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Geminin cooperates with Polycomb to restrain multi-lineage commitment in the early embryo

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SUMMARY

Transient maintenance of a pluripotent embryonic cell population followed by the onset of multi-lineage commitment is a fundamental aspect of development. However, molecular regulation of this transition is not well characterized *in vivo*. Here, we demonstrate that the nuclear protein Geminin is required to restrain commitment and spatially restrict mesoderm, endoderm and non-neural ectoderm to their proper locations in the *Xenopus* embryo. We used microarray analyses to demonstrate that Geminin overexpression represses many genes associated with cell commitment and differentiation, while elevating expression levels of genes that maintain pluripotent early and immature neurectodermal cell states. We characterized the relationship of Geminin to cell signaling and found that Geminin broadly represses Activin-, FGF- and BMP-mediated cell commitment. Conversely, Geminin knockdown enhances commitment responses to growth factor signaling and causes ectopic mesodermal, endodermal and epidermal fate commitment in the embryo. We also characterized the functional relationship of Geminin with transcription factors that had similar activities and found that Geminin represses commitment independent of Oct4 ortholog (Oct25/60) activities, but depends upon intact Polycomb repressor function. Consistent with this, chromatin immunoprecipitation assays directed at mesodermal genes demonstrate that Geminin promotes Polycomb binding and Polycomb-mediated repressive histone modifications, while inhibiting modifications associated with gene activation. This work defines Geminin as an essential regulator of the embryonic transition from pluripotency through early multi-lineage commitment, and demonstrates that functional cooperativity between Geminin and Polycomb contributes to this process.

KEY WORDS: *Xenopus*, Commitment, Embryo, Mesoderm, Neural, Pluripotent

INTRODUCTION

Embryonic patterning involves a complex regulatory relationship between non-cell autonomous signaling and cell-intrinsic factors. Prior to gastrulation, vertebrate embryos contain many uncommitted cells that respond to instructive cues to acquire different fates. For example, ectoderm of *Xenopus laevis* embryos remains pluripotent through the late blastula stages, while embryonic cells from the mouse blastocyst are used to derive embryonic stem cell (ESC) lines that remain pluripotent indefinitely *in vitro* (Tiedemann et al., 2001; Yamanaka et al., 2008). Growth factor signaling at blastula and gastrula stages induces embryonic cells to undergo commitment and patterns the embryo, with Activin/Nodal, FGF and BMP signaling playing essential roles. Nodal signaling through Smad2/3 directly transactivates expression of transcription factors that regulate mesendodermal commitment, including Goosecoid and Brachyury (Koide et al., 2005; Loose and Patient, 2004; Wardle and Smith, 2006). BMP signaling also patterns the germ layers at these stages, inducing expression of genes that regulate formation of ventral mesoderm and non-neural ectoderm/epidermis, through Smad1-mediated activation of direct BMP target genes, including Vent2.

Conversely, neurectoderm formation requires inhibition of both Nodal and BMP signaling during gastrulation (Chang and Harland, 2007; Vonica and Brivanlou, 2006).

Embryos must maintain pluripotent cell populations and control when cells undergo commitment to ensure correct spatial and temporal patterning. Cell-intrinsic activities regulate this process by counteracting commitment-promoting signals. In ESCs, Oct4 and Sox2 maintain pluripotency (Yamanaka et al., 2008). Oct4-null mice are early embryonic lethal, precluding further analysis (Nichols et al., 1998), so work in *Xenopus* has been useful for defining Oct4 roles in embryonic cell commitment and patterning. Three functional Oct4 orthologs are present in *Xenopus* (Oct25/Oct60/Pou91) and have overlapping expression in blastula ectoderm, gastrula neurectoderm and non-involuting mesoderm (Morrison and Brickman, 2006). Like their mouse counterparts, they control pluripotency maintenance in embryos, suppressing BMP-mediated ventral fate acquisition, inhibiting Activin- and FGF-induced mesodermal commitment, and promoting neural tissue formation. This activity contributes to maintaining proper tissue boundaries and preventing expansion of mesodermal and endodermal territories into pluripotent ectoderm (Cao et al., 2006; Henig et al., 1998; Morrison and Brickman, 2006; Snir et al., 2006; Takebayashi-Suzuki et al., 2007).

Epigenetic regulation, which modulates transcription through effects on chromatin structure, also centrally controls cell commitment. Actively transcribed genes are marked by trimethylation of lysine 4 on Histone H3 (H3K4me3), while gene activation is balanced by Polycomb-mediated repression. Polycomb group (PcG) proteins act in two repressive complexes to control developmental gene expression: PRC2 (Suz12/Eed/Ezh2) trimethylates lysine 27 of histone H3 (H3K27me3), which recruits

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PRC1 to mediate gene silencing (Pietersen and Van Lohuizen, 2008). In *Xenopus tropicalis* embryos, these modifications occur sequentially and correlate with transcriptional status (Akkers et al., 2009): H3K4me3 is deposited from mid-blastula and correlates with early activation of zygotic gene expression. H3K27me3 deposition begins during mid-gastrulation, occurs at many developmental genes and preferentially marks genes with regionally restricted expression, blocking expression elsewhere in the embryo (Akkers et al., 2009). In ESCs, Sox2/Oct4 transcriptional regulation and Polycomb-mediated epigenetic regulation play distinct roles: Sox2/Oct4 govern a transcriptional network essential for pluripotency, whereas Polycomb is dispensable for pluripotency but instead restrains commitment (Boyer et al., 2006; Chamberlain et al., 2008; Pietersen and Van Lohuizen, 2008). Polycomb may epigenetically buffer cells from erroneous transcriptional responses to sub-threshold commitment-promoting signals (Chi and Bernstein, 2009). However, cell intrinsic regulation of the transition from pluripotency to lineage commitment is not well characterized during embryonic lineage commitment in vivo.

We previously defined Geminin (Gem or Gmn) as another putative cell intrinsic regulator of early embryogenesis. Geminin is a small predominantly nuclear protein that can promote neural gene expression at the expense of non-neural epidermis (Kroll et al., 1998). Geminin can also regulate the fidelity of DNA replication by binding and inhibiting the pre-replication complex protein Cdt1 (McGarry and Kirschner, 1998; Tada et al., 2001; Wohlschlegel et al., 2000). Geminin is highly enriched in many progenitor cell types and regulates proliferation-differentiation decisions through interactions with multiple protein partners including Brg1, the SWI/SNF remodeling complex catalytic subunit, and Polycomb PRC1 (Del Bene et al., 2004; Karamitros et al., 2010; Luo et al., 2004; Seo et al., 2005). However, the molecular basis for the early activities of Geminin in blastula and gastrula stage embryos remained obscure. Mice null for geminin die at pre-implantation before embryonic cell lineage commitment (Gonzalez et al., 2006). Therefore, here we used a combination of approaches in *Xenopus laevis* embryos to determine the roles of Geminin in early embryogenesis.

We found that Geminin is an essential cell intrinsic regulator of the pluripotency to multi-lineage commitment transition and acts by broadly antagonizing transcriptional responses to three signaling pathways (BMP, FGF and Activin/Nodal) that promote cell commitment and embryonic patterning at blastula-gastrula stages. As the activities of Geminin resembled those of the Oct4 orthologs, we tested its functional relationship to other transcriptional and epigenetic regulators that restrain commitment. We found that the repression of commitment by Geminin is Oct4 ortholog-independent, instead depending on intact Polycomb repressor complex activity. Likewise, Geminin enhances Polycomb complex retention and associated histone modifications on chromatin, while blocking modifications associated with transactivation of mesodermal genes. Together, this work demonstrates that Geminin is required to spatially restrict mesoderm, endoderm and non-neural ectoderm to their proper embryonic locations, and supports a role for Geminin-Polycomb cooperativity in controlling spatial and temporal patterning of the early embryo.

MATERIALS AND METHODS

Capped RNA synthesis

Capped RNAs were made using mMessage mMachine (SP6; Ambion) and these plasmids: CS2MT-xGeminin Δ coil, CS2MT-xGeminin-full-length/FL (Seo et al., 2005), CS2NLS- β Gal, CS2-Sox2, SP64T-Activin β (Thomsen

et al., 1990), SP64T-tALK4 (Chang and Harland, 2007), CS2-xSIP1 (Postigo et al., 2003), CS2-CA-ALK3 (Akiyama et al., 1997), CS2-Smad1 (Faure et al., 2000), CS2-Smad2 (Grammer et al., 2000), CS2-Fast-VPI6/Foxh1 (Watanabe and Whitman, 1999) and Oct25/Oct60/Ezh2/Suz12 full-length cDNAs in CMV-Sport6 (Open Biosystems).

Microarray analysis

Xenopus embryos were obtained by in vitro fertilization (Kroll et al., 1998), with staging after (Nieuwkoop and Faber, 1967). xGeminin Δ coil RNA/300 pg was injected into both blastomeres of two-cell stage pigmented embryos (animal hemisphere). Injected embryos were raised until stage 8-9 and 80 ectodermal (animal cap) explants/sample were isolated and incubated in 0.7 \times MMR/25°C. When sibling embryos reached stage 10.5 or 12, total RNA (20 μ g/sample) was isolated using Trizol (Invitrogen). For each experiment, paired Geminin-injected versus uninjected samples were used for probe synthesis and hybridization to Affymetrix *Xenopus laevis* genome v1.0 arrays (Washington University Genechip facility). Raw CEL and DAT files were analyzed using dChip (<http://biosun1.harvard.edu/complab/dchip/>) (Li and Hung Wong, 2001) after normalization as described (see Table S3 in the supplementary material). Microarray data are deposited in the Gene Expression Omnibus (GSE25158).

Ectodermal explants and quantitative RT-PCR (qRT-PCR)

To confirm microarray results in explants, Geminin Δ coil RNA (300 pg), full-length Geminin (30 pg), Geminin antisense morpholino (MO; 2.5 ng; 5'-ATCTCTGCTTCTTGTGGTATTCAT) (Seo et al., 2005), or CMV-promoter plasmids encoding full-length or Geminin Δ coil (125 pg each) were injected into both blastomeres of two-cell stage embryos. Explants and RNA were obtained as above. For growth factor treatment, uninjected or mRNA/MO injected explants were cultured for 2 hours in 10 ng/ml Activin (R&D Systems/338-AC) or 25 ng/ml bFGF (R&D systems/233-FB) before total RNA preparation. Total RNA (1 μ g) was used per 20 μ l cDNA synthesis reaction with oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was performed with the 7500 Fast Real-time PCR System (Applied Biosystems) and iQSYBRGreen Supermix (Bio-Rad). Table S1 in the supplementary material lists primers. Relative gene expression was calculated by the $\Delta\Delta$ Ct method (Stappenbeck et al., 2002) with normalization to EF1 α . Each qPCR was carried out in triplicate and three to six independent experiments of each type were performed; representative examples are presented as fold changes with the control condition set to 1.0 in Figs 3-5 and Figs S6, S8 and S10 in the supplementary material. For each primer pair, the melt curve was analyzed and PCR product was examined using 2% agarose gel to ensure a single fragment of the predicted size was amplified.

Microinjection and whole-mount in situ hybridization

To examine expression changes of target genes by Geminin overexpression or MO knockdown, one blastomere of eight-cell stage albino embryos was injected with Geminin Δ coil/120 pg RNA or GemininMO/1.0 ng+20 pg β -galactosidase RNA. Embryos were grown to stage 11-14, fixed, stained [X-Gal/Sigma-Aldrich or Red-Gal/Research Organics] and in situ hybridized as described previously (Seo et al., 2005). H1FOO, Foxi1 and Grhl1 cDNAs were purchased from OpenBiosystems. Morpholino (Gene Tools) sequences were: Oct25MO-5'-TTGGGAAGGGCTGTTGGCTGTACAT, Oct60MO-5'-TCCATCTCCAGCACTTGCTCAGGCC (Cao et al., 2006), Sox2MO-5'-GCTCGGTCTCCATCATGCTGTACA, Ezh2MO-5'-CAG-ATTTCTCCCCGTCTGGCCAT, Suz12MO-5'-CCATGCGGGATACTACGAGTGATAA and SIP1MO-5'-CTTGCTTCATTGATAAGAG-TGGGAT (Nitta et al., 2004).

Luciferase assay

Luciferase constructs were: Mix.2-luc (Watanabe and Whitman, 1999), xBra2.1pGL2 (Latinkic et al., 1997), -266gsc-luc (Watabe et al., 1995), pE-bambi-xcol-luc, pE1'-Samd7-xcol-luc, pId1(-1.0kb)-xcol-luc, and pId2(-3.2kb)-xcol-luc (Karaulanov et al., 2004). *Xenopus* embryos were injected with 100 pg of each construct+CS2NLS- β -Gal plasmid (for normalization) at the two-cell stage. At stage 8-9, ectodermal explants were isolated, treated with 10 ng/ml Activin or 25 ng/ml bFGF, and cultured 2 hours. Activities in 20 explants/150 μ l reporter lysis buffer (Promega) were

measured using Luciferase and β -Galactosidase Enzyme Assay Systems (Promega). At least three independent experiments were performed; Fig. 5 and Fig. S6C in the supplementary material show representative technical replicates (luciferase assay performed in triplicate \pm s.e.m.).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation in *Xenopus* early embryos was modified from published methods (Blythe et al., 2009). Geminin Δ coil (120 pg), tAlk4 (120 pg), Actin β (12 pg) RNA, Geminin morpholino (1 ng) or Ezh2+Suz12 morpholino (8 ng each) was injected laterally into each blastomere of 150-200 four-cell stage embryos/immunoprecipitation. Injected embryos and uninjected controls were raised to stage 10.5 or 12.5-13 and crosslinked in 1% formaldehyde/PBS for 45 minutes. Crosslinking was stopped (10 minutes, 0.125 M Glycine/PBS), followed by three PBS washes. Embryos were homogenized in 1 ml RIPA buffer, incubated for 10 minutes on ice, and centrifuged (14,000 g). The pellet was re-suspended in 1 ml RIPA buffer and chromatin sheared to \sim 250-500 bp (Branson Sonifier250, power 2.5/50% duty cycle/10 second pulse, 20 second restX10 rounds). Sheared chromatin (3 ml; 150 embryo-equivalents) was incubated with 5 ng antibody and immunoprecipitated using 20 μ l ProteinG Dynabeads (Invitrogen). Antibodies used were: normal rabbit IgG/Millipore/12-370, anti-H3K4me3/Cell Signaling/9751, anti-H3K4me3/Abcam/Ab8580, anti-H3K27me3/Millipore/07-449, anti-H3K27me3/Cell Signaling/9733, anti-Ezh2/Active Motif/39639, anti-Acetyl-histone3/Millipore/06-599 and anti-Smad2/Cell Signaling/3211. Eluted DNA was purified by Phenol/Chloroform extraction and PCR/Gel Purification (Macherey-Nagel NucleoSpin ExtractII). Purified DNA (2 μ l; 100 μ l elution/ChIP) was used for qPCR. Table S1 in the supplementary material lists ChIP primers. For qPCR determination of ChIP enrichment (Blythe et al., 2009), each sample was immunoprecipitated with specific antibody and non-specific IgG control. Immunoprecipitated values were normalized against 1% input values for each specific antibody and control IgG pull-down [$\Delta C(t) = \text{ChIP } C(t) - 1\% \text{ input } C(t)$]. The percentage input for each sample was then calculated using the formula $[\% \text{ input} = 2^{-\Delta C(t)}]$. Fold enrichment over IgG was calculated by dividing $\% \text{ input}_{\text{antibody}}$ by $\% \text{ input}_{\text{IgG}}$.

RESULTS

Geminin antagonizes mesoderm and endoderm formation during early embryogenesis

We previously identified Geminin based on its ability to expand the neural plate at the expense of non-neural epidermis (Fig. 1) (Kroll et al., 1998). Overexpression of low levels of full-length Geminin (<30 pg RNA) or of two Cdt1 non-binding variants (the Geminin amino terminus or Geminin Δ coil, which lacks the central coiled-coil) can both elicit this effect without affecting cell proliferation or apoptosis (see Fig. S1 in the supplementary material) (Kroll et al., 1998; Seo et al., 2005). Conversely, Geminin knockdown in embryos induces epidermal differentiation at the expense of neural tissue formation, without affecting cell proliferation or viability (Seo et al., 2005) (see Fig. S1 in the supplementary material). However, it is not known whether Geminin also regulates other cell fates, such as mesoderm and endoderm, in embryos. We therefore used the experimental conditions defined above and found that Geminin overexpression suppresses mesodermal (Xpo, Mix.2, Brachyury) and endodermal (Sox17, Endodermin, Hex) gene expression (Fig. 1; see Fig. S2A in the supplementary material). Both full-length Geminin and Geminin Δ coil suppress mesodermal gene expression when overexpressed from early embryogenesis as injected mRNAs (Fig. 1; see Fig. S3A in the supplementary material). These variants also suppress mesodermal gene expression when overexpression is limited to gastrulation, using CMV promoter-driven expression from injected plasmids (see Fig. S3A in the supplementary material). This suggests that Geminin overexpression during blastula-gastrula

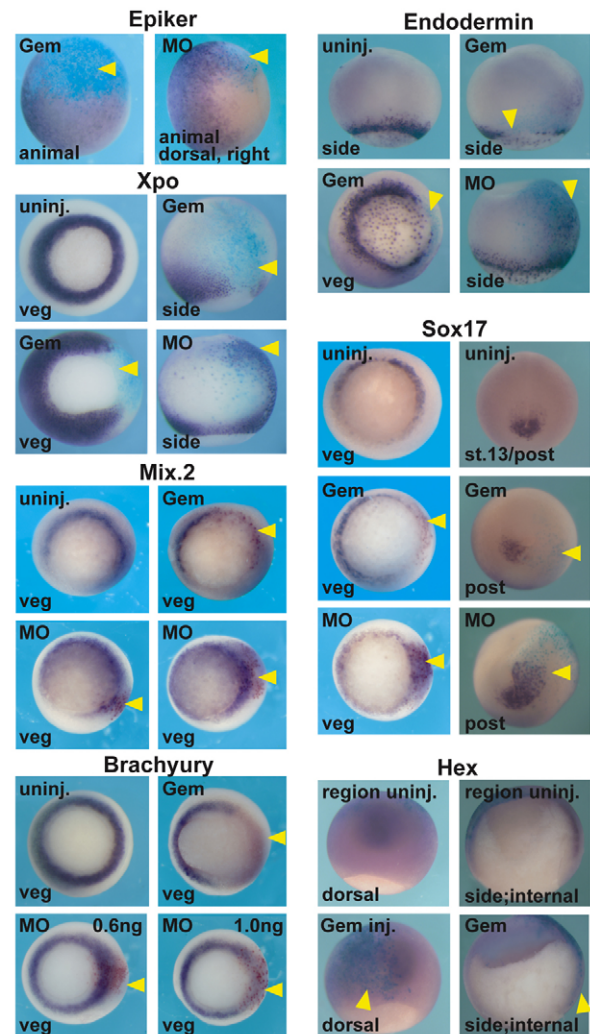


Fig. 1. Geminin regulates epidermal, mesodermal and endodermal gene expression. Geminin RNA or morpholino/MO-injected regions are oriented rightwards, indicated by yellow arrowheads, and lineage is labeled by β -galactosidase RNA co-injection and Red-gal (Mix.2, Brachyury/Xbra, and Sox17 at stage 11) or X-gal staining. Embryos are stage 10.5-11, except for Epidermal keratin/Epiker and Sox17 (right), which are stage 12-13. Views are indicated: (veg, vegetal; post, posterior).

stages is sufficient to suppress mesodermal fate acquisition. The repression of mesodermal gene expression by Geminin is also restricted to mRNA-injected, lineage-labeled cells, suggesting that this activity occurs in a cell autonomous manner (Fig. 1; see Fig. S3B in the supplementary material).

We likewise tested the effects of Geminin knockdown in embryos, using a previously described morpholino oligo (Seo et al., 2005) and found that Geminin knockdown instead promotes ectopic expression of Xpo, Mix.2, Endodermin and Sox17 (Fig. 1; see Fig. S2B in the supplementary material). In Geminin knockdown embryos, Xpo (a pan-mesodermal and ventral/posterior ectodermal marker) is ectopically expressed broadly in the animal hemisphere, corresponding to the morpholino-injected cell territory (Fig. 1). Geminin knockdown embryos also ectopically express Mix.2, Endodermin and Sox17, but ectopic expression is restricted to morpholino-injected tissue adjacent to the endogenous expression

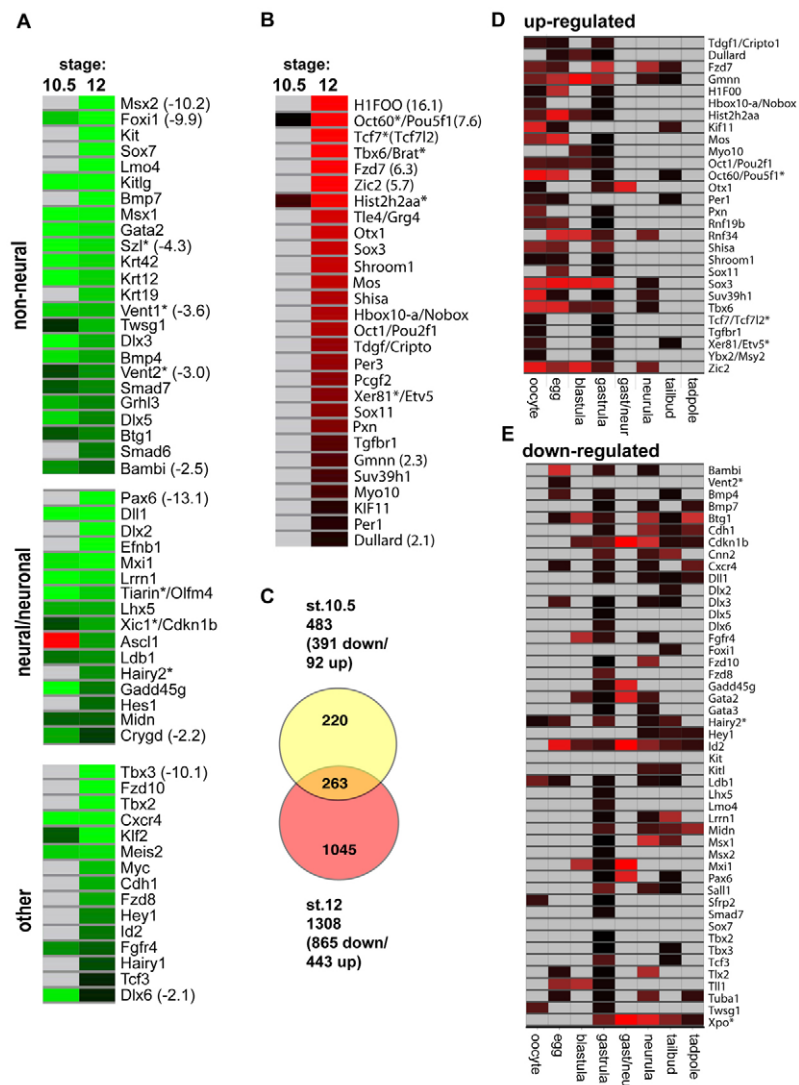


Fig. 2. Geminin-regulated gene programs in gastrula embryos. (A,B) Heat maps of selected genes from microarrays whose expression (A) decreased or (B) increased upon Geminin overexpression, categorized by expression/function. Genes not present in dataset are gray. Some fold changes are in parentheses. Asterisks indicate *Xenopus*-specific gene symbols. (C) Overlap between datasets. (D,E) Temporal expression of Geminin-regulated genes during embryogenesis. Relative transcript abundance from NCBI expression data (defined as transcripts/million) and expressed in heat map view. Gray, 0 TPM; black, lower; red, higher expression.

domain. This indicates that Geminin knockdown alters normal gene expression boundaries for these genes, but is insufficient to evoke ectopic gene expression throughout animal pole ectoderm, where endogenous mesoderm/endoderm-inducing signals are absent.

Unlike the other mesodermal and endodermal markers examined, by late gastrulation Brachyury is lost in both Geminin overexpressing and knockdown embryos, resembling the effects of Oct4 ortholog overexpression and knockdown (Cao et al., 2006; Morrison and Brickman, 2006). One explanation for this finding is that Geminin knockdown may enhance expression of a negative regulator of Brachyury, such as Mix or Gsc (Latinkic et al., 1997). In this case, Brachyury expression may respond differently to Geminin knockdown as gastrulation proceeds. To test this, we compared effects of Gem knockdown on Brachyury from late blastula-late gastrula stages. By mid-late gastrulation, Gem knockdown always results in loss of Brachyury expression. However, some early gastrula embryos instead have expanded or unaffected Brachyury expression, or only mild reduction (see Fig. S2C in the supplementary material). These data suggest that knockdown of Gem can initially increase Brachyury expression, like the other mesodermal markers examined. Together, these data indicate that Geminin is required to maintain ectoderm, mesoderm and endoderm in their correct spatial locations in the embryo.

We compared these activities with the embryonic expression profile of Geminin in order to consider their relationship to the function of Geminin. Through the blastula stages, maternal Geminin is expressed throughout pluripotent ectoderm and overlaps the mesodermal territory expressing Brachyury (see Fig. S4 in the supplementary material). However, at early gastrulation, the expression of Geminin rapidly becomes localized to prospective neurectoderm and non-overlapping with regions that contribute to non-neural ectoderm and mesendoderm (see Fig. S4 in the supplementary material). This restriction of expression could therefore protect non-neural tissue from Geminin-mediated repression and facilitate boundary formation between these tissue types.

Geminin is required to restrict ventral and mesodermal gene expression to the correct embryonic territories and to block neural maturation

To more comprehensively define the programs of gene expression under the control of Geminin, we conducted microarray experiments. We over-expressed Geminin Δ coil in embryos by mRNA injection at the two-cell stage, isolated ectodermal (animal cap) explants from blastulae and cultured these until either early

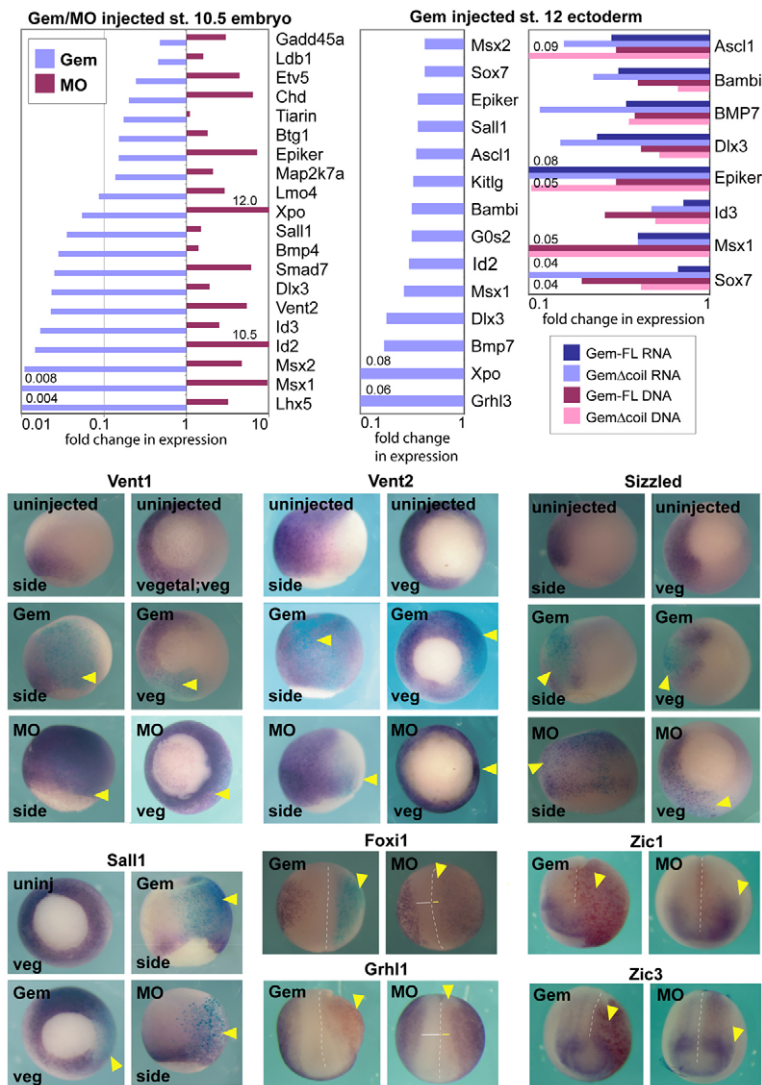


Fig. 3. Geminin suppresses mesoderm and non-neural ectoderm formation.

Top: downregulated genes from microarrays were assayed by qRT-PCR for expression changes upon Geminin overexpression or knockdown in embryos (left) or stage 12 ectodermal explants (center/right). Right panel compares effects of plasmid-driven (125 pg) versus mRNA-based overexpression of full-length-FL/30 pg or Δ coil/300 pg Geminin on gene expression. Relative expression normalized to EF1 α is shown, control (uninjected embryo/explant) values=1.0. A representative result from at least three independent experiments is shown. Bottom: confirmation of Geminin-downregulated genes by in situ hybridization. Injected region is marked by Red-gal (Grhl1/Zic1/Zic3) or X-gal staining and yellow arrowheads. For Foxi1/Grhl1/Zic1/Zic3, stage 13/neural plate view is shown with dorsal midlines indicated (lines).

(stage 10.5) or late (stage 12) gastrula. We used Geminin Δ coil to eliminate the possibility that cell cycle perturbation could have caused gene expression changes. These explants were compared with uninjected animal cap explants, which have low endogenous Geminin RNA levels during gastrulation, and differentially expressed genes were defined using Affymetrix genome arrays. Three independent experiments of each type were performed and probe sets meeting cut-off values with over twofold expression changes in the same direction in at least two experiments were designated as putatively Geminin regulated (see Materials and methods; Tables S3 and S4 in the supplementary material). Subsets of these Geminin-regulated datasets are shown in Fig. 2A,B.

We found that Geminin overexpression reduces the expression of many genes involved in ventral ectoderm and mesoderm formation by early gastrulation (Fig. 2A). These include direct transcriptional targets of BMP signaling (e.g. *Bambi*/*Bmp7*/*Smad7*/*Vent2*) and other genes that regulate or mark ventral (e.g. *Bmp4*/*Foxi1*/*Grhl1*/*Sizzled*/*Vent1*) or mesodermal/endodermal (e.g. *Sall1*/*Sox7*/*Sox18*/*Cxcr4*) cells (Fig. 2A). Geminin also reduces expression of genes that regulate later events such as primary neurogenesis (e.g. *Cbfa2t2*/*Dll1*/*Gadd45g*/*Ascl1*/*Pax6*). We validated the ability of Geminin to repress a range of these target genes by quantitative RT-PCR (Fig. 3; see Tables S1,S2 in the

supplementary material): nearly all Geminin downregulated genes defined by microarray are also repressed by Geminin overexpression in gastrula embryos or ectodermal explants. Geminin knockdown also increases expression levels of many of these genes in the embryo, suggesting that they are negatively regulated by endogenous Geminin in vivo (Fig. 3). Both full-length Geminin and Geminin Δ coil can repress the genes examined in ectodermal explants. Plasmid-based overexpression of Geminin variants during gastrulation is also sufficient to repress expression of these genes (Fig. 3).

We further confirmed these microarray results by performing in situ hybridization analysis in Geminin overexpressing and knockdown embryos: Geminin Δ coil suppresses *Vent1*, *Vent2*, *Sizzled* (ventral ectoderm and/or mesoderm markers) and pan-mesodermal *Sall1* expression. Upon Geminin knockdown, these markers are ectopically expressed throughout the animal hemisphere, while other non-neural ectoderm markers (*Foxi1*, *Grhl1*) also spread into the neural plate territory (Fig. 3; see Table S2 in the supplementary material). As seen previously, Geminin-mediated repression of these markers is restricted to mRNA-injected, lineage-labeled cells, consistent with cell autonomous regulation (Fig. 3 and see Fig. S3B in the supplementary material). Also consistent with the microarray results, Geminin suppresses

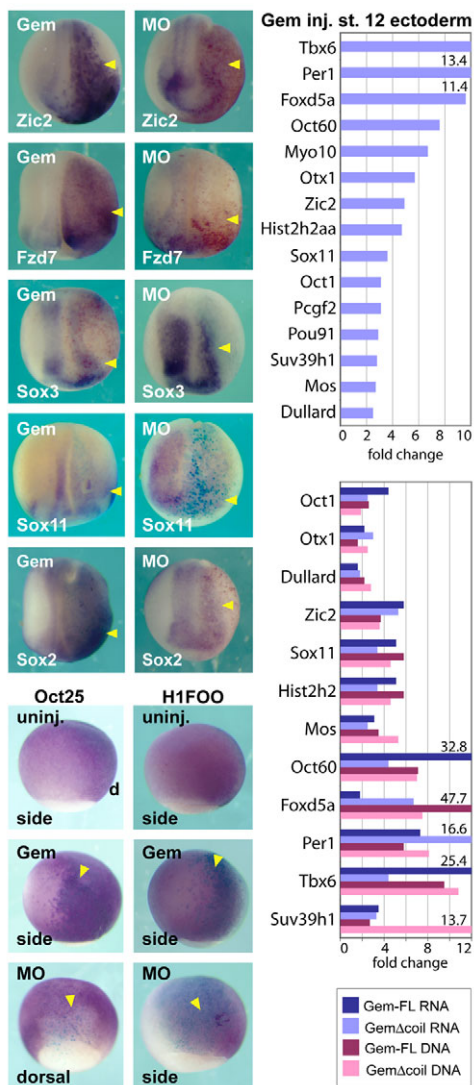


Fig. 4. Geminin promotes expression of genes that mark pluripotent ectoderm and neuroectoderm. Left: in situ hybridization of Geminin-overexpressing or MO knockdown embryos with injected area marked by Red-gal (Zic2/Fzd7/Sox2-MO/Sox3-Geminin) or X-gal (yellow arrowhead). Right: genes were assayed by qRT-PCR for changes in expression in stage 12 ectoderm and comparison of Geminin FL versus Δ coil after Fig. 3.

expression of Zic1 and Zic3, which regulate later neural plate patterning, while Geminin knockdown expands their expression domains. Together, these data suggest that Geminin broadly represses many genes that mark or regulate cellular commitment during blastula gastrulation.

Geminin promotes pluripotent and immature neuroectodermal gene expression

In our microarrays, Geminin overexpression elevated expression levels of a smaller, functionally distinct group of genes, including the three Oct4 orthologs (Oct25/60, Pou91), genes that regulate early neuroectoderm formation (Foxd5a/Zic2/Sox2/3/11/Dullard), and genes predominantly expressed during early development. Using qRT-PCR and in situ hybridization analysis, we found that Geminin Δ coil overexpression elevates

and expands expression domains of many of these genes, while Geminin knockdown reduces their expression (Fig. 4; see Table S2 in the supplementary material). As before, overexpression of either full-length or Geminin Δ coil from injected mRNA or expression plasmids yielded comparable effects (Fig. 4). Therefore, Geminin elevates expression levels of genes that mark and regulate pluripotent and immature neuroectodermal gene expression and is also required for their expression in vivo. However, unlike the Geminin-repressed genes, Geminin overexpression only affects their transcript levels by late but not early gastrulation (compare Fig. 2A with 2B). This delay suggests that Geminin may play a less proximal or direct role in their regulation. Also in contrast to results for Geminin-repressed genes, Geminin overexpression broadly expands neural marker expression beyond mRNA-injected territories, suggesting that these effects are at least partially non-cell autonomous and involve the modulation by Geminin of secreted signaling activities that affect fate in neighboring non-injected cells (Fzd7/Oct25, see Fig. S3B in the supplementary material). Maternal transcripts for some of these genes are pan-ectodermal, while zygotic expression occurs in presumptive neuroectoderm during gastrulation. Therefore, their steady-state transcript levels decline in uninjected ectodermal explants during gastrulation, as cells commit to an epidermal fate. This decline is evident for many genes examined here (e.g. Fzd7, Oct25/60/91, Sox2/3/11, Zic2) by microarray-based comparison of relative mRNA abundance in uninjected ectodermal explants at early versus late gastrulation. We hypothesize that the ability of Geminin to block epidermal fate acquisition may maintain late gastrula ectoderm in a cell state resembling that normally seen at early gastrula, resulting in elevated steady state levels of these transcripts relative to uninjected ectoderm. This does not preclude the possibility that Geminin also plays a more direct role in promoting expression of some of these genes. For example, Gem-Brahma interactions can directly regulate Sox2 expression by competing HP1 α repressor from a Sox2 early neural enhancer (Papanayotou et al., 2008).

To further characterize genes whose expression decreased rather than increased upon Geminin overexpression, we compared their developmental expression profiles. Geminin overexpression increases expression levels of genes that are highly expressed from oocyte/egg through gastrula stages, whereas many genes have little or no post-gastrula expression. Expression of many Geminin downregulated genes instead begins from gastrulation and, for some, extends to later stages (Fig. 2, compare D with E). We also used Gene Ontology (GO)-based algorithms to define higher-order patterns of gene expression in the Geminin-regulated gene set (Doherty et al., 2006; Doherty et al., 2008). This approach defines GO terms most frequently associated with the entire Geminin up- or downregulated dataset and compares those with GO profiles from many stem and differentiated cell types. The fractional representation in a gene expression profile of the GO term 'nucleus' is universally higher in stem/progenitor cells relative to differentiated cells, whereas the term 'integral to membrane' is more frequently associated with genes in a profile of differentiated cells. Thus, plotting the fractional representation of 'nucleus' versus 'integral to membrane' allows analysis of the relative differentiation state of a gene expression profile (Doherty et al., 2008). In these analyses, the Geminin upregulated dataset also has greater similarity to GO profiles for stem cells, whereas the Geminin downregulated dataset more closely resembles their differentiated derivatives (see Fig. S5 in the supplementary

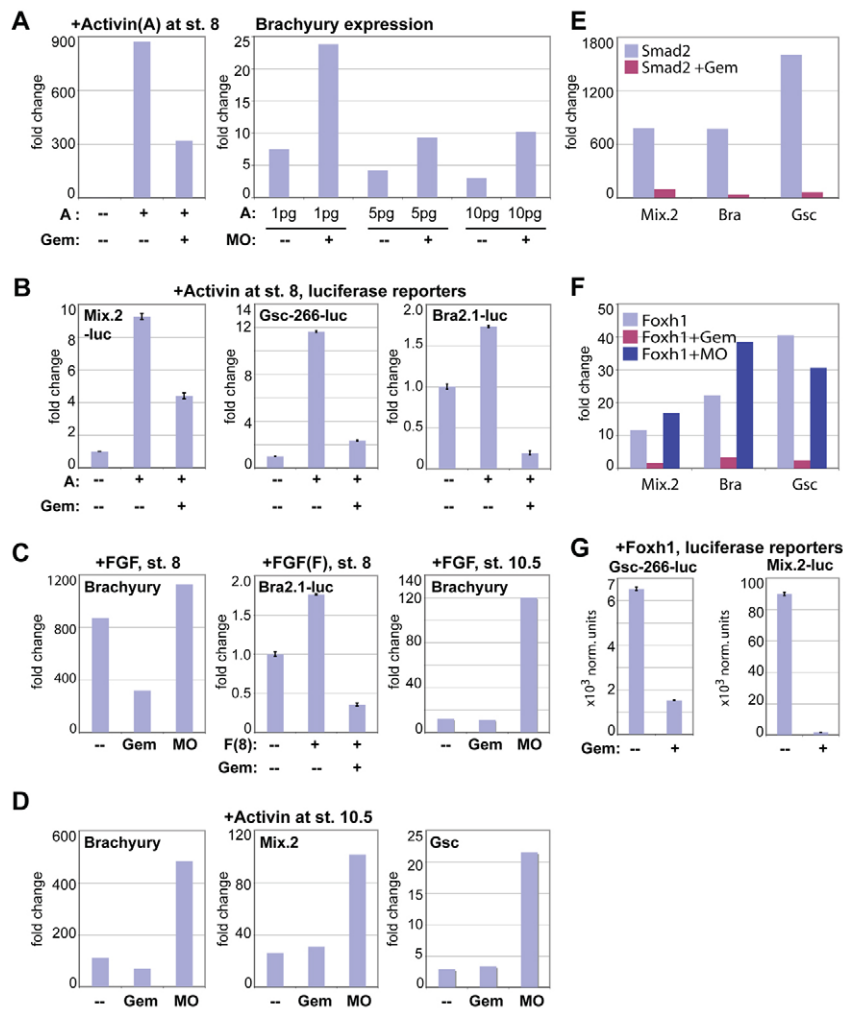


Fig. 5. Geminin overexpression antagonizes and knockdown enhances transcriptional responses to Activin and FGF signaling. (A,B,D) Ectodermal explants were obtained from embryos injected as indicated, Activin(A)-treated (10 ng/ml) for 2 hours/25°C from stages indicated, and harvested for qRT-PCR (see Materials and methods). In A, right panel, Activin RNA (1/5/10 pg) was injected at the two-cell stage instead. Relative expression is shown, uninjected/untreated samples=1.0. (A) Left: Geminin suppresses Activin-induced Brachyury expression. Right: Geminin knockdown enhances Activin-induced Brachyury. (B) Geminin suppresses Activin-induced luciferase reporter responses. (C) Geminin suppresses and knockdown enhances FGF responses. Animal caps were cut at stage 8 and treated with FGF (25 ng/ml) from blastula (stage 8; left/center) or gastrula (stage 10.5; right) stages. Geminin also suppressed FGF-mediated induction of a Brachyury-luciferase reporter (center) (see Materials and methods). (D) Animal caps were treated with Activin from stage 10.5; Geminin knockdown enhanced mesodermal responses of gastrula stage ectoderm to Activin. (E-G) Geminin suppresses mesodermal gene expression and luciferase reporter responses induced by Smad2/150pg and Foxh1/30pg mRNA overexpression in ectodermal explants.

material). Together, these data support a role for Geminin in repressing genes associated with commitment and differentiation, while elevating expression levels of genes associated with pluripotent and immature neuroectodermal cell states.

Geminin antagonizes transcriptional responses to Activin, FGF and BMP signaling

During blastula-gastrula stages, Activin/Nodal, FGF and BMP signaling regulates commitment and patterning of mesoderm, endoderm and non-neural ectoderm. As Geminin overexpression antagonizes these cell fates, while Geminin knockdown promotes ectopic fate acquisition, we hypothesized that Geminin might act by antagonizing transcriptional responses to one or more of these signaling pathways. To test this, we injected embryos with Geminin Δ coil mRNA, isolated ectodermal explants and treated these with Activin from the blastula stage. We found that Geminin overexpression represses Activin-mediated Brachyury induction. MO knockdown conversely enhances the Brachyury response to Activin (Fig. 5A). Performing similar animal cap experiments, we found that Geminin Δ coil also represses Activin-mediated induction of Mix.2, Gsc and Brachyury promoter-luciferase reporter constructs (Fig. 5B). Geminin Δ coil likewise represses FGF-mediated induction of Brachyury and a Brachyury promoter-luciferase construct, whereas Geminin knockdown enhances the Brachyury response to FGF (Fig. 5C, left and center). Together,

these data suggest that Geminin can repress transcriptional responses to more than one type of growth factor signaling. In this regard, the activity of Geminin resembles that of the *Xenopus* Oct4 orthologs.

Competence of embryonic cells to form mesoderm is high at blastula stages (stage 8) and declines by mid-gastrulation (stage 10.5-11). For example, FGF or Activin treatment from stage 10.5 evokes lower mesodermal gene responses than treatment from stage 8 (for FGF, compare Fig. 5C, left versus right panels; for Activin, compare Fig. 5A, left panel versus 5D). Therefore, we wondered whether Geminin could also affect growth factor responsiveness of gastrula stage ectoderm. To assess this, we performed Geminin knockdown in embryos, isolated ectodermal explants and treated these with FGF and Activin from stage 10.5. We found that Geminin knockdown can still enhance mesodermal responses of gastrula cells to growth factor treatment (Fig. 5C, right panel, FGF; Fig. 5D, Activin). These results suggest that in addition to restraining mesodermal commitment at blastula stages, Geminin continues to buffer embryonic cells against overresponsiveness to mesoderm-inducing growth factor signals during gastrulation.

As Geminin antagonizes Activin-dependent transcription, we also tested whether Geminin can modulate activities of its nuclear effectors Smad2 and Foxh1. We found that overexpression of either Smad2 or constitutively active Foxh1 promotes mesodermal gene

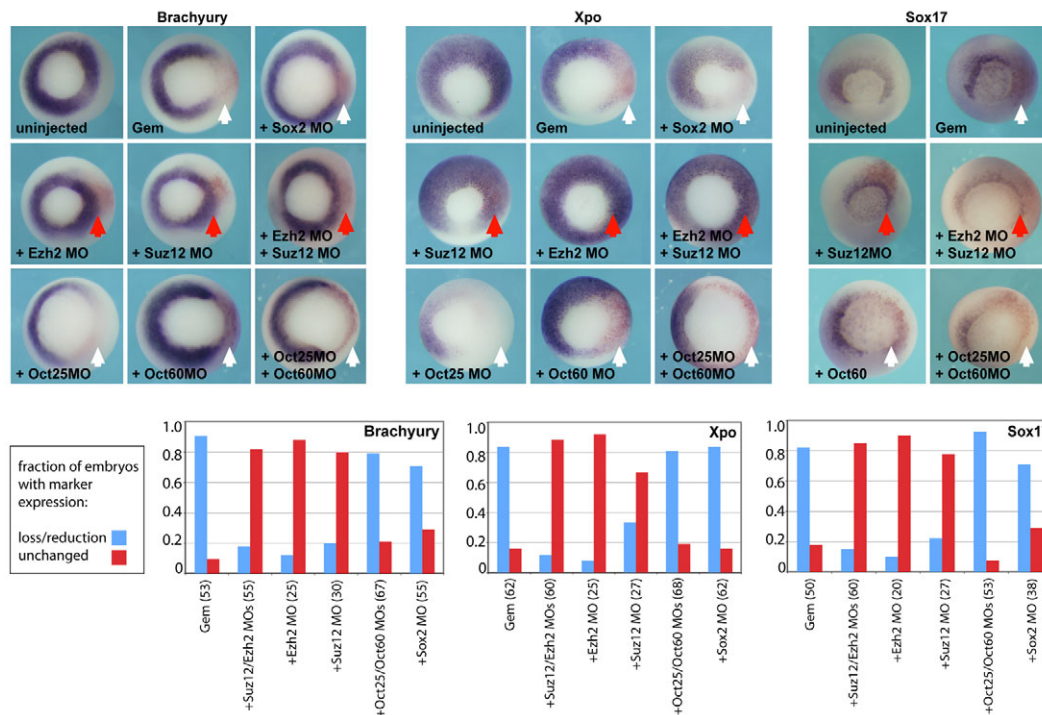


Fig. 6. Geminin suppression of mesoderm and endoderm depends on Polycomb but is independent of Oct25/60 and Sox2 activities.

Geminin RNA (120 pg) with or without 8 ng of MOs (16 ng Oct60MO) + 30 pg β -galactosidase RNA was injected into one cell of eight-cell embryos, with in situ hybridization at stage 11.5. Red-gal staining and white or red arrowheads mark injected regions. Below are additive results for four experiments shown as fractions of embryos with indicated effects (embryo numbers analyzed are in parentheses).

expression in ectodermal explants, while Geminin Δ coil co-expression antagonizes this response (Fig. 5E-F). Geminin Δ coil also antagonizes Foxh1-driven activation of Gsc and Mix.2 promoter-luciferase reporter constructs (Fig. 5G). Therefore, Geminin can block transcriptional responses by acting downstream of these nuclear effectors of Activin signaling.

Finally, because many Geminin-repressed genes are direct targets of BMP signaling, we also tested the ability of Geminin to modulate transcriptional effects of BMP signaling. In co-injected animal cap explants, Geminin Δ coil can block ventral/non-neural gene expression in the presence of a constitutively active BMP receptor (CA-ALK3) or Smad1, and Geminin Δ coil also suppresses BMP responsive enhancer-luciferase reporter construct activation by CA-ALK3 (see Fig. S6 in the supplementary material). As Geminin is a predominantly nuclear protein and can suppress responses to CA-ALK3 and Smad1, these results are consistent with a role for Geminin in blocking BMP/Smad1-induced transcriptional responses. Together, these data indicate that Geminin can act broadly to restrain transcriptional responses to several signaling pathways that promote cell commitment and embryonic patterning at blastula-gastrula stages.

Geminin requires Polycomb but not Oct4 ortholog or Sox2 activities to suppress mesodermal and endodermal gene expression

Geminin acts during blastula-gastrula stages to restrain transcriptional responses to several growth factor signals and their nuclear effectors, and is required in vivo for proper allocation of cells to and boundary formation between neurectoderm versus mesendodermal/non-neural territories. By what mechanism does

this occur? Geminin could modulate binding of nuclear effectors (Smad/Foxh1) to their targets or could affect their activities. Alternatively, Geminin could cooperate with transcription factors with similar activities, such as the Oct4 orthologs and Sox2, or with complexes that regulate cell fate through effects on chromatin structure, such as Polycomb. We therefore initially assayed whether Geminin Δ coil could still repress mesendodermal gene expression in gastrula embryos following knockdown of Oct25/60, Sox2 or Polycomb activities. Morpholino knockdown of Oct25 or Oct60 individually or in combination, or of Sox2, does not interfere with the ability of Geminin to repress mesoderm (Brachyury and Xpo) or endoderm (Sox17) (Fig. 6). By contrast, morpholino knockdown of the Polycomb proteins Suz12 or Ezh2 separately, or more effectively in combination, strongly attenuates the ability of Geminin to repress all three markers (Fig. 6). These data indicate that Geminin is either acting downstream or independent of Oct25/60 and Sox2 activities but, conversely, Geminin requires intact Polycomb repressor activity to inhibit mesendodermal gene expression in vivo.

The prior results indicated that Geminin either acts downstream or is functionally independent of Oct25/Oct60/Sox2 activity. To distinguish between these possibilities, we tested whether Oct25/60 or Sox2 activities are sensitive to Geminin knockdown by co-injecting Geminin morpholino with mRNAs encoding Oct25, Oct60 or Sox2 into embryos. We found that Geminin knockdown does not inhibit Oct25, Oct60 or Sox2-mediated mesendodermal gene suppression in vivo (see Fig. S7 in the supplementary material) or Oct25-mediated repression of non-neural and induction of neural markers in ectodermal explants (see Fig. S8A,B in the supplementary material). The third Oct4 ortholog, Pou91, is

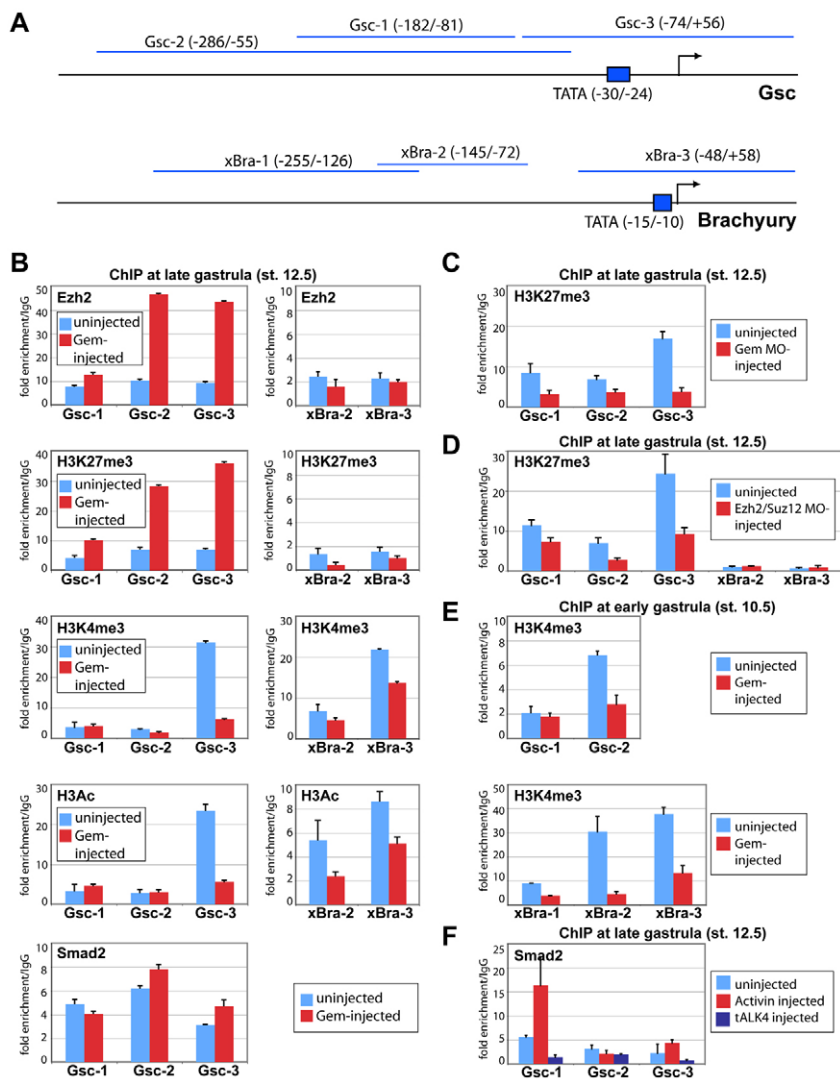


Fig. 7. Geminin regulates Polycomb binding and Polycomb-dependent histone modification.

(A) Quantitative ChIP was performed using late (stage 12.5; B-D,F) or early (stage 10.5; E) gastrulae, with PCR primers amplifying regions shown.

(B) Geminin overexpression increases Ezh2 and H3K27me3 but does not affect Smad2 enrichment at *Gsc*. Geminin decreases H3K4me3 and pan-H3 acetylation at *Gsc/Brachyury*.

(C) Gem MO knockdown reduces H3K27me3 at *Gsc*. (D) Ezh2 and Suz12 knockdown diminishes H3K27me3 at *Gsc*. (E) At early gastrula, Geminin overexpression also decreases H3K4me3 at *Gsc/Brachyury*.

(F) Activin overexpression or tALK4 dominant-interference affects Smad2 enrichment at *Gsc*. For each antibody and stage presented, three to five independent experiments were performed and a representative technical replicate (triplicate qPCR analysis of one experimental sample set (\pm s.e.m.)) is shown.

exclusively expressed during gastrulation and acts upstream of the zinc-finger protein Churchill to induce the transcription factor SIP1, which directly inhibits Brachyury expression to promote the transition from mesodermal to neural cell competence (Sheng et al., 2003; Snir et al., 2006). We therefore also tested whether the repression of mesodermal genes by Geminin was sensitive to SIP1 knockdown and vice versa. We found that SIP1 can suppress Activin-induced mesodermal gene expression in ectodermal explants, even under conditions of Gem knockdown (see Fig. S8C in the supplementary material). Geminin Δ coil also suppresses Smad2- or Fast1-induced mesodermal gene expression, and this is not blocked by SIP1 morpholino knockdown (see Fig. S8D-E in the supplementary material). Together, these results suggest that Geminin and Oct/Sox2/SIP1 repressive effects are functionally independent.

Geminin regulates the chromatin modification state of mesodermal genes

As Geminin depends on intact Polycomb activity to repress mesodermal gene expression in embryos, we tested whether Geminin also affects Polycomb complex binding and Polycomb-mediated histone modification (H3K27me3) of mesodermal genes. We performed quantitative chromatin immunoprecipitation (ChIP)

at the *Brachyury* and *Gsc* genes in whole embryos, using the qPCR primer pairs shown (Fig. 7A). We performed ChIP for Ezh2, H3K27me3, H3K4me3, Smad2 and pan-H3 acetylation in either early and/or late gastrula embryos, both under normal conditions and following Geminin Δ coil overexpression. As previously described for *Xenopus tropicalis* (Akkers et al., 2009), we do not detect enrichment for Ezh2 or H3K27me3 at *Brachyury* or *Gsc* in *Xenopus laevis* early gastrulae (not shown). However, by late gastrulation, we detect four- to 10-fold enrichment for both of these modifications at *Gsc*, consistent with Polycomb-mediated regulation during gastrulation [potentially regional restriction of expression (Akkers et al., 2009)]. Moreover, Geminin overexpression causes four- to fivefold enrichment of both Ezh2 and H3K27me3, relative to control conditions (Fig. 7B). Conversely, Geminin morpholino knockdown diminishes H3K27me3 levels (Fig. 7C). As a control for antibody specificity, we also demonstrated that *Gsc* enrichment for H3K27me3 was sensitive to morpholino knockdown of Ezh2/Suz12 (Fig. 7D).

We also assayed histone modifications associated with gene activation, H3K4me3 and pan-H3 acetylation. Enrichment for both modifications is detected at *Gsc* and *Brachyury* in both early and late gastrula embryos and Geminin overexpression strongly reduces chromatin enrichment for both modifications (Fig. 7B,E). This is

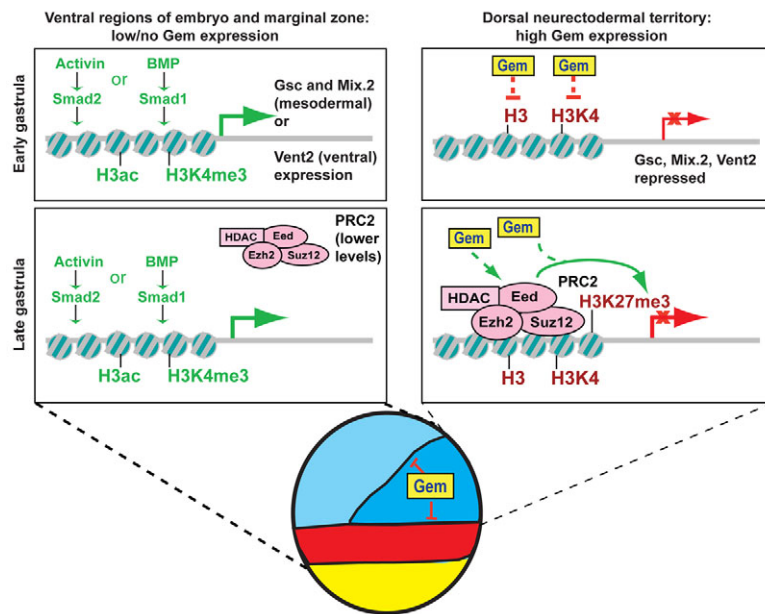


Fig. 8. Model for regulation of embryonic patterning by Geminin.

Right: Geminin is highly expressed in prospective neurectoderm in the gastrula embryo. Geminin blocks non-neural gene expression in this region by antagonizing histone modifications associated with transcriptional activation (H3ac/H3K4me3). In the late gastrula embryo, Geminin maintains non-neural gene repression in a Polycomb-dependent manner by facilitating Polycomb binding and/or activity. Left: Activin- and BMP-dependent target gene transactivation is not inhibited in ventral ectodermal and mesodermal regions of the embryo, where Geminin expression levels are low.

consistent with the ability of Geminin to repress mesodermal gene expression and demonstrates that this is manifested by failure to acquire a chromatin signature compatible with active gene transcription. Finally, although Smad2 was enriched at *Gsc*, Geminin overexpression did not alter Smad2 enrichment (Fig. 7B). By contrast, Smad2 enrichment was enhanced by Activin overexpression and suppressed by a dominant-interfering receptor (TALK4), showing antibody specificity (Fig. 7F). This suggests that, although Geminin can block Smad2-induced transcriptional responses (Fig. 5E), it does not do so by blocking Smad2 recruitment to enhancers. Together, these data demonstrate that Geminin enhances retention of Polycomb proteins and Polycomb-mediated repressive histone modifications, while antagonizing histone modifications associated with gene activation at mesodermal genes. These data are congruent with the dependence of Geminin on intact Polycomb repressor activity to repress mesodermal genes in vivo.

DISCUSSION

Geminin restrains cell commitment during embryogenesis

Balancing cellular controls that maintain pluripotency versus those that promote commitment provides the foundation for early embryonic patterning. However, molecular mechanisms that regulate this transition from pluripotency to multi-lineage commitment remain largely uncharacterized in vivo. Here, we defined Geminin as an essential regulator of this process. Geminin overexpression represses commitment to mesoderm, endoderm and epidermis, while promoting pluripotent and immature neurectodermal cell states. Geminin knockdown instead causes ectopic mesoderm, endoderm and non-neural ectoderm formation, disrupts normal boundaries between these tissues, and reduces expression of pluripotent and early neurectodermal markers and regulators. Geminin antagonizes transcriptional responses to three major signaling pathways that regulate cell commitment and embryonic patterning: Activin/Nodal, FGF and BMP. Conversely, Geminin knockdown enhances mesodermal induction by FGF and Activin in blastula and mid-gastrula cells. Although similar to Oct4 ortholog, Sox2 and SIP1 transcription factor activities, the effects of Geminin are functionally independent, instead requiring intact Polycomb repressor activity to

block mesoderm and endoderm formation. Congruent with this, Geminin enhances Polycomb binding and H3K27me3 modification, while antagonizing chromatin modifications associated with transcriptional activation at mesodermal genes.

Together, these data suggest a model for regulation of embryonic patterning by Geminin (Fig. 8). By early gastrulation, Geminin is highly expressed in presumptive neurectoderm and is required to block non-neural fate acquisition within this region. To achieve this, Geminin suppresses transcriptional responses to growth factor signaling that induce mesendoderm in the marginal zone (e.g. Activin/Nodal) and pattern the ventral and epidermal territory (e.g. BMP) (Fig. 8, right). This repression is manifested on chromatin as inhibition of histone H3K4 tri-methylation and H3 acetylation associated with active transcription. During gastrulation, Geminin continues to suppress mesendodermal and epidermal commitment to maintain and refine boundaries between these cell territories and presumptive neurectoderm. The maintenance of repression by Geminin depends on intact Polycomb activity, and Geminin enhances retention of Polycomb proteins and H3K27me3 methylation on chromatin. Ventral and marginal zone territories have low/no expression of Geminin (and Polycomb, by late gastrulation) and Activin- or BMP-dependent gene expression is not inhibited (Fig. 8, left).

Geminin regulates gene expression through both Polycomb-dependent and independent activities

Polycomb, like Geminin, induces neurectoderm and regionally restricts developmental gene expression in gastrula and post-gastrula *Xenopus* embryos (Akkers et al., 2009; Satijn et al., 2001). Likewise, in ESCs Polycomb loss renders cells differentiation-prone, suggesting a role in restraining multi-lineage commitment (Boyer et al., 2006). In *Xenopus*, Geminin and PRC2 have similar expression profiles, with *Suz12* and *Eed* expressed maternally and zygotically, while *Ezh2* expression begins at late blastula and all genes are enriched in presumptive neurectoderm (Aldiri and Vetter, 2009; Barnett et al., 2001).

Here, we found that Geminin requires Polycomb activity to restrain mesodermal gene expression and enhances Polycomb retention and H3K27me3 modification at mesodermal genes in vivo.

We also compared Geminin-regulated gene programs with H3K27me3-modified and Polycomb-bound genes in *Xenopus tropicalis* gastrulae and mouse ESCs (Akkers et al., 2009; Bernstein et al., 2006; Boyer et al., 2006). Geminin-downregulated genes are frequently enriched for Polycomb binding and H3K27me3 modification, relative to Geminin-upregulated genes (Fig. S9). For example, in ESC comparisons, Geminin-downregulated genes (stage 10.5) overlap with 38% (54/142) of H3K27me3-modified and 29% (55/190) of Polycomb-bound genes, while these constitute only 23% (8/35) and 11% (5/44) of Geminin-upregulated genes.

This Geminin-Polycomb functional cooperativity may involve direct protein interactions: Geminin can interact with the PRC1 protein Smh1 and represses Hox gene expression in the chick neural tube both through antagonistic interactions with Hox proteins and by exerting a 'polycomb-like activity' at Hox regulatory elements (Luo et al., 2004). Our data demonstrate that functional cooperativity between Geminin and Polycomb is also involved in early embryogenesis, and contributes to spatially restricting commitment-related gene expression to pattern gastrula embryos. Although Geminin depends upon intact Polycomb function to maintain repression at late gastrula, the initial transcriptional repression of mesendodermal genes appears to occur through a Polycomb-independent mechanism. By early gastrula, Geminin blocks enrichment of histone modifications associated with transcriptional activation at mesodermal genes, whereas Geminin knockdown enhances induction and ectopic expression of these genes. This activity therefore precedes H3K27me3 modification, which occurs from mid-gastrulation in *Xenopus tropicalis* (Akkers et al., 2009). In *Xenopus laevis*, we likewise find H3K27me3 and Ezh2 enrichment at *Gsc* by late, but not early, gastrulation.

What is the mechanism for the early gastrula activity of Geminin? One possibility would involve cooperativity with histone deacetylase (HDAC) activities, e.g. by promoting recruitment or activity of an HDAC-dependent co-repressor at these genes. Geminin can bind a repressor complex containing the co-repressor SMRT and associated HDAC3 (Kim et al., 2006). However, we found that Geminin still represses Brachyury when embryos are co-treated with the class I/II HDAC inhibitor Trichostatin A (TSA) from the two-cell stage (immediately after Geminin injection) to early gastrulation (see Fig. S10 in the supplementary material). Geminin also represses Activin-induced Brachyury expression in ectodermal explants in a TSA-independent manner (see Fig. S10 in the supplementary material). These data suggest that Geminin-mediated mesoderm repression does not depend on class I/II HDAC activities.

Transcriptional and epigenetic regulation of pluripotency and multi-lineage commitment

The activity of Geminin closely resembles that of Polycomb, Sox2 and the Oct4 orthologs, transcriptional and epigenetic regulators that restrain commitment at blastula-gastrula stages. Other molecules also restrain mesendodermal commitment at these stages, but their activities are distinct. For example, the Smad4 ubiquitin ligase Ectodermin regulates threshold responses to TGF β signaling (Dupont et al., 2005). However, Ectodermin does not also modulate transcriptional responses to non-TGF β signaling pathways, such as FGF. Ectodermin also regulates germ layer formation at blastula stages but its expression diminishes during gastrulation. By contrast, Geminin, the Oct4 orthologs, Sox2 and Polycomb restrain transcriptional responses downstream of multiple growth factor pathways throughout gastrulation. This establishes general transcriptional and epigenetic thresholds for commitment-related gene transactivation. Oct4/Sox2 and Polycomb play distinct roles in

ESCs: Oct4/Sox2 control a transcriptional network that transactivates pluripotency-regulatory genes and represses commitment/differentiation-promoting genes (Loh et al., 2008). Polycomb instead appears to be dispensable for pluripotency but represses commitment/differentiation-associated gene expression (Akkers et al., 2009; Boyer et al., 2006; Chamberlain et al., 2008). Polycomb might epigenetically buffer transcriptional 'noise', with competition between the chromatin structure and commitment-promoting transcription factors defining threshold responses for gene transactivation (Chi and Bernstein, 2009). Geminin depends on intact Polycomb function for repression while acting independently of Oct4 ortholog/Sox2 activities. Therefore, Geminin-Polycomb cooperation could facilitate a repressive chromatin state to prevent subthreshold transcriptional responses associated with multi-lineage commitment. Altering Geminin levels would then disrupt the balance between repressive versus permissive states for gene transactivation, perturbing spatial and temporal aspects of the transcriptional response to commitment-inducing signals.

Geminin-null mice are early embryonic lethal, as are mice lacking PRC2 proteins or Oct4, supporting essential roles in early embryogenesis (Faust et al., 1998; Gonzalez et al., 2006; Nichols et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004). The phenotype of Geminin-null mice resembles that of Oct4, as the inner cell mass fails to form, whereas all cells instead differentiate into extra-embryonic trophoderm (Gonzalez et al., 2006; Nichols et al., 1998). However, early lethality precluded assessing Geminin requirements for embryonic cell commitment. Here, we instead manipulated Geminin activity in blastula-gastrula stage *Xenopus* embryos to define distinct requirements for transcriptional control of embryonic development. This work demonstrated that Geminin is an essential regulator of the transition from pluripotency through early multi-lineage commitment, defined gene programs under the control of Geminin, placed its activity relative to growth factor signaling and cell intrinsic regulatory activities that act at these stages, and assessed the activities of Geminin at the chromatin level. Together, these data provide new insights into molecular events that govern the transition from pluripotency to commitment during embryonic development and define essential roles for Geminin and Geminin-Polycomb cooperativity in these processes.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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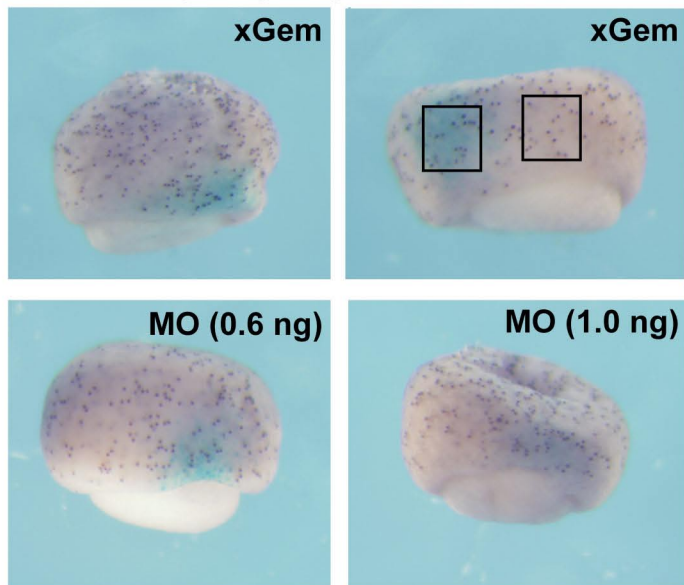
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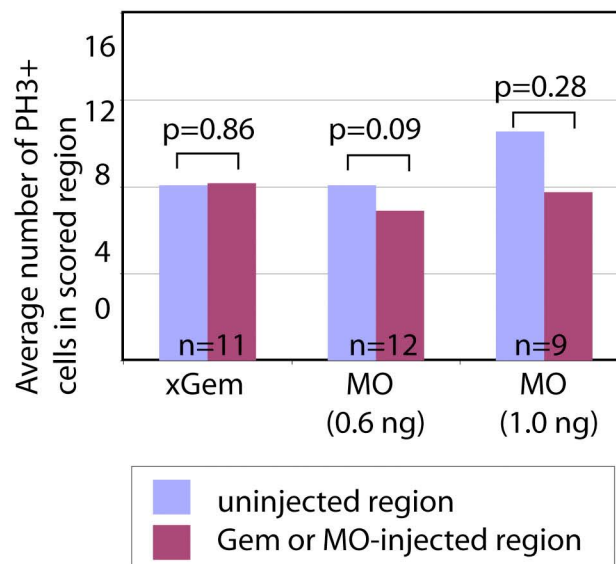
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A

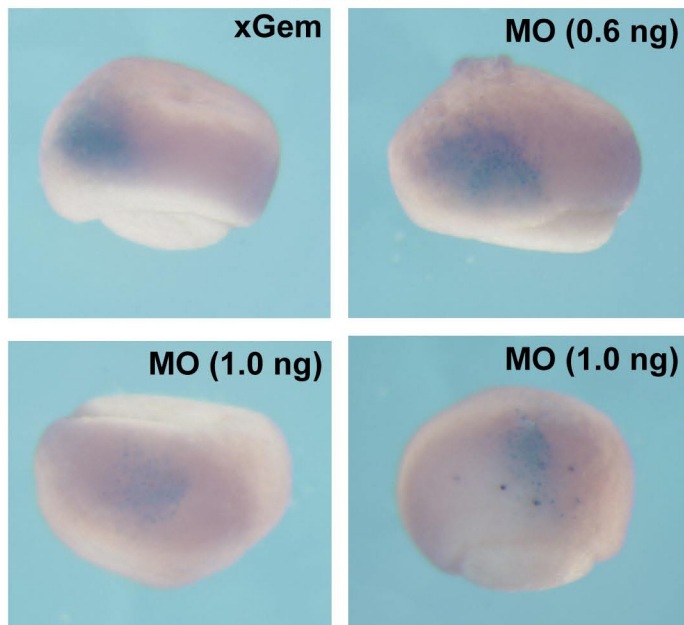
anti-phosphorylated histone H3

**B**

anti-phosphorylated histone H3

**C**

TUNEL

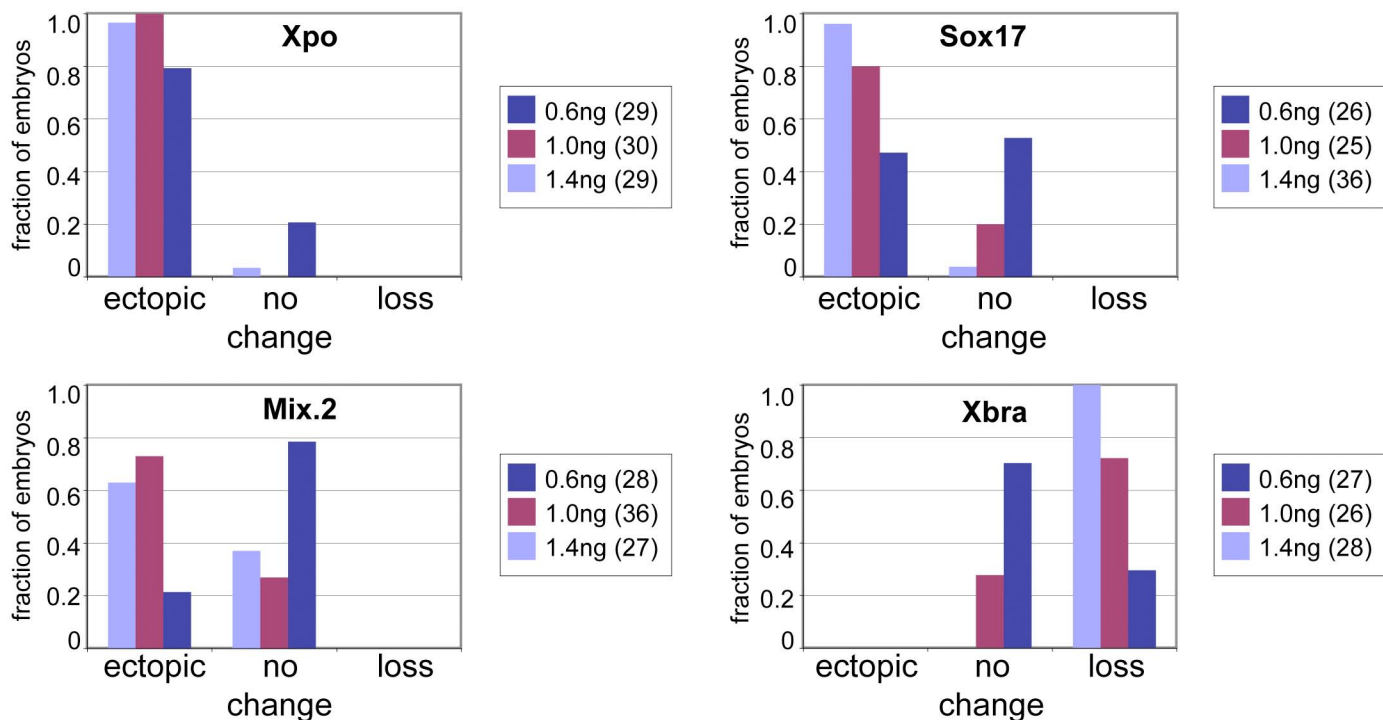
**D**

Sample	total TUNEL + cells	number of embryos scored	Average TUNEL + cells/embryo
xGem	2	14	0.14
xGemMO (0.6 ng)	6	15	0.40
xGemMO (1.0 ng)	9	10	0.90

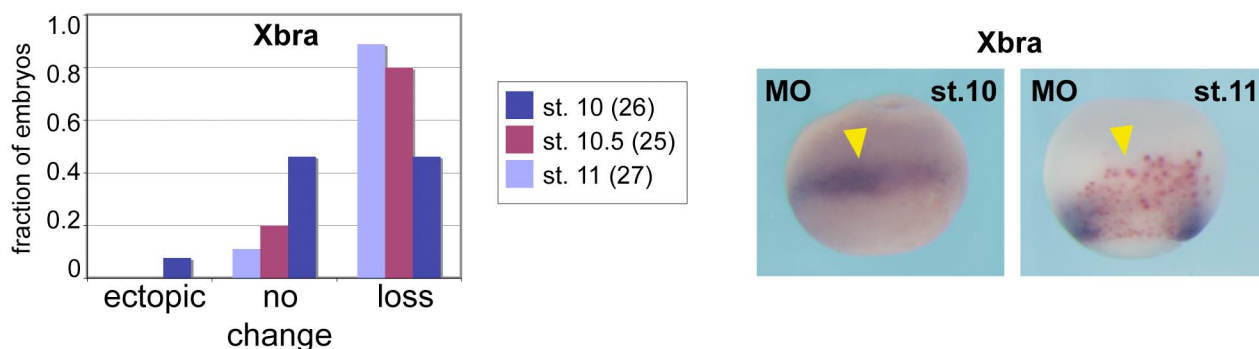
A Gene expression changes in embryos resulting from Gem over-expression or MO knockdown

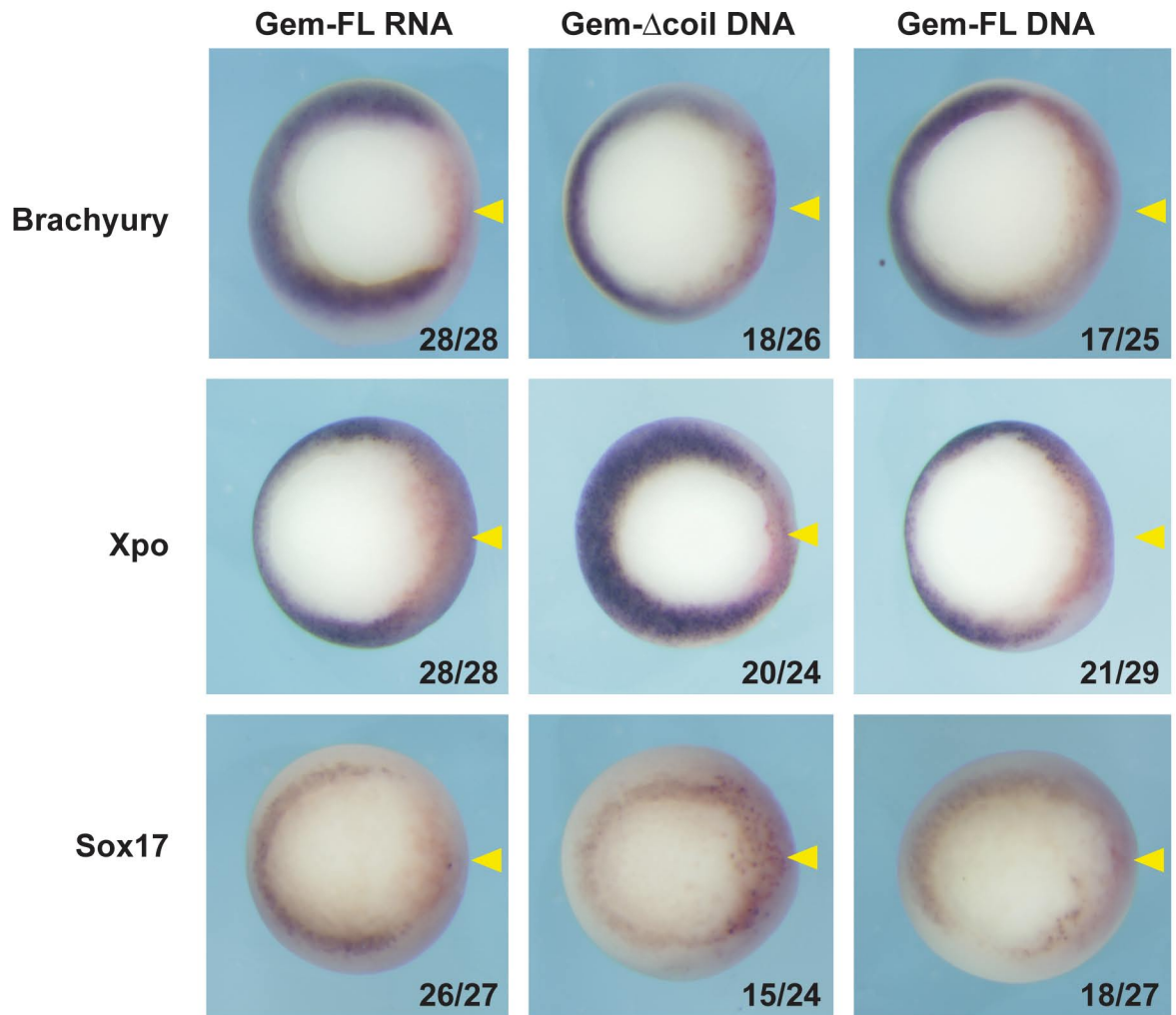
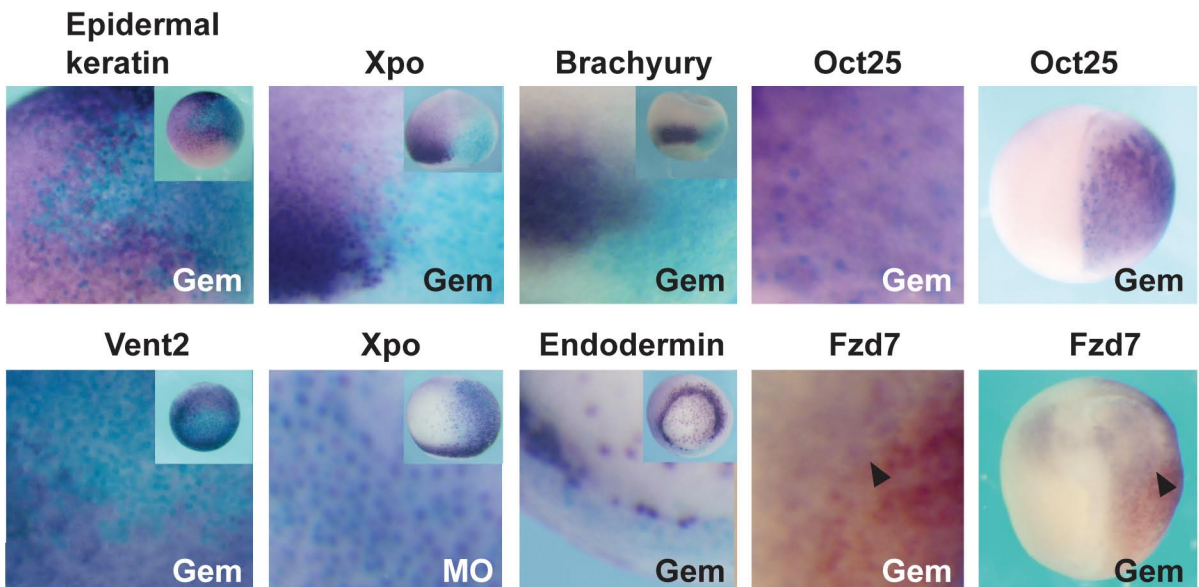
Sample	Marker	Loss of expression		No Change		Increased and/or ectopic expression		# of embryos
		#	%	#	%	#	%	
xGem	Xpo	41	98	1	2	0	0	42
GemMO	Xpo	0	0	6	13	40	87	46
xGem	Mix.2	38	90	4	10	0	0	42
GemMO	Mix.2	1	5	5	25	14	70	20
xGem	Brachyury	45	85	8	15	0	0	53
GemMO	Brachyury	38	88	5	12	0	0	43
xGem	Endodermin	36	86	6	14	0	0	42
GemMO	Endodermin	7	14	7	14	36	72	50
xGem	Sox17	29	81	7	19	0	0	36
GemMO	Sox17	0	0	8	23	27	77	35
xGem	xHex	21	100	0	0	0	0	21

B Gene expression changes in embryos resulting from Gem MO knockdown, dose curve



C Gene expression changes in embryos resulting from Gem MO knockdown, time course



A**B**

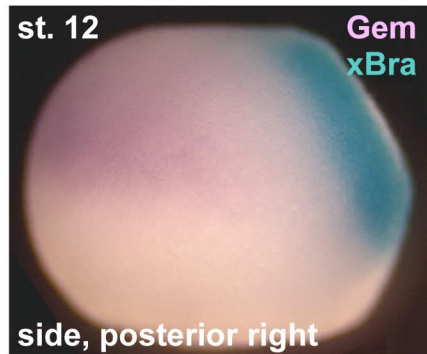
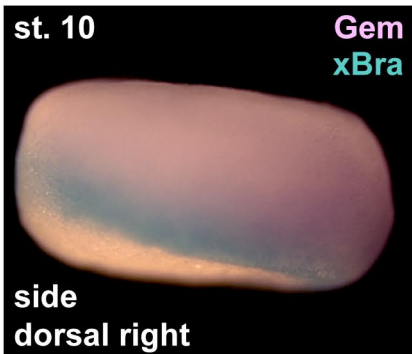
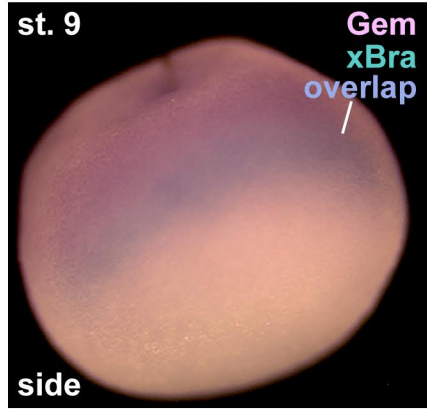
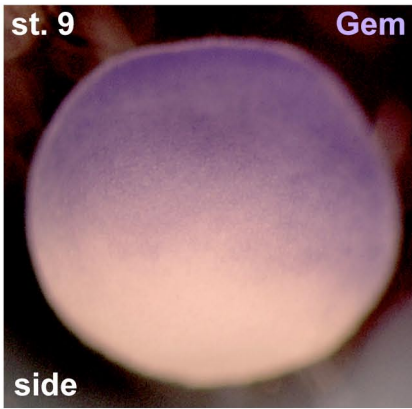
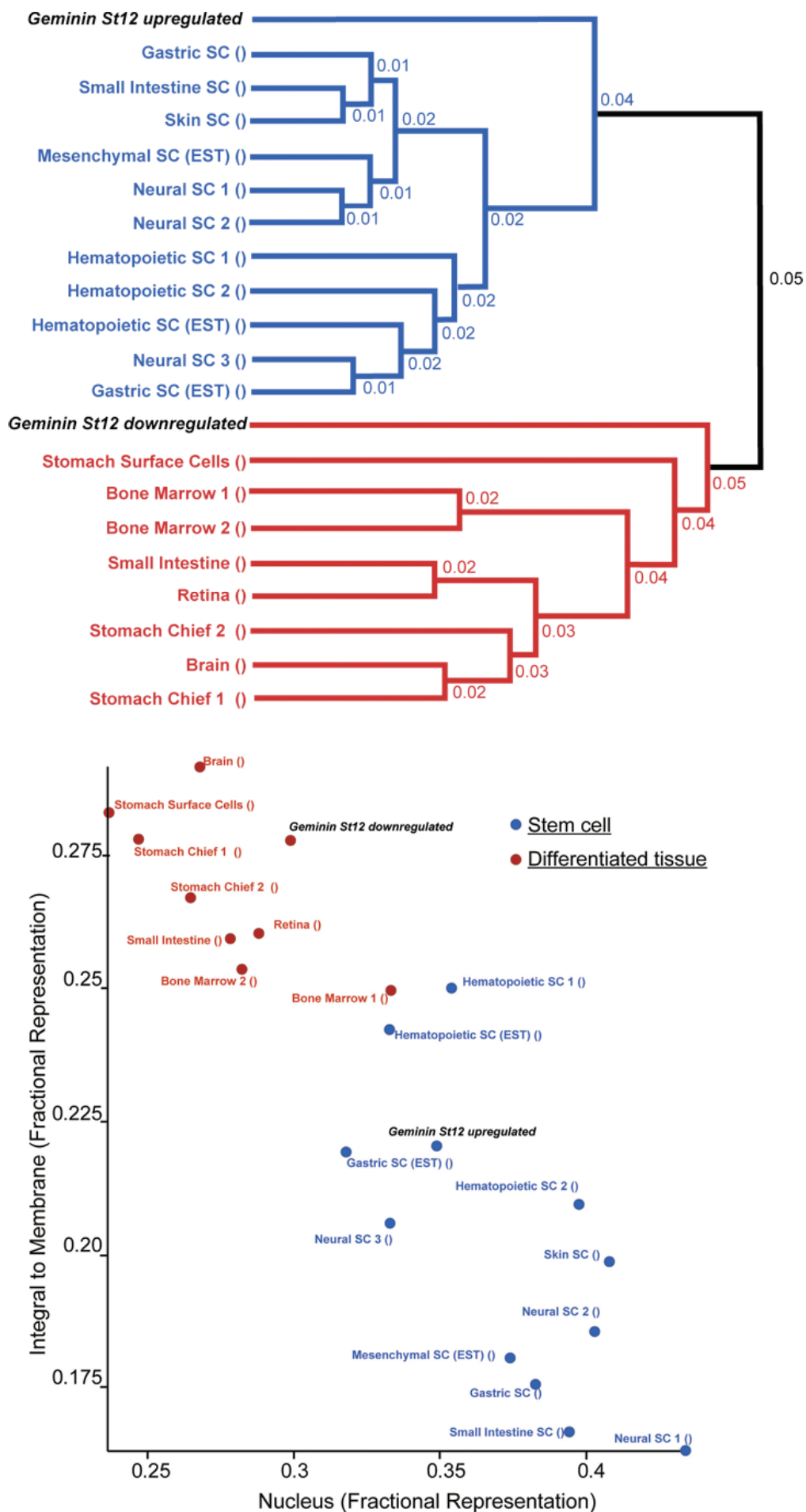
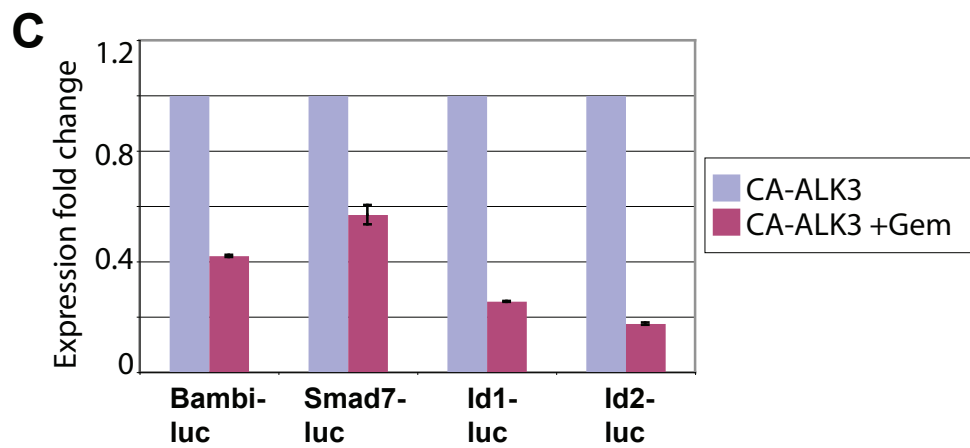
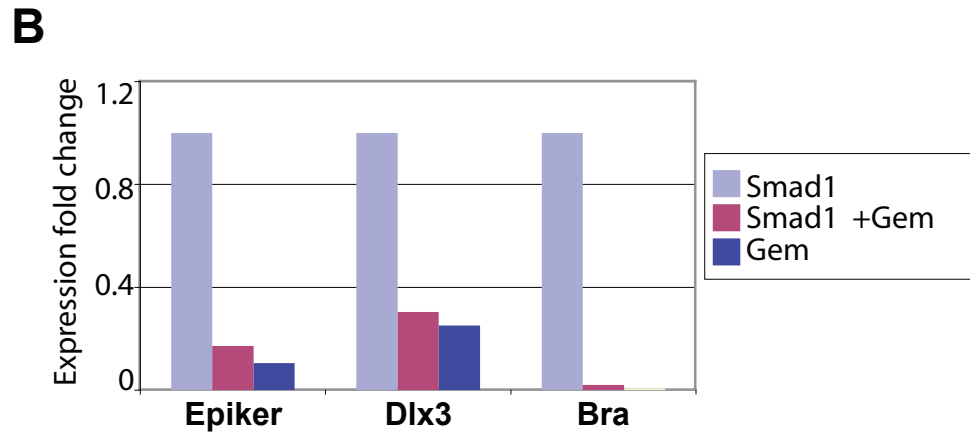
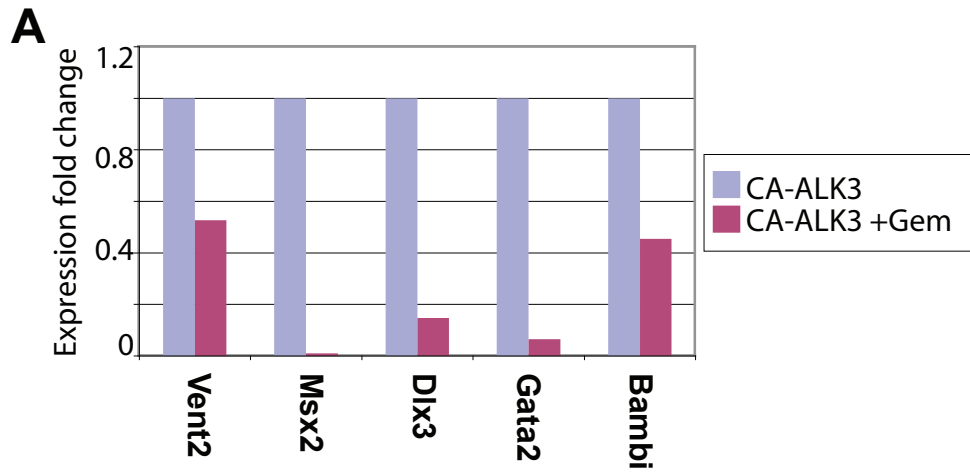
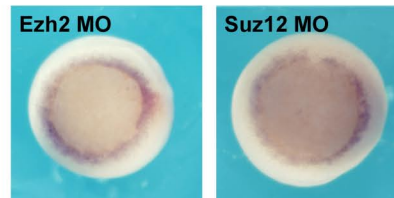
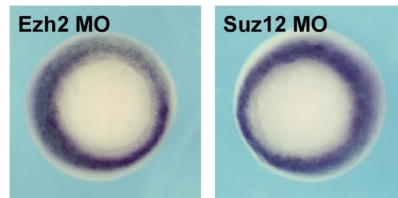
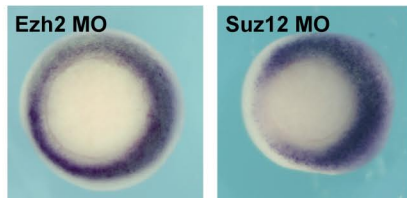
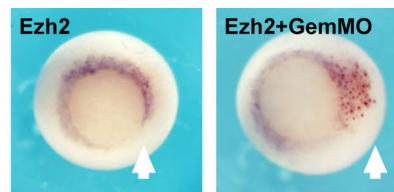
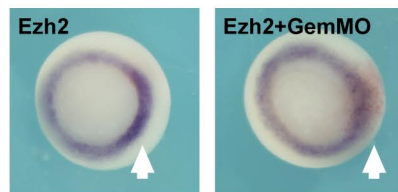
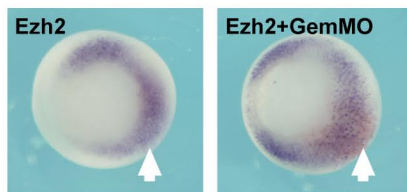
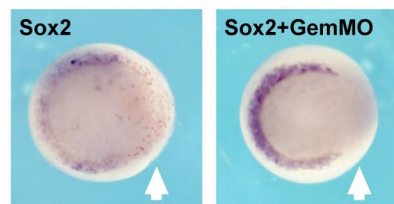
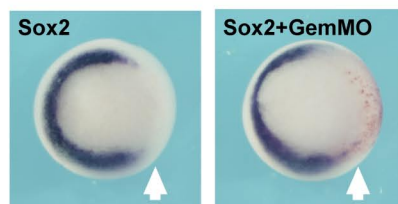
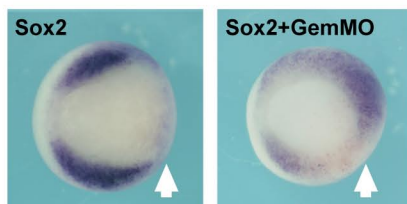
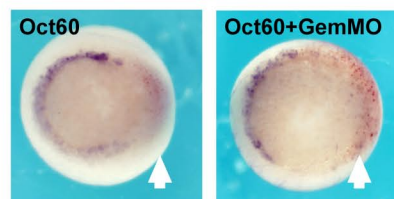
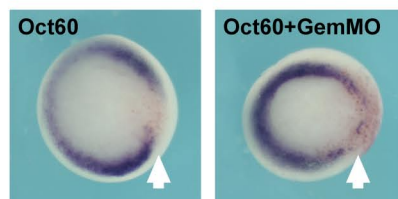
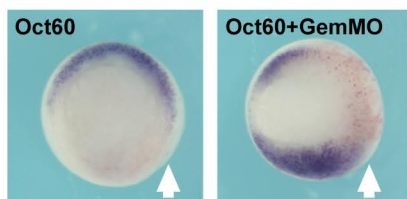
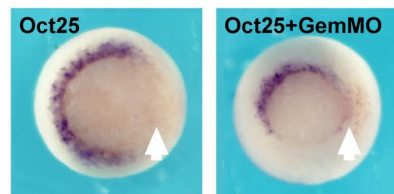
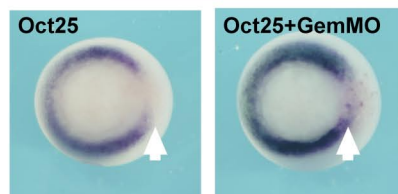
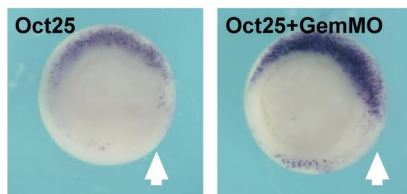
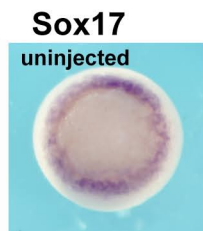
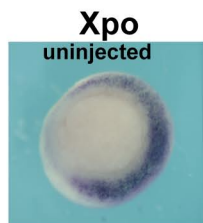


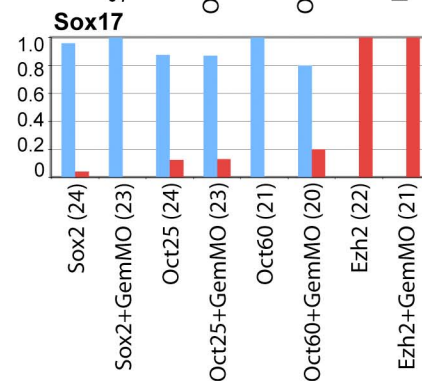
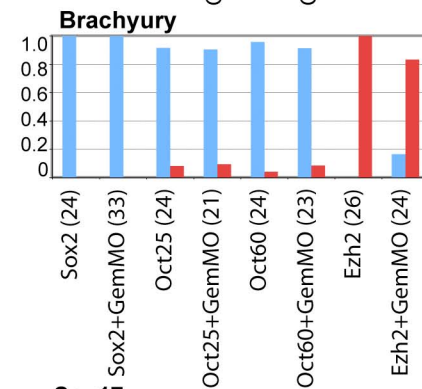
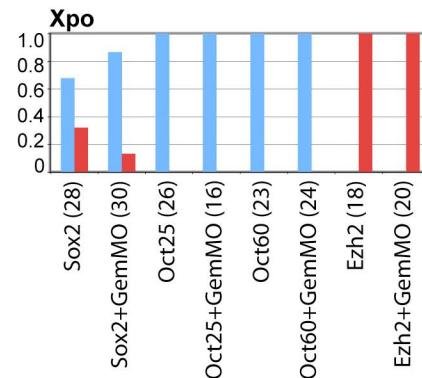
Fig. S5

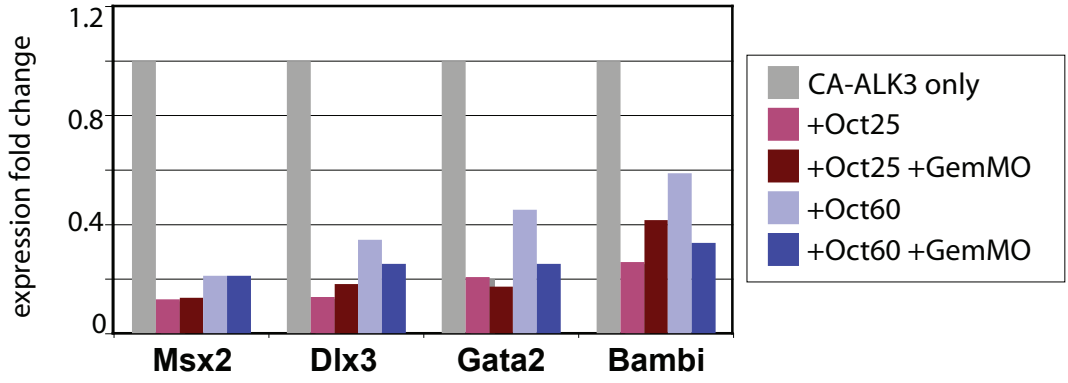
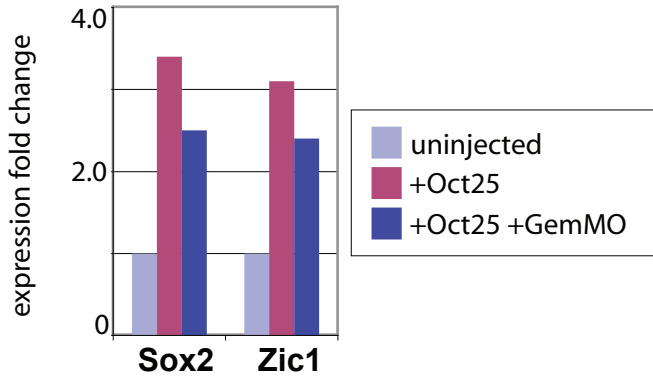
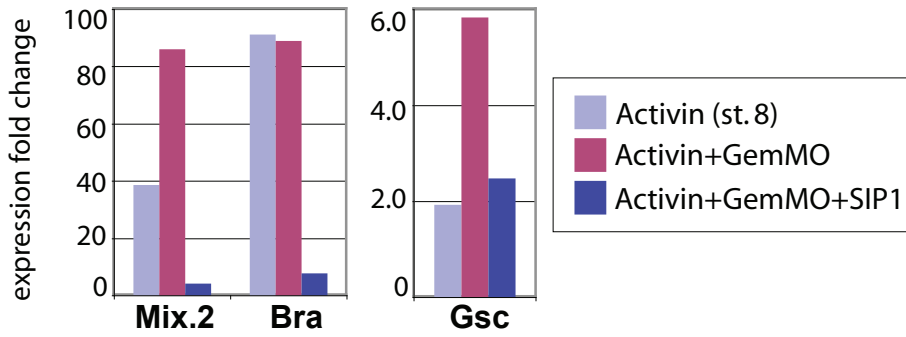
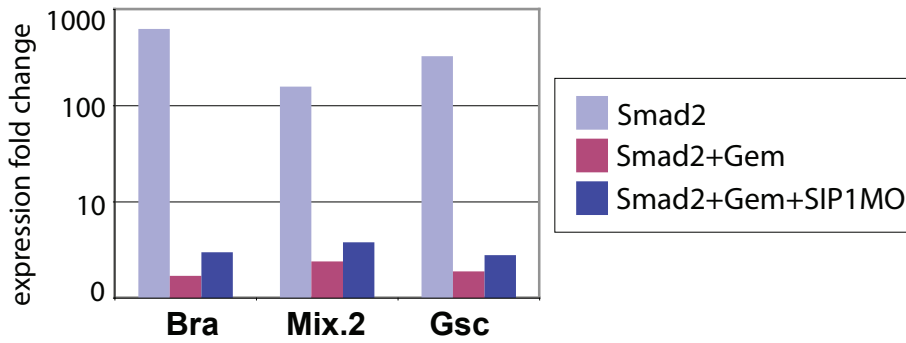
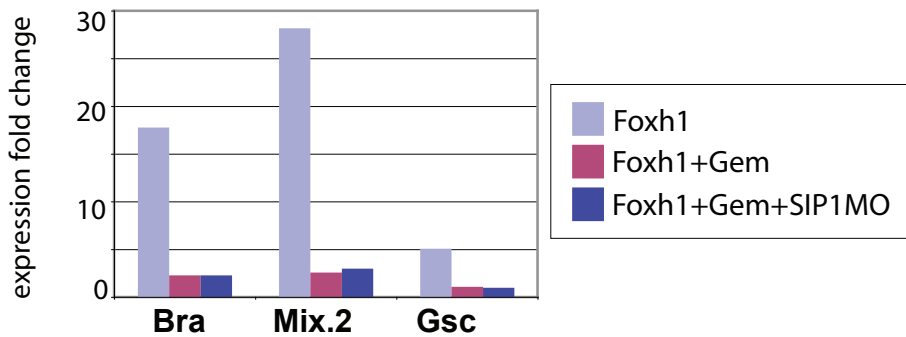


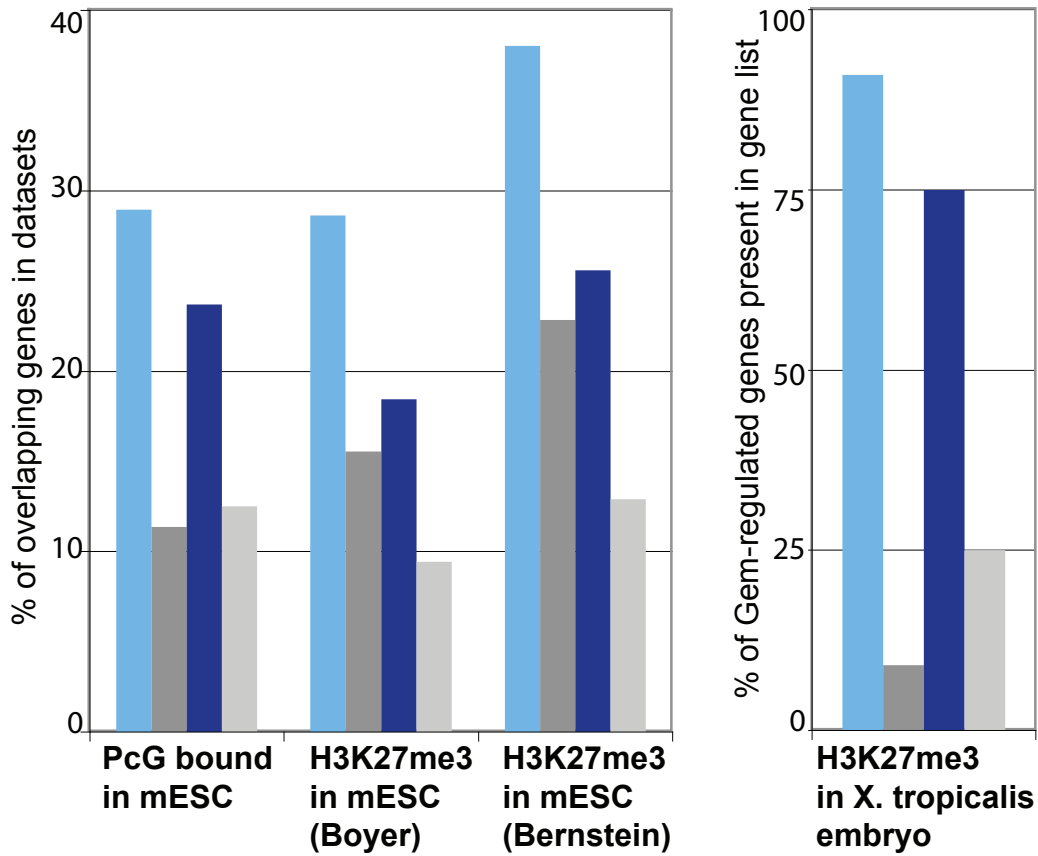




fraction of embryos with expression:
 loss/reduction
 unchanged

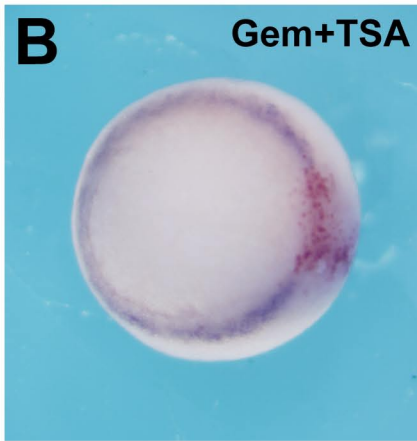
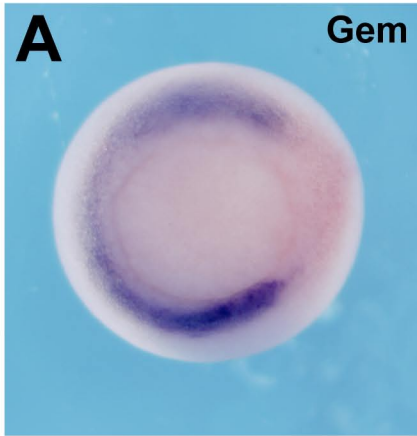


A**B****C****D****E**

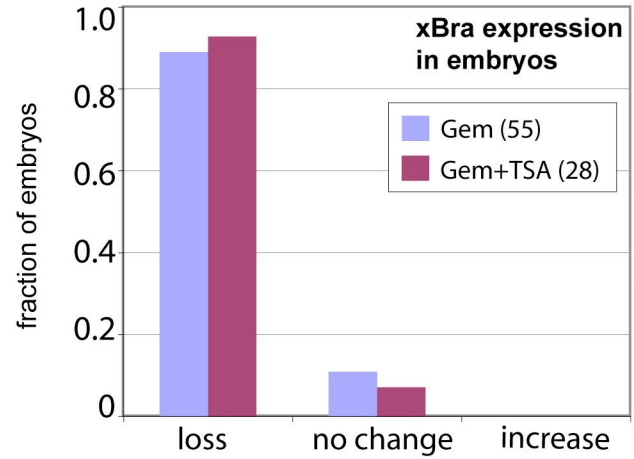


% overlap of Gem-regulated and PcG bound or H3K27me3 modified genes:





C



D

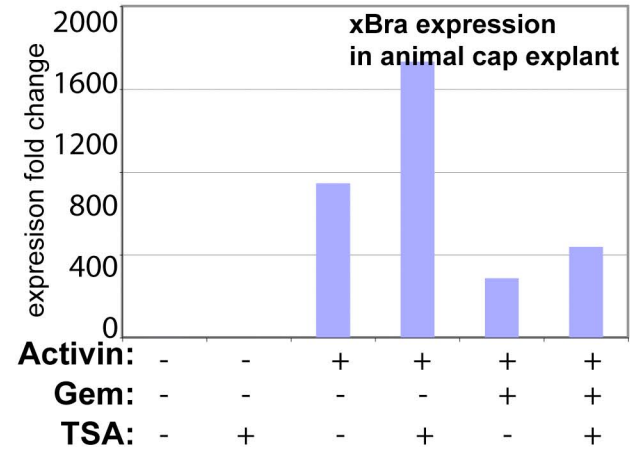


Table S1. Primers used

A Quantitative RT-PCR		
Gene	Primer name	Sequence
Ascl1/Xash1	Fxash1qRT	CCCCAACTATTCCCACGATA
Ascl1/Xash1	Rxash1qRT	TGCTACTCCGCATCTCAGAA
Bambi	F xBambi	AAAAGCTGCTGCCTGACCTGT
Bambi	R xBambi	CTGTACATCCCCCAATGGAC
Bmp4	xBmp4 F	ACCCATAGCTGCAAATGGAC
Bmp4	xBmp4 R	CATGCTTCCCCTGATGAGTT
Bmp7	xBmp7 F	TGAGTTCTTTCCGCACA
Bmp7	xBmp7 R	CAGTCTTCTCCAGCATTATT
Brachyury	Xbra F	AGCTCACCAACGAGATGATCG
Brachyury	Xbra R	AACCGTATACATTGCATTGGGAT
Brat	xBrat F	GAGAACTGCTGTCCGGGAAT
Brat	xBrat R	GGCAGGTAGAGGGAGTCTTG
Btg1	xBtg1 F	GCCAACATGAAGCCAGAGA
Btg1	xBtg1 R	TTATAGTGATCGGCCAGCAT
Ccnd1	xCcnd1 F	TGATCTCCTTCTGGCAACAT
Ccnd1	xCcnd1 R	GTGTCCACTTCGAGCATAG
Chd	Fx Chd3	ATGCCTATGGGCTCTTTCCT
Chd	Rx Chd3	CCAAGCAAATCCTTCTCTCG
Cxcr4	Fx Cxcr4	ACTGCATTTGGGAGAACACC
Cxcr4	Rx Cxcr4	CTGAGGTGAATGCGTTCTGA
Dlx3	Fx Dlx3	GGTTCAGCGAAATGTTCCAT
Dlx3	Rx Dlx3	ATGGAGTGGCACTGGATTTC
Dlx5	F xDlx5	GCGCTGAATGCGTATCAGTA
Dlx5	R xDlx5	AGGGCTCCCATAGCCATAGT
Dlx6	F xDlx6	ATCCGGTTTAAATGGGAAAGG
Dlx6	R xDlx6	AGTGCCAGGTAAGGGTTTG
Dullard	xDullard F	CTGGAGCTTCGTCTCTACC
Dullard	xDullard R	TTCCGTTTCACTGACTGAG
Dusp1	xDusp1 F	GGTCGTTCTTCTCTTCAGC
Dusp1	xDusp1 R	CACCTCTGCTCCTCATTGG
Ef1a	xEf1a F	CACCATGAAGCCCTACTGA
Ef1a	xEf1a R	ACCTGTGCGGTAAAAGAACC
Efna1	Fx Efna1	TGAGTGGAGTGTTGGAGCTG
Efna1	Rx Efna1	ACCGTGAATCCTCCTGCAC
Elf1	xElf1 F	CGCCTGAAAGCAAACAGAC
Elf1	xElf1 R	GGAGAAGGAAACCCGTTAGG
Epiker	FxEpiKerqRT	CACCAGAACACAGAGTAC
Epiker	RxEpiKerqRT	CAACCTTCCCATCAACCA
Etv5/Xer81	xER81/ETV5 F	CGAGCCGTGCAATTCTTT
Etv5/Xer81	xER81/ETV5 R	CCTTGTGGAGGAAACTGAATG
Foxd5a	xFoxD5a F	CCAATCTGTGACCCTCCACT
Foxd5a	xFoxD5a R	CTTCCCGGACAACCTTGTA
Foxi1	F xFoxi1	TCCTCAGCGACCTTCAAAC
Foxi1	R xFoxi1	TCCTGGCAGATATGGAGGAG
Fzd10	Fx Fzd10b	GACGCAAGACAAGTGCAAAA
Fzd10	Rx Fzd10b	CTGCTGGGATAGAGCTGGTC
Fzd7	Fx Fzd7	AAACACACCTTGTGCCTTCC
Fzd7	Rx Fzd7	GGCCCATTTAAAGCAAAACA
Gadd45a	xGadd45a F	AGTCAGCAAGCGAAGAACAC
Gadd45a	xGadd45a R	AGGTTGCACTCCTCTGCAC
Gnb1	Fx Gnb1	GCCGGTTTCTTGATGACAAT
Gnb1	Rx Gnb1	GTCTTCTGCTGTCCCCTCTC
Goosecoid	xGoosecoid F	TTCACCGATGAACAACCTGGA
Goosecoid	xGoosecoid R	TTCCACTTTTGGGCATTTTC
Gos2	Fx G0S2	CCGATATGGAAACCATCCAC
Gos2	Rx G0S2	AATGACCACTCCGAACAAGG
GPR143	Fx Gpr143	TCCAATGCAAGGCTTTCTCT
GPR143	Rx Gpr143	CCATTGTGTGACGCTTCAGT
Grhl1	xGrhl1 F	AAGACGGTGCCTTTAAACAT
Grhl1	xGrhl1 R	CTCTGGGATTCGGTTTTGAT
Grhl3	xGrhl3 F	GGCACGAGACCTCAGTTTTAC
Grhl3	xGrhl3 R	CAATCATCTGTGACCCTCCTC
Hist2h2aa	xHist2h2aa F	GCCAGGGACAACAAGAAACT
Hist2h2aa	xHist2h2aa R	CAGCAGAGCAGATTGGATGT
Id2	xId2 F	GCATCGTCAGTCTCCATCAT

Id2	xld2 R	GGATTTGCTCTCGTCTGTGA
Id3	Fx Id3	GTTATTTGCCACCCCATCTG
Id3	Rx Id3	TTACTAGCCAAGCCCCACAC
Kitlg	Fx Kitlg	CTTGTACCATGCCTGCAGAA
Kitlg	Rx Kitlg	GACCTGGCAGAGGAATCAAA
Klf2	F xKlf2	GCTGGTTGTGGGAAGACCTA
Klf2	R xKlf2	GTTATCATGATCGGGCAAAT
Ldb1	Fx ldb1	TTGGAGGATGGACCAAAGAG
Ldb1	Rx ldb1	CTTGGGGTGCTTCAAGACAT
Lhx5	Fx Lhx5	GTGCAACCTGACCGAAAAAT
Lhx5	Rx Lhx5	CTCGCTTTCCTGACCAAGTC
Lmo4	xLMO4 F	TCATCCCAGCTTCTTCTTT
Lmo4	xLMO4 R	GATCAACTCGGCGCTATTCT
Map2k7	xMap2k7-A F	GAGCAGAAACTCTCCCGTCT
Map2k7	xMap2k7-A R	GAATGTCCATCATTGGCAAG
MGC81002	Fx MGC81002	GAACGTGGAGACCTCAGAGC
MGC81002	Rx MGC81002	AAGAAACGAGGCCACTGAGA
Mix.2	Fx Mix.2	GAA GGA CTT CCA GCA GCA TC
Mix.2	Rx Mix.2	GCG TCT TTG GGT CTG ACA TT
Mos	Fx mosxe	TGGGGCAATTCATACCAAGT
Mos	Rx mosxe	GGCCATTACACTTCTGAT
Msx1	FxMsx1qRT	TTATGCAACTGCCAGAGGAG
Msx1	RxMsx1qRT	GGGCTTTCTGTTGGTTTTGT
Msx2	xMsx2 F	TCATTTAGGGATCAGGGACAGC
Msx2	xMsx2 R	TGGTGCCATCTTCGGAGTTTTT
Myo10	xMyo10 F	GTGAAGAAGCGTTGCAGTGT
Myo10	xMyo10 R	CTCGATGAAGGATGATGACG
Oct1/Pou2f1	xOct-1 F	AACTACCTCAGCAAAGCCAAG
Oct1/Pou2f1	xOct-1 R	GTACAGGGGTGGCAGCTATT
Oct25/Pou5f1	xOct-25 F	CCTTCACCTTTTGTTTTTA
Oct25/Pou5f1	xOct-25 R	TTTTTGGGTTTGGGAATG
Oct60/Pou5f1	xOct-60 F	ACTACAATGCCCGTCCTACCC
Oct60/Pou5f1	xOct-60 R	ACTCCCCGGCGTTTTCTCCT
Oct91/Pou5f1	xOCT-91 F	ATGAAGGGCAGTTATGGTA
Oct91/Pou5f1	xOCT-91 R	CAGTGGGGGTGGTGCTCA
Otx1	Fx Otx1	CTTGGTACAGCCCTGGGATA
Otx1	Rx Otx1	GCCAGAAGCTACACGAGTCC
Pax6	Fx Pax6	CCAATCAGCCTAGGATTCCA
Pax6	Rx Pax6	CGCTAAGATCCTGTGTGCAA
Pcgf2	xPcgf2 F	CCTGCAAGAGGTTGAGGTTT
Pcgf2	xPcgf2 R	CTGGGGAGAGAGGATGATGT
Per1	xPer1-A F	GGGCTTCTTCTCTCTCTCT
Per1	xPer1-A R	GAACTTGGGCTGGTGTCTCT
Rab27A	Fx Rab27a	TCCGATGGGGATTACGATTA
Rab27A	Rx Rab27a	TCAATGCCACTGTTGTGAT
Sall1	F xSall1	TGAACAAATCCGTACCAAA
Sall1	R xSall1	GGGACAAATGGGAACCTAGA
Sizzled/Szl	xSz1 F	GGCAGAACCTCCTACAAACC
Sizzled/Szl	xSz1 R	GAACGTGTCCAGGCAGACT
Smad7	Fx Smad7	GGCGACTTTAGAGCGACATC
Smad7	Rx Smad7	TAGCAACAGCATCAGCATCC
Sox11	xSox11 F	GGCAGAAGGCACATCATAC
Sox11	xSox11 R	CATTAGGAAAACAGGCCAAAAG
Sox2	Xsox2 F	CCAGTCCACCTGTAGTCACCTCT
Sox2	Xsox2 R	CACTTCTGCCCCAGGTAGGTAC
Sox7	F xSox7	CAGAGAGCCTGGACTGTTCC
Sox7	R xSox7	GTCTTCGGATACGGGTCTCA
Suv39h1	xSuv39h1 F	GCAACAATCTCCTGAAGCAA
Suv39h1	xSuv39h1 R	TAGTGGGAAAGGCTCTGGTC
Szl	xSz1 F	GGCAGAACCTCCTACAAACC
Szl	xSz1 R	GAACGTGTCCAGGCAGACT
Tbx3	F xTbx3	GATCCCAGAGTCTCCATCCA
Tbx3	R xTbx3	CTGACACAGCCAGGAGTTCA
Tbx6/Brat	xBrat	GAGAAACTGGTGTGCGGAAT
Tbx6/Brat	xBrat	GGCAGGTAGAGGGAGTCTTG
Tead1	Fx Tead1	CGTTTCTTTACCGAATCCA
Tead1	Rx Tead1	GTGTCCCGTTTGTAAACCAC
Tiarin	xTiarin F	GGAACCTGGCAAACATGGAG
Tiarin	xTiarin R	TGCAGAGGAGCTACCCAAT
Tmem45b	xTmem45b	AGTGGGACCAGACTGACCAC

Tmem45b	xTmem45b	TGCCAGACAACCTTCTGTGC
Vdac1	Fx Vdac1	TGGTGTCCGCAACATAGAA
Vdac1	Rx Vdac1	TGGGTGTTTGCATACCAGA
Vent1	xVent1 F	TGGTTCAACAGGGATTCTTC
Vent1	xVent1 R	CTGCTAAGGAAGGATTTGC
Vent2	FxVent2qRT	GCTACACAGGGACACAACCTC
Vent2	RxVent2qRT	GCCTGAGTCAGTGCTAGTGC
Xer81/Etv5	XER81/ETV5 F	CGAGCCGTGCAATTCTTT
Xer81/Etv5	XER81/ETV5 R	CCTTGTGGAGGAAACTGAATG
Xhel	F xXhel	CACATTCTGTGGACCCCTCT
Xhel	R xXhel	AGCCGTTCTTCTGCACTGAT
Xpo	xXpo F	GTGCAGGAGATCAAAAAGAGAG
Xpo	xXpo R	CAATGTGAGACCAACTGCTGTA
Zic1	xZic1 F	TCCGTTACATGAGGCAGCC
Zic1	xZic1 R	TTGTTGCACGACTTTTGGG
Zic2	Fx Zic2	TCAACAACCCCAAGAAAAGC
Zic2	Rx Zic2	ACACTCCTCCAGAAGCAGA
Zic3	xZic3 F	CCTGTGTGCACCCTGATATG
Zic3	xZic3 R	TACATGAGGCAACCCATCAA

B Chromatin immunoprecipitation

Gene	Primer name	Sequence
Goosecoid	Fx Gsc-1	CAGGGAGAGTCTGCGATTTT
Goosecoid	Rx Gsc-1	GGATGGGGTGAGAGGAGACT
Goosecoid	Fx Gsc-2	TAATGTCCCATCACGCTCAA
Goosecoid	Rx Gsc-2	AAGCACAGCAGCTCCACTCT
Goosecoid	Fx Gsc-3	AGAGTGGAGCTGCTGTGCTT
Goosecoid	Rx Gsc-3	CGACTTTGTGTGTGCGTCTC
Brachyury	Fx Bra-1	GGGTGACACACCACTTCCTC
Brachyury	Rx Bra-1	GAGCACAGAAACCCAGAGA
Brachyury	Fx Bra-2	TCTCTGGGGTTTCTGTGCTC
Brachyury	Rx Bra-2	AGGCAGCTCATTGGTCAGTT
Brachyury	Fx Bra-3	CACAGCTTGTCCAGGCCTAT
Brachyury	Rx Bra-3	TGCGATTGATCAGCAGGTAG

Each primer set was designed using the Primer 3 program and parameters recommended for quantitative PCR (qPCR). Some primer sequences were also analyzed using the NCBI primer-BLAST program to assure that they only recognized the intended gene and not related genes. PCR products were run on a 2% agarose gel and tested by melting curve to ensure that they produced a single product of the expected size.

Table S2. Confirmation of Geminin down- and upregulated genes from microarrays: expression changes in embryos after Geminin overexpression or morpholino (MO) knockdown**A Effect of Geminin overexpression and knockdown on downregulated genes from microarrays**

Sample	Marker	Loss of expression		No change		Increased and/or ectopic expression		Number of embryos
		Number	Percentage	Number	Percentage	Number	Percentage	
xGem	Sall1	38	95	2	5	0	0	40
GemMO	Sall1	6	12	6	12	39	76	51
xGem	Szl	37	100	0	0	0	0	37
GemMO	Szl	0	0	5	19	21	81	26
xGem	Vent1	20	100	0	0	0	0	20
GemMO	Vent1	0	0	3	13	20	87	23
xGem	Vent2	13	87	2	13	0	0	15
GemMO	Vent2	0	0	5	17	24	83	29
xGem	Zic1	37	88	5	12	0	0	42
GemMO	Zic1	0	0	10	22	36	78	46
xGem	Zic3	29	83	6	17	0	0	35
GemMO	Zic3	3	7	20	48	19	45	42
xGem	Foxi1	13	100	0	0	0	0	13
GemMO	Foxi1	0	0	0	0	12	100	12
xGem	Grhl1	21	100	0	0	0	0	21
GemMO	Grhl1	4	14	0	0	24	86	28

B Effect of Geminin overexpression and knockdown on upregulated genes from microarrays

Sample	Marker	Loss of expression		No change		Increased and/or ectopic expression		Number of embryos
		Number	Percentage	Number	Percentage	Number	Percentage	
xGem	Fzd7	15	29	7	13	30	58	52
GemMO	Fzd7	26	49	27	51	0	0	53
xGem	H1F00	0	0	20	41	29	59	49
GemMO	H1F00	19	70	7	26	1	4	27
xGem	Oct25	0	0	16	20	64	80	80
GemMO	Oct25	55	85	10	15	0	0	65
xGem	Sox2	7	27	0	0	19	73	26
GemMO	Sox2	25	66	12	32	1	3	38
xGem	Sox3	4	13	0	0	28	88	32
GemMO	Sox3	18	56	13	41	1	3	32
xGem	Sox11	4	13	2	6	25	81	31
GemMO	Sox11	24	86	4	14	0	0	28
xGem	Zic2	0	0	0	0	53	100	53
GemMO	Zic2	12	26	35	74	0	0	47

Geminin Δ coil mRNA or MO were co-injected with β -galactosidase lineage tracer mRNA. After lineage labeling and in situ hybridization, embryos were selected for scoring only if the lineage-labeled region overlapped the marker gene. For each gene analyzed, numbers represent additive data from two to six experiments.