Development 138, 33-44 (2011) doi:10.1242/dev.059824
© 2011. Published by The Company of Biologists Ltd

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

Jong-Won Lim[1], Pamela Hummert[1], Jason C. Mills[2] and Kristen L. Kroll[1,*]

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-involuted 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 tri-methylation 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

[1]Departments of Developmental Biology, Washington University School of Medicine, 660 South Euclid Avenue, Saint Louis, MO 63110, USA. [2]Pathology and Immunology, Washington University School of Medicine, 660 South Euclid Avenue, Saint Louis, MO 63110, USA.

*Author for correspondence (kkroll@wustl.edu)

Accepted 19 October 2010

DEVELOPMENT

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 Gmnn) 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-xGemininΔcoil, CS2MT-xGeminin-full-length/FL (Seo et al., 2005), CS2NLS-βGal, CS2-Sox2, SP64T-Activinβb (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-VP16/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). xGemininΔcoil 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× MMR/25°C. When sibling embryos reached stage 10.5 or 12, total RNA (20 μg/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, GemininΔcoil RNA (300 pg), full-length Geminin (30 pg), Geminin antisense morpholino (MO; 2.5 ng; 5′-ATCTCTGCTTCTTGTTGGTATTCAT) (Seo et al., 2005), or CMV-promoter plasmids encoding full-length or GemininΔcoil (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 μg) was used per 20 μl 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 ΔΔCt method (Stappenbeck et al., 2002) with normalization to EF1α. 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 GemininΔcoil/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-ATTTCTTCCCCGTCTGGCCCAT, Suz12MO-5′-CCATGCGGGATA-CTACGAGTGATAA 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 μl reporter lysis buffer (Promega) were

DEVELOPMENT

measured using Luciferase and β-Galatosidase 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 ±s.e.m.).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation in *Xenopus* early embryos was modified from published methods (Blythe et al., 2009). GemininΔcoil (120 pg), tAlk4 (120 pg), Activinβb (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 ~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 µl 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 µl; 100 µl 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 [$\Delta C(t)$=ChIP $C(t)$ – 1% input $C(t)$]. The percentage input for each sample was then calculated using the formula [%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 GemininΔcoil, 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 GemininΔcoil suppress mesendodermal gene expression when overexpressed from early embryogenesis as injected mRNAs (Fig. 1; see Fig. S3A in the supplementary material). These variants also suppress mesendodermal 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 stages is sufficient to suppress mesendodermal fate acquisition. The repression of mesendodermal 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

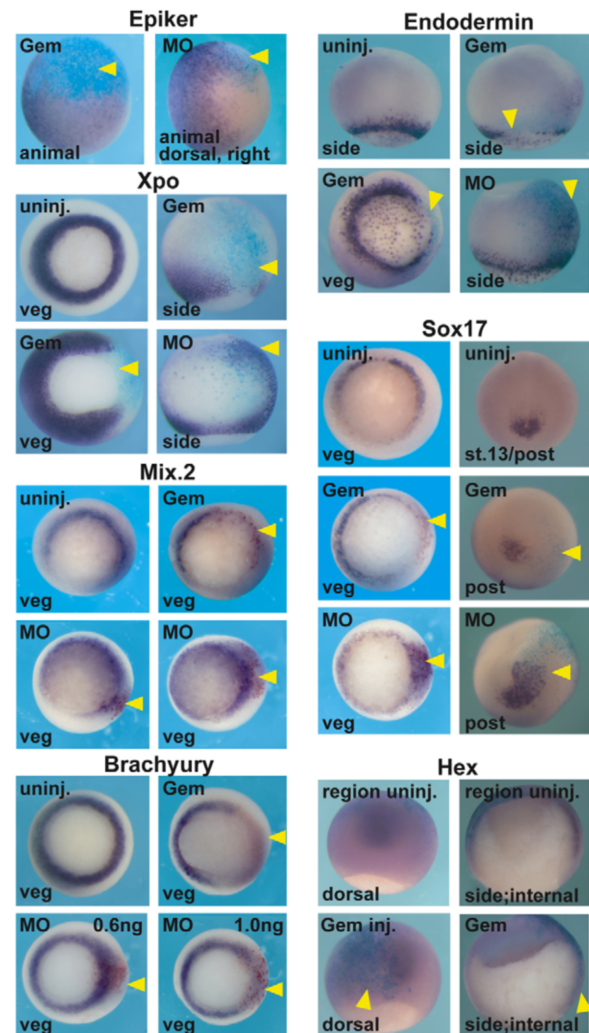

Fig. 1. Geminin regulates epidermal, mesodermal and endodermal gene expression. Geminin RNA or morpholino/MO-injected regions are oriented rightwards, indicated by yellow arrowheads, and lineage is labeled by β-galactosidase RNA co-injection and Red-gal (Mix.2, Brachyury/Xbra, and Sox17 at stage 11) 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).

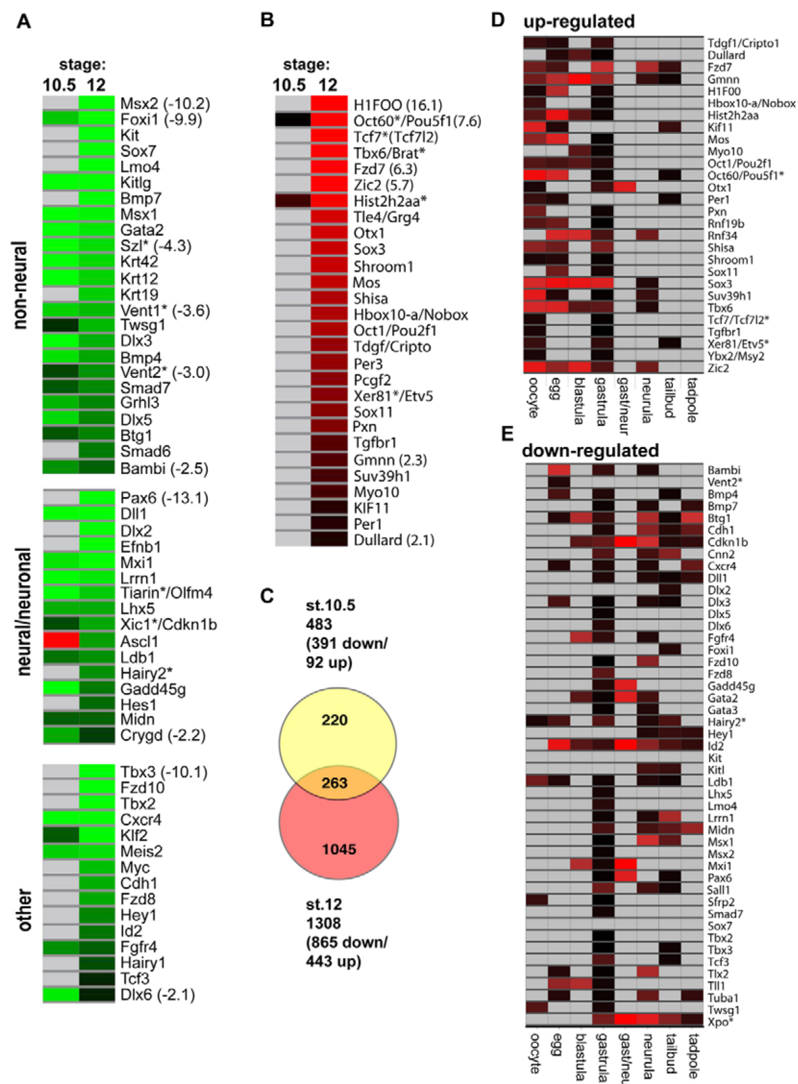

Fig. 2. Geminin-regulated gene programs in gastrula embryos. (**A**,**B**) Heat maps of selected genes from microarrays whose expression (A) decreased or (B) increased upon Geminin overexpression, categorized by expression/function. Genes not present in dataset are gray. Some fold changes are in parentheses. 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 GemininΔcoil in embryos by mRNA injection at the two-cell stage, isolated ectodermal (animal cap) explants from blastulae and cultured these until either early

DEVELOPMENT

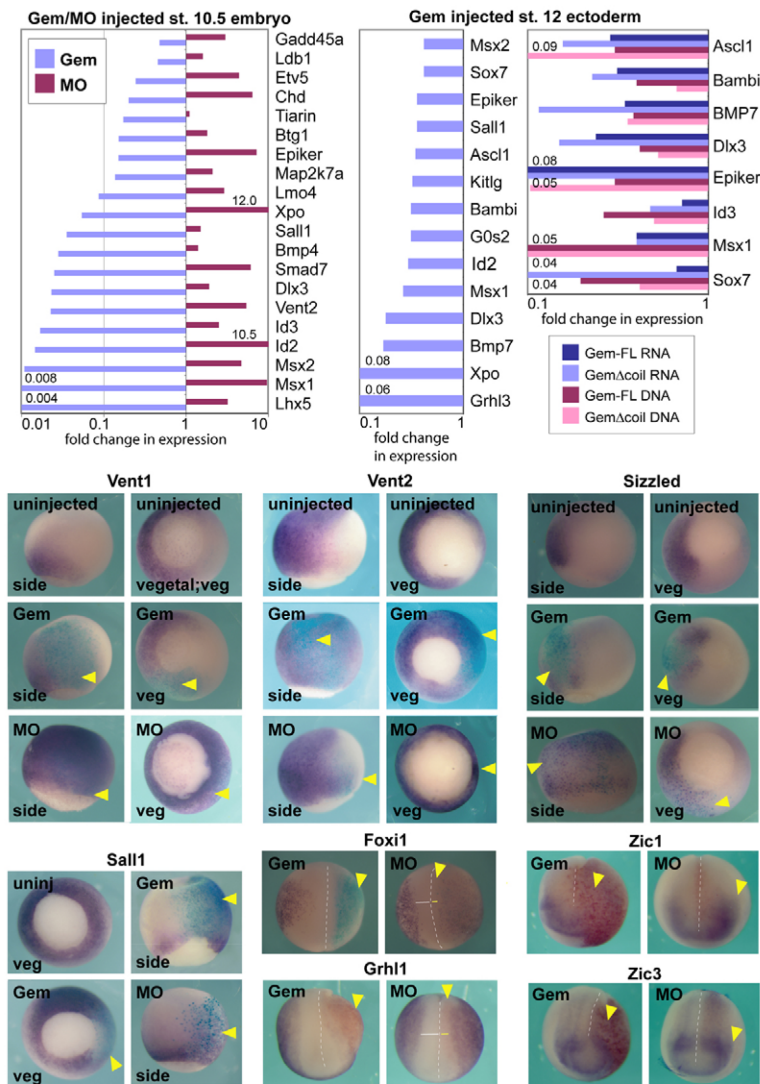

Fig. 3. Geminin suppresses mesoderm and non-neural ectoderm formation. Top: downregulated genes from microarrays were assayed by qRT-PCR for expression changes upon Geminin overexpression or knockdown in embryos (left) or stage 12 ectodermal explants (center/right). Right panel compares effects of plasmid-driven (125 pg) versus mRNA-based overexpression of full-length-FL/30 pg or Δcoil/300 pg Geminin on gene expression. Relative expression normalized to EF1α 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 GemininΔcoil 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 GemininΔcoil 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: GemininΔcoil 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

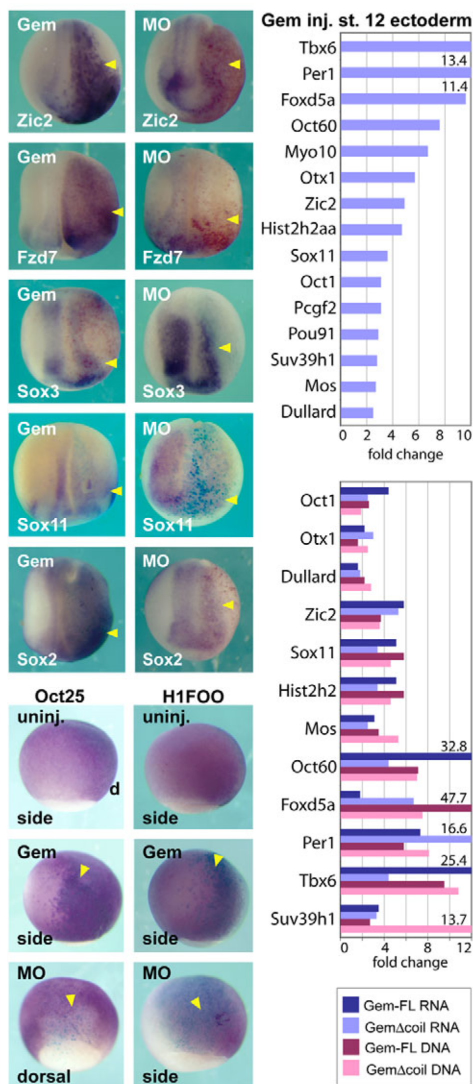

Fig. 4. Geminin promotes expression of genes that mark pluripotent ectoderm and neurectoderm. Left: in situ hybridization of Geminin-overexpressing or MO knockdown embryos with injected area marked by Red-gal (Zic2/Fzd7/Sox2-MO/Sox3-Geminin) or X-gal (yellow arrowhead). Right: genes were assayed by qRT-PCR for changes in expression in stage 12 ectoderm and comparison of Geminin FL versus Δcoil 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 neurectodermal 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 neurectoderm 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 GemininΔcoil 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 GemininΔcoil 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 neurectodermal 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 neurectoderm 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 HP1α 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

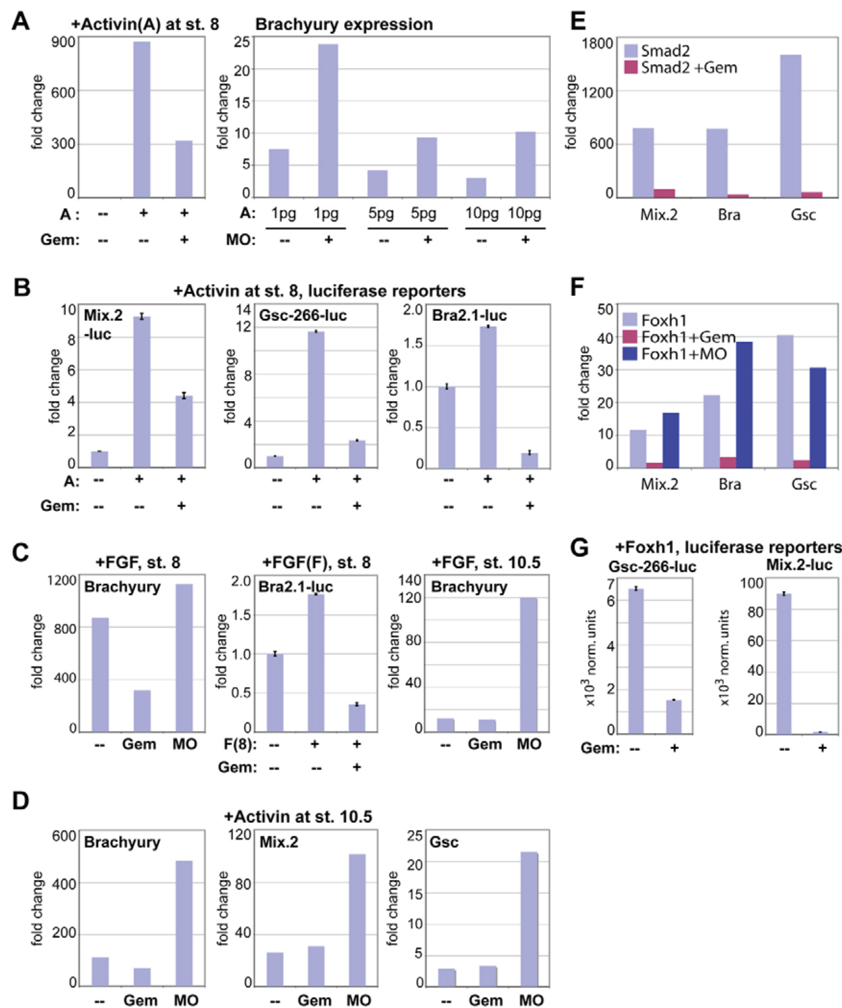

Fig. 5. Geminin overexpression antagonizes and knockdown enhances transcriptional responses to Activin and FGF signaling. (A,B,D) Ectodermal explants were obtained from embryos injected as indicated, Activin(A)-treated (10 ng/ml) for 2 hours/25°C from stages indicated, and harvested for qRT-PCR (see 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 neurectodermal 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 GemininΔcoil 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 GemininΔcoil also represses Activin-mediated induction of Mix.2, Gsc and Brachyury promoter-luciferase reporter constructs (Fig. 5B). GemininΔcoil 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 over-responsiveness 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

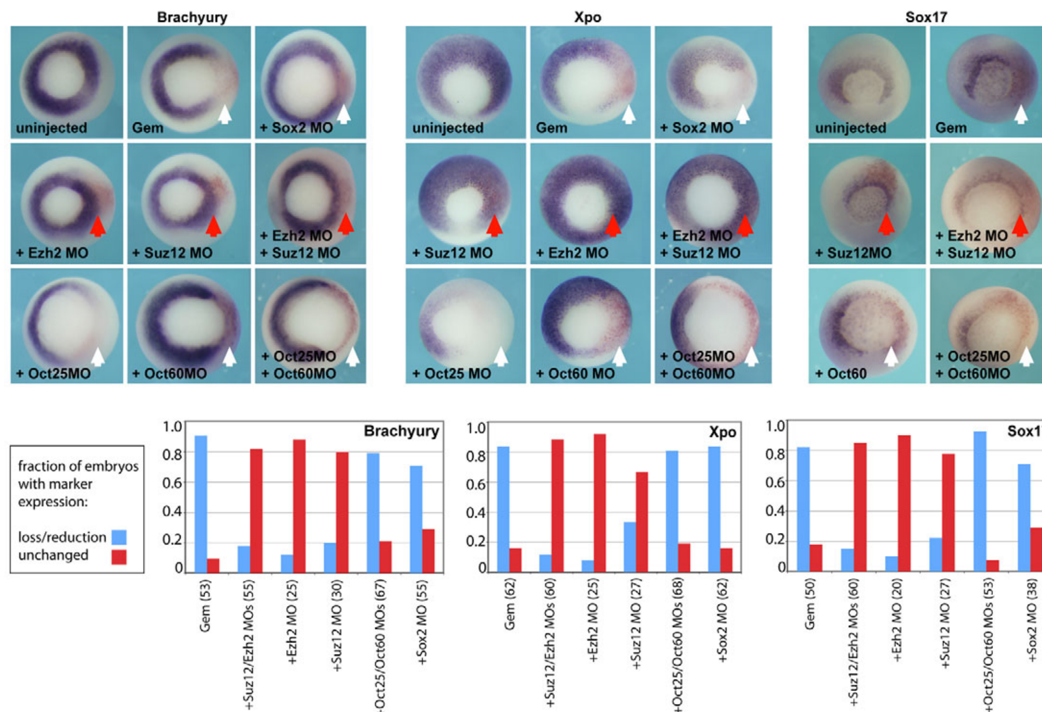

Fig. 6. Geminin suppression of mesoderm and endoderm depends on Polycomb but is independent of Oct25/60 and Sox2 activities. Geminin RNA (120 pg) with or without 8 ng of MOs (16 ng Oct60MO) + 30 pg β-galactosidase RNA was injected into one cell of eight-cell embryos, with in situ hybridization at stage 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 GemininΔcoil co-expression antagonizes this response (Fig. 5E-F). GemininΔcoil 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, GemininΔcoil can block ventral/non-neural gene expression in the presence of a constitutively active BMP receptor (CA-ALK3) or Smad1, and GemininΔcoil 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 GemininΔcoil 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

DEVELOPMENT

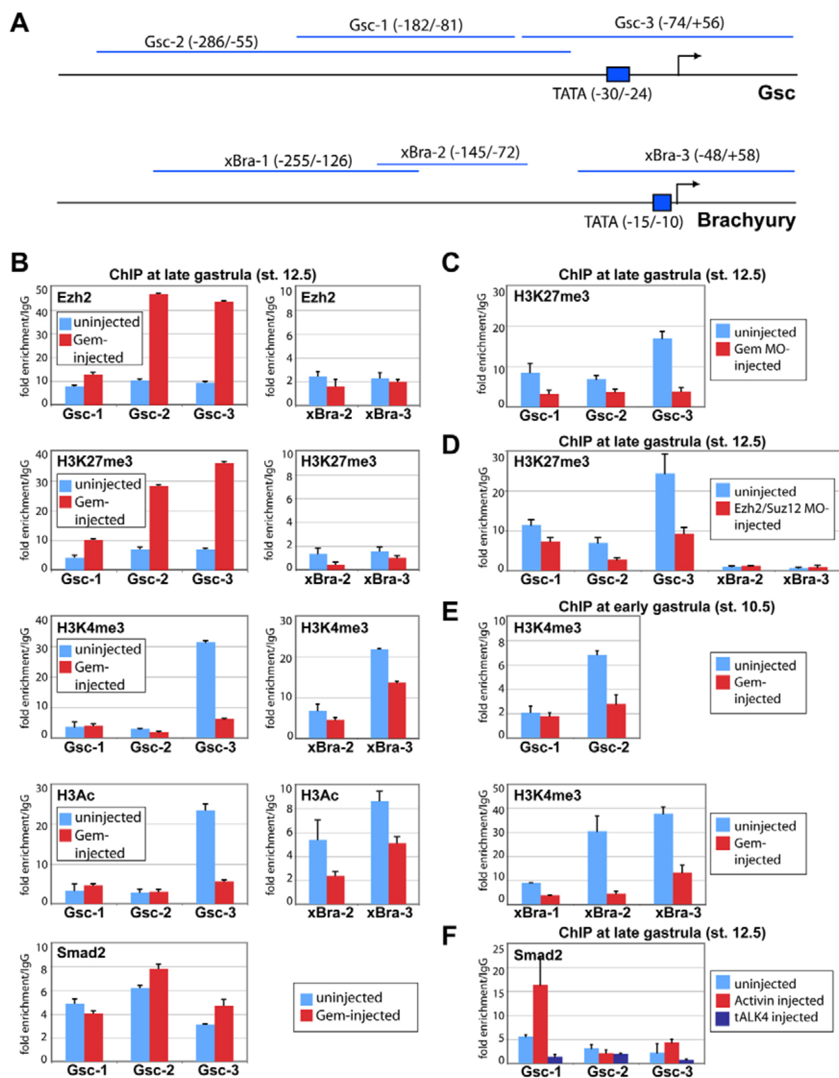

Fig. 7. Geminin regulates Polycomb binding and Polycomb-dependent histone modification. (**A**) Quantitative ChIP was performed using late (stage 12.5; B-D,F) or early (stage 10.5; E) gastrulae, with PCR primers amplifying regions shown. (**B**) Geminin overexpression increases Ezh2 and H3K27me3 but 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 (±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). GemininΔcoil 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 mesendodermal 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 GemininΔcoil 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

DEVELOPMENT

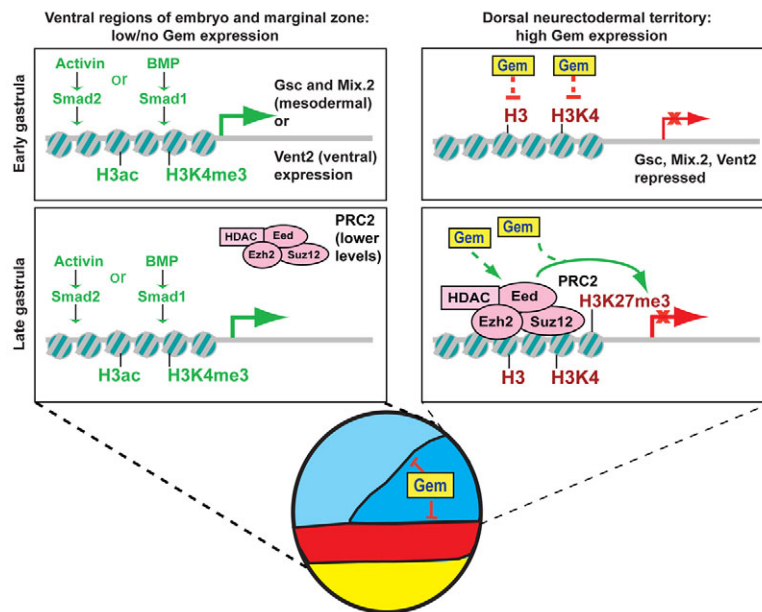

Fig. 8. Model for regulation of embryonic patterning by Geminin. Right: Geminin is highly expressed in prospective neurectoderm in the gastrula embryo. Geminin blocks non-neural gene expression in this region by antagonizing histone modifications associated with transcriptional activation (H3ac/H3K4me3). 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.

DEVELOPMENT

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 Scmh1 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 TGFβ signaling (Dupont et al., 2005). However, Ectodermin does not also modulate transcriptional responses to non-TGFβ 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 trophectoderm (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.

Acknowledgements
We thank Mustafa Khokha, Sally Moody, Dongkook Park, Changwon Park, Ethan Patterson, Dan Yellajoshyula and Lyle Zimmerman for critical review of the manuscript; David Goodstein and Itai Yanai for bioinformatics assistance to define mammalian orthologs of Xenopus genes; Malcolm Whitman, Ken Cho, Christof Niehrs, Jan Christian, Richard Harland and Sally Moody for plasmids; and the Siteman Cancer Center (WUSM, supported by P30 CA91842) for microarray hybridization services. This work was supported by grants from the March of Dimes, American Cancer Society and NIH-GM066815 to K.L.K. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.059824/-/DC1

References

Akiyama, S., Katagiri, T., Namiki, M., Yamaji, N., Yamamoto, N., Miyama, K., Shibuya, H., Ueno, N., Wozney, J. M. and Suda, T. (1997). Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. *Exp. Cell Res.* **235**, 362-369.

Akkers, R. C., van Heeringen, S. J., Jacobi, U. G., Janssen-Megens, E. M., Françoijs, K.-J., Stunnenberg, H. G. and Veenstra, G. J. C. (2009). A hierarchy of H3K4me3 and H3K27me3 acquisition in spatial gene regulation in Xenopus embryos. *Dev. Cell* **17**, 425-434.

Aldiri, I. and Vetter, M. L. (2009). Characterization of the expression pattern of the PRC2 core subunit Suz12 during embryonic development of Xenopus laevis. *Dev. Dyn.* **238**, 3185-3192.
Barnett, M. W., Seville, R. A., Nijjar, S., Old, R. W. and Jones, E. A. (2001). Xenopus Enhancer of Zeste (XEZ); an anteriorly restricted polycomb gene with a role in neural patterning. *Mech. Dev.* **102**, 157-167.
Bernstein, B. E., Mikkelsen, T. S., Xie, X., Kamal, M., Huebert, D. J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K. et al. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315-326.
Blythe, S. A., Reid, C. D., Kessler, D. S. and Klein, P. S. (2009). Chromatin immunoprecipitation in early Xenopus laevis embryos. *Dev. Dyn.* **238**, 1422-1432.
Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K. et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**, 349-353.
Cao, Y., Siegel, D. and Knochel, W. (2006). Xenopus POU factors of subclass V inhibit activin/nodal signaling during gastrulation. *Mech. Dev.* **123**, 614-625.
Chamberlain, S. J., Yee, D. and Magnuson, T. (2008). Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. *Stem Cells* **26**, 1496-1505.
Chang, C. and Harland, R. M. (2007). Neural induction requires continued suppression of both Smad1 and Smad2 signals during gastrulation. *Development* **134**, 3861-3872.
Chi, A. S. and Bernstein, B. E. (2009). Developmental biology. Pluripotent chromatin state. *Science* **323**, 220-221.
Del Bene, F., Tessmar-Raible, K. and Wittbrodt, J. (2004). Direct interaction of geminin and Six3 in eye development. *Nature* **427**, 745-749.
Doherty, J. M., Carmichael, L. K. and Mills, J. C. (2006). GOurmet: a tool for quantitative comparison and visualization of gene expression profiles based on gene ontology (GO) distributions. *BMC Bioinformatics* **7**, 151.
Doherty, J. M., Geske, M. J., Stappenbeck, T. S. and Mills, J. C. (2008). Diverse adult stem cells share specific higher-order patterns of gene expression. *Stem Cells* **26**, 2124-2130.
Dupont, S., Zacchigna, L., Cordenonsi, M., Soligo, S., Adorno, M., Rugge, M. and Piccolo, S. (2005). Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. *Cell*, **121**, 87-99.
Faure, S., Lee, M. A., Keller, T., ten Dijke, P. and Whitman, M. (2000). Endogenous patterns of TGFbeta superfamily signaling during early Xenopus development. *Development* **127**, 2917-2931.
Faust, C., Lawson, K. A., Schork, N. J., Thiel, B. and Magnuson, T. (1998). The Polycomb-group gene eed is required for normal morphogenetic movements during gastrulation in the mouse embryo. *Development* **125**, 4495-4506.
Gonzalez, M. A., Tachibana, K. E., Adams, D. J., van der Weyden, L., Hemberger, M., Coleman, N., Bradley, A. and Laskey, R. A. (2006). Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development. *Genes Dev.* **20**, 1880-1884.
Grammer, T. C., Liu, K. J., Mariani, F. V. and Harland, R. M. (2000). Use of large-scale expression cloning screens in the Xenopus laevis tadpole to identify gene function. *Dev. Biol.* **228**, 197-210.
Henig, C., Elias, S. and Frank, D. (1998). A POU protein regulates mesodermal competence to FGF in Xenopus. *Mech. Dev.* **71**, 131-142.
Karamitros, D., Kotantaki, P., Lygerou, Z., Veiga-Fernandes, H., Pachnis, V., Kioussis, D. and Taraviras, S. (2010). Differential geminin requirement for proliferation of thymocytes and mature T cells. *J. Immunol.* **184**, 2432-2441.
Karaulanov, E., Knöchel, W. and Niehrs, C. (2004). Transcriptional regulation of BMP4 synexpression in transgenic Xenopus. *EMBO J.* **23**, 844-856.
Kim, M. Y., Jeong, B. C., Lee, J. H., Kee, H. J., Kook, H., Kim, N. S., Kim, Y. H., Kim, J. K., Ahn, K. Y. and Kim, K. K. (2006). A repressor complex, AP4 transcription factor and geminin, negatively regulates expression of target genes in nonneuronal cells. *Proc. Natl. Acad. Sci. USA* **103**, 13074-13079.
Koide, T., Hayata, T. and Cho, K. W. (2005). Xenopus as a model system to study transcriptional regulatory networks. *Proc. Natl. Acad. Sci. USA* **102**, 4943-4948.
Kroll, K. L., Salic, A. N., Evans, L. M. and Kirschner, M. W. (1998). Geminin, a neuralizing molecule that demarcates the future neural plate at the onset of gastrulation. *Development* **125**, 3247-3258.
Latinkic, B. V., Umbhauer, M., Neal, K. A., Lerchner, W., Smith, J. C. and Cunliffe, V. (1997). The Xenopus Brachyury promoter is activated by FGF and low concentrations of activin and suppressed by high concentrations of activin and by paired-type homeodomain proteins [published erratum appears in *Genes Dev*. 1998 Apr 15;12(8):1240]. *Genes Dev.* **11**, 3265-3276.
Li, C. and Hung Wong, W. (2001). Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol.* **2**, RESEARCH0032.
Loh, Y. H., Ng, J. H. and Ng, H. H. (2008). Molecular framework underlying pluripotency. *Cell Cycle* **7**, 885-891.
Loose, M. and Patient, R. (2004). A genetic regulatory network for Xenopus mesendoderm formation. *Dev. Biol.* **271**, 467-478.
Luo, L., Yang, X., Takihara, Y., Knoetgen, H. and Kessel, M. (2004). The cell-cycle regulator geminin inhibits Hox function through direct and polycomb-mediated interactions. *Nature* **427**, 749-753.
McGarry, T. J. and Kirschner, M. W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93**, 1043-1053.
Morrison, G. M. and Brickman, J. M. (2006). Conserved roles for Oct4 homologues in maintaining multipotency during early vertebrate development. *Development* **133**, 2011-2022.
Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H. and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379-391.
Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis (daudin).* Amsterdam: North-Holland Publishing Company.
Nitta, K. R., Tanegashima, K., Takahashi, S. and Asashima, M. (2004). XSIP1 is essential for early neural gene expression and neural differentiation by suppression of BMP signaling. *Dev. Biol.* **275**, 258-267.
O'Carroll, D., Erhardt, S., Pagani, M., Barton, S. C., Surani, M. A. and Jenuwein, T. (2001). The polycomb-group gene Ezh2 is required for early mouse development. *Mol. Cell. Biol.* **21**, 4330-4336.
Papanayotou, C., Mey, A., Birot, A. M., Saka, Y., Boast, S., Smith, J. C., Samarut, J. and Stern, C. D. (2008). A mechanism regulating the onset of Sox2 expression in the embryonic neural plate. *PLoS Biol.* **6**, e2.
Pasini, D., Bracken, A. P., Jensen, M. R., Lazzerini Denchi, E. and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *EMBO J.* **23**, 4061-4071.
Pietersen, A. M. and Van Lohuizen, M. (2008). Stem cell regulation by polycomb repressors: postponing commitment. *Curr. Opin. Cell Biol.* **20**, 201-207.
Postigo, A. A., Depp, J. L., Taylor, J. J. and Kroll, K. L. (2003). Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. *EMBO J.* **22**, 2453-2462.
Satijn, D. P., Hamer, K. M., den Blaauwen, J. and Otte, A. P. (2001). The polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in Xenopus embryos. *Mol. Cell. Biol.* **21**, 1360-1369.
Seo, S., Herr, A., Lim, J. W., Richardson, G. A., Richardson, H. and Kroll, K. L. (2005). Geminin regulates neuronal differentiation by antagonizing Brg1 activity. *Genes Dev.* **19**, 1723-1734.
Sheng, G., dos Reis, M. and Stern, C. D. (2003). Churchill, a zinc finger transcriptional activator, regulates the transition between gastrulation and neurulation. *Cell* **115**, 603-613.
Snir, M., Ofir, R., Elias, S. and Frank, D. (2006). Xenopus laevis POU91 protein, an Oct3/4 homologue, regulates competence transitions from mesoderm to neural cell fates. *EMBO J.* **25**, 3664-3674.
Stappenbeck, T. S., Hooper, L. V., Manchester, J. K., Wong, M. H. and Gordon, J. I. (2002). Laser capture microdissection of mouse intestine: characterizing mRNA and protein expression, and profiling intermediary metabolism in specified cell populations. *Methods Enzymol.* **356**, 167-196.
Tada, S., Li, A., Maiorano, D., Mechali, M. and Blow, J. J. (2001). Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nat. Cell Biol.* **3**, 107-113.
Takebayashi-Suzuki, K., Arita, N., Murasaki, E. and Suzuki, A. (2007). The Xenopus POU class V transcription factor XOct-25 inhibits ectodermal competence to respond to bone morphogenetic protein-mediated embryonic induction. *Mech. Dev.* **124**, 840-855.
Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. *Cell* **63**, 485-493.
Tiedemann, H., Asashima, M., Grunz, H. and Knochel, W. (2001). Pluripotent cells (stem cells) and their determination and differentiation in early vertebrate embryogenesis. *Dev. Growth Differ.* **43**, 469-502.
Vonica, A. and Brivanlou, A. H. (2006). An obligatory caravanserai stop on the silk road to neural induction: inhibition of BMP/GDF signaling. *Semin. Cell Dev. Biol.* **17**, 117-132.
Wardle, F. C. and Smith, J. C. (2006). Transcriptional regulation of mesendoderm formation in Xenopus. *Semin. Cell Dev. Biol.* **17**, 99-109.
Watabe, T., Kim, S., Candia, A., Rothbacher, U., Hashimoto, C., Inoue, K. and Cho, K. W. (1995). Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between Xenopus and mouse. *Genes Dev.* **9**, 3038-3050.
Watanabe, M. and Whitman, M. (1999). FAST-1 is a key maternal effector of mesoderm inducers in the early Xenopus embryo. *Development* **126**, 5621-5634.
Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C. and Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science* **290**, 2309-2312.
Yamanaka, S., Li, J., Kania, G., Elliott, S., Wersto, R. P., Van Eyk, J., Wobus, A. M. and Boheler, K. R. (2008). Pluripotency of embryonic stem cells. *Cell Tissue Res.* **331**, 5-22.

DEVELOPMENT