a binding to the upstream sequences of *Zic-r.b* was greatly reduced when Gata.a protein was co-incubated with β-catenin and Tcf7 (Fig. 3A,B). Thus, the *in vitro* binding activity of Gata.a protein to the Gata sites of the *Zic-r.b* upstream region was negatively regulated by the presence of β-catenin and Tcf7.

We have previously shown that treatment of *Ciona* embryos with a pharmacological inhibitor of Gsk3 (BIO, GSK-3 inhibitor IX) stabilizes nβ-catenin and results in loss of *Zic-r.b* expression in NN and aMM cells (Hudson et al., 2013) (Fig. 3C,D). Based on the results presented so far, we predicted that in embryos treated with BIO, Gata.a would exhibit reduced binding to the upstream regulatory sequences of *Zic-r.b*. To test this, we performed a ChIP experiment with anti-Gata.a antibodies followed by quantitative

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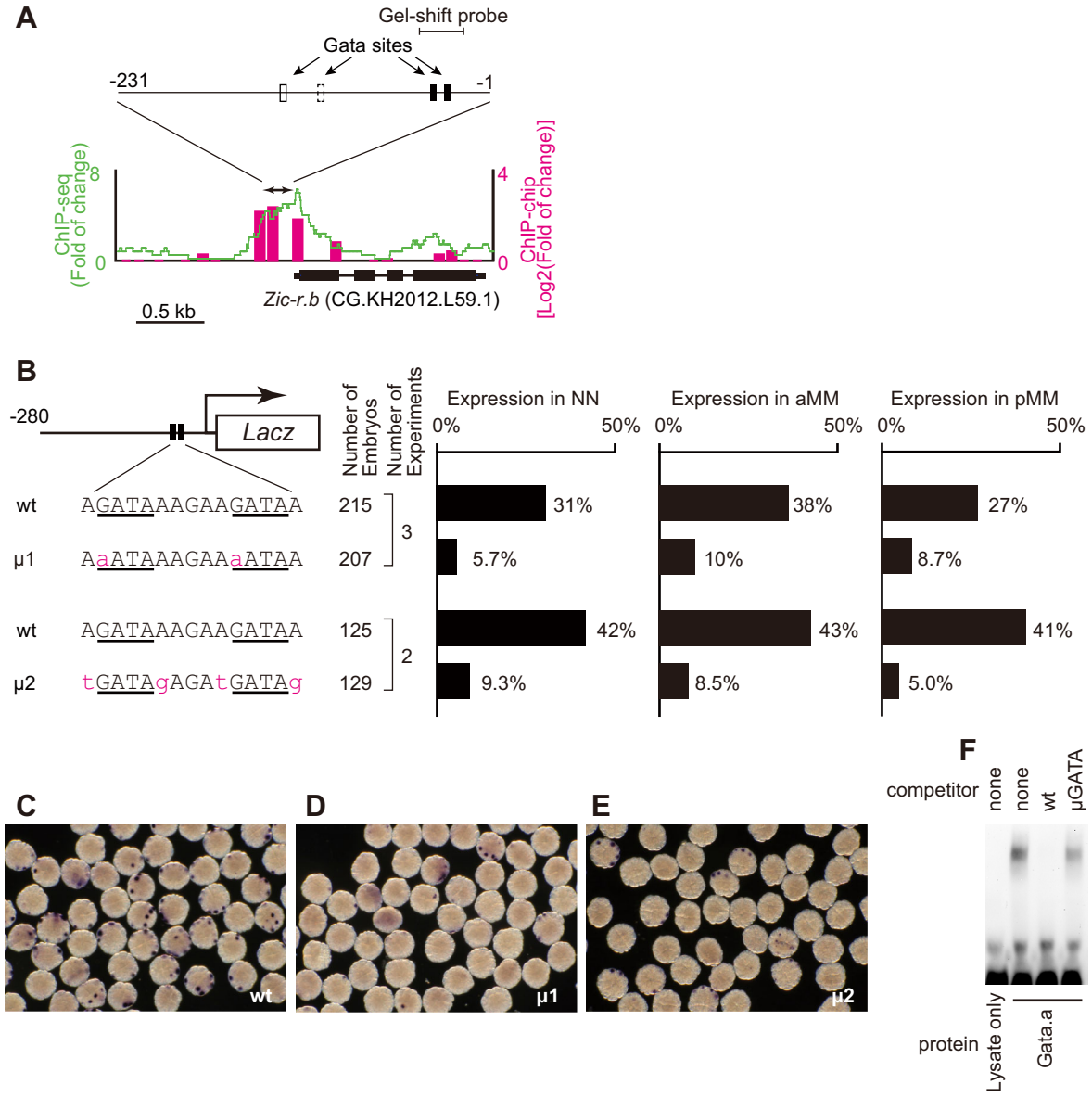

Fig. 2. Gata.a directly regulates *Zic-r.b* expression. (A) Mapping of Gata.a ChIP data, which were published previously (Oda-Ishii et al., 2016), onto a genomic region consisting of the exons and upstream region of *Zic-r.b*. The ChIP-chip data are shown in bars and the ChIP-seq data are shown as a green line. Each graph shows the fold enrichment (*y*-axis) for the chromosomal region (*x*-axis). A double-headed arrow indicates the upstream region that is highly conserved among four copies of *Zic-r.b* (Fig. S2A). This region contains four conserved Gata sites. The proximal Gata sites are shown by black boxes, and the distal Gata sites are shown by white boxes. The second site is not conserved in this copy, and is indicated with a dashed line. Mapping data onto genomic regions containing the other three copies are shown in Fig. S2B. (B-E) Analysis of a regulatory region of *Zic-r.b*. (B) The *lacZ* reporter constructs are depicted on the left with the two proximal Gata sites indicated by the black boxes. The sequences of intact (wt) and mutated (μ1, μ2) Gata sites are shown below. The numbers indicate the relative nucleotide positions from the transcription start site of *Zic-r.b*, which was determined previously (Satou et al., 2006). The graph shows the percentage of NN, aMM and pMM cells that expressed *lacZ* mRNA at the 32-cell stage. Note that although there are four MM and four NN cells in a 32-cell stage embryo, not all cells or embryos will express the reporter because of mosaic incorporation of the electroporated constructs. (C-E) Photographs showing expression of *lacZ* mRNA (detected by *in situ* hybridization) in embryos electroporated with wt (C), μ1 (D) and μ2 (E) constructs. (F) Gel-shift analysis showing that the proximal Gata sites in the upstream region of *Zic-r.b* bound Gata.a protein *in vitro*. The shifted band disappeared by incubation with a specific competitor (wt), but not the competitor with mutant Gata.a-binding sites (μGATA). The mutated sequence is the same as the one shown in Fig. S3.

PCR (Fig. 3E). In all three independent experiments, Gata.a binding to the upstream region of *Zic-r.b* was reduced in embryos cultured in BIO. We conclude that β-catenin/Tcf activity reduces *in vivo* binding of Gata.a to the target sites in the *Zic-r.b* upstream region.

Conclusions

Our results show that antagonism between Gata.a and β-catenin/Tcf7 are central to both rounds of β-catenin-dependent switches that segregate the germ layers during early ascidian embryogenesis (Fig. 4). This antagonistic relationship first segregates the ectodermal lineages (Gata.a dependent) from the endomesodermal lineages (nβ-catenin dependent) at the 16-cell stage, and then the mesodermal/neural lineages (Gata.a dependent) from the endodermal lineages (nβ-catenin dependent) at the 32-cell stage. Thus, the two nβ-catenin/Gata.a switches at the 16- and 32-cell stages subdivide the *Ciona* embryo into three broad domains that correspond to the segregating germ layers. The second nβ-catenin/Gata.a switch cooperates with the lineage-specific factors inherited

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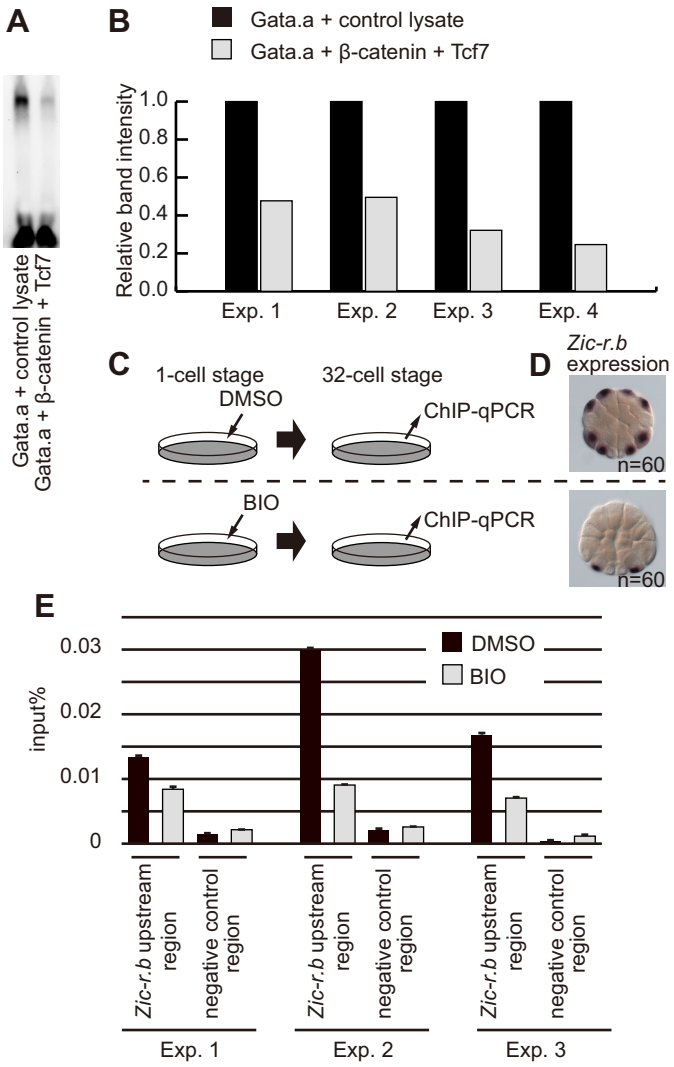

Fig. 3. Gata.a binding activity is reduced by interaction with Tcf7 and β-catenin. (A) Gel-shift analysis showing suppression of Gata.a binding activity by Tcf7 and β-catenin. Whereas Gata.a protein produced *in vitro* bound to the upstream sequences containing the Gata.a sites (left lane), co-incubation of Gata.a with β-catenin and Tcf7 reduced this binding (right lane). In the left lane, the same amount of a rabbit reticulocyte lysate was added for control instead of β-catenin and Tcf7. (B) Quantification of the shifted bands in four independent gel-shift experiments. (C-E) ChIP followed by quantitative PCR showing that BIO treatment of embryos reduces Gata-a binding to the *Zic-r.b* upstream region. (C) The experimental design. BIO treatment ectopically stabilizes β-catenin. (D) BIO treatment from the one-cell stage abolished *Zic-r.b* expression in NN and aMM cells, as does BIO treatment from the eight-cell or 16-cell stage (Hudson et al., 2013). (E) Three biological replicates indicate that BIO treatment reduced Gata.a binding to the *Zic-r.b* upstream region. Error bars indicate s.d. among qPCR technical replicates.

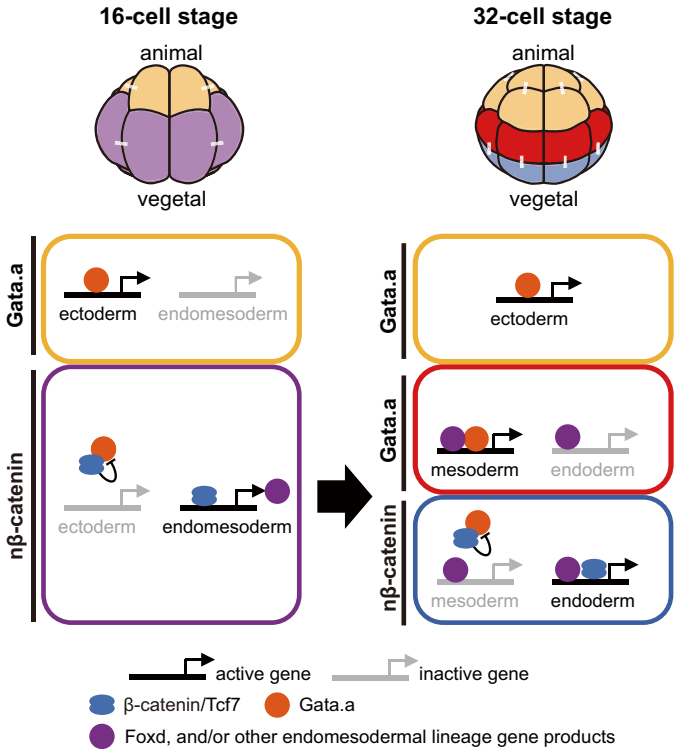

Fig. 4. Antagonistic action of Gata.a and β-catenin/Tcf7 during early germ layer segregation. The diagrams at the top represent an anterior (notochord side) view of 16- and 32-cell embryos. In 16-cell embryos, β-catenin is localized to nuclei of the vegetal hemisphere (purple) (Hudson et al., 2013; Imai et al., 2000) where it activates 'endomesoderm' target genes such as *Foxd* (Imai et al., 2002a), and suppresses Gata.a activity by physical interaction (Oda-Ishii et al., 2016). In the animal hemisphere (yellow), Gata.a is free from suppression by β-catenin/Tcf7, and activates its 'ectoderm' target genes, which include *Efna.d* and *Tfap2-r.b* (Oda-Ishii et al., 2016; Rothbächer et al., 2007). In 32-cell embryos, nβ-catenin is activated in E cells (blue) but not margin cells (red). In E cells, β-catenin/Tcf7, together with the endomesoderm gene products, activates 'endoderm' genes, which include *Lhx3/4* (Hudson et al., 2013; Imai et al., 2006), and suppresses Gata.a activity. In the margin, Gata.a is now free from β-catenin/Tcf7-mediated inhibition and, together with 'endomesoderm' gene products, it activates 'mesoderm' genes such as *Zic-r.b*.

from the endomesoderm precursors, during activation of the mesoderm- and endoderm-specific target genes (Fig. 4). *Foxd*, activated by the first nβ-catenin/Gata.a switch in the endomesoderm precursors of the 16-cell embryo (Imai et al., 2002a), is inherited by the both daughter cells of the 32-cell embryo (Hudson et al., 2013) where it co-operates with the second nβ-catenin/Gata.a switch to activate distinct gene expression. Consistent with this idea, previous studies have shown that *Foxd* is required for activation of *Zic-r.b* (Imai et al., 2002b), and can bind directly to its upstream sequences (Kubo et al., 2010). Indeed, there are four putative Fox binding sites in the upstream regions (Fig. S2A) (Anno et al., 2006), and mutations introduced into these Fox sites abolished reporter gene expression in NN and aMM cells (Fig. S4A-C), matching the required role of *Foxd* for *Zic-r.b* expression (Fig. S4D). Similarly, *Foxa.a* is activated in the endomesoderm precursors of the 16-cell embryo (Imai et al., 2004), and is required for correct *Zic-r.b* expression in NN and aMM cells (Fig. S4E) (Hudson et al., 2016).

The mechanism of nβ-catenin-mediated transcriptional repression described in the present and previous (Oda-Ishii et al., 2016) studies is different from other mechanisms described in *Caenorhabditis elegans* and *Drosophila melanogaster* (Bertrand, 2016; Blauwkamp et al., 2008; Murgan et al., 2015). Thus, nuclear β-catenin-dependent transcriptional repression appears to be mediated by diverse mechanisms.

MATERIALS AND METHODS

Animals

C. intestinalis adults were obtained from the National Bio-Resource Project for *Ciona* in Japan or purchased from the Station Biologique de Roscoff in France.

In situ hybridization

Whole-mount *in situ* hybridization was performed as described previously (Hudson et al., 2013; Imai et al., 2004). Identifiers for genes examined in the

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present study were as follows (Satou et al., 2008; Stolfi et al., 2015): CG.KH2012.C8.396/CG.KH2012.C8.890 for *Foxd*, CG.KH2012.L20.1 for *Gata.a*, CG.KH2012.C9.53 for *β-catenin*, CG.KH2012.C6.71 for *Tcf7*, and CG.KH2012.L59.12/CG.KH2012.L59.1/CG.KH2012.S816.1/CG.KH2012.S816.4 for *Zic-r.b*.

Knockdown studies

The MO (Gene Tools, LLC) against *Gata.a*, which blocks translation, has been used previously (5′-GGGTTAGGCATATACATTCTTTGGA-3′) (Bertrand et al., 2003; Oda-Ishii et al., 2016; Rothbächer et al., 2007). A second MO that differs by two nucleotides was also used (5′-GGTTAGGCATATACATTCTTTGGAA-3′) and gave similar results (Fig. 1D). We also used a MO against *E. coli lacZ* as a negative control (5′-TACGCTTCTTCTTTGGAGCAGTCAT-3′). These MOs were introduced by microinjection under a stereo-microscope. The conserved upstream region present in each of our reporter assay constructs is depicted in Fig. S2A. Reporter constructs were introduced into fertilized eggs by electroporation (Corbo et al., 1997). All knockdown experiments and reporter assays were performed at least twice independently.

ChIP experiments

Mapping of our previously published ChIP-chip (GEO accession number: GSE70902) and ChIP-seq (SRA accession number: DRA003742) data was performed as described previously (Oda-Ishii et al., 2016). Gel-shift assays were also performed as described previously (Oda-Ishii et al., 2016), using a digoxigenin-labeled probe and Gata.a protein synthesized with a rabbit reticulocyte lysate system (Promega). The sequence of the probe is shown in Fig. S2A. Chromatin-immunoprecipitation followed by quantitative PCR was performed as described previously (Oda-Ishii et al., 2016). The negative control primer set was the same as that used in a previous study (Oda-Ishii et al., 2016). The primer sequences to amplify the upstream sequence of *Zic-r.b* are shown in Fig. S2A. BIO (Merck Millipore) was used at 2.5 µM.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.S.I, C.H., I.O., H.Y. and Y.S. performed the experiments. C.H. and Y.S. wrote the paper. K.S.I., H.Y. and Y.S. conceived the project.

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Supplementary information

Supplementary information available online at
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