Whole-mount antibody staining

After *in situ* detection of *Rx3* expression using Fast Red (Boehringer Mannheim) as the fluorescent substrate, phosphorylation of histone H3 at Ser 10 was revealed using a polyclonal antibody (Upstate Biotechnology, 1:1,000 dilution). Secondary anti-rabbit antibody fluorescein-conjugate was used, and embryos were analysed using a confocal microscope (Leica TCS-SP). Optical sections of 4 μm were recorded, and positive cells were counted. Alternatively anti-phospho-histone H3 antibody was detected with a secondary peroxidase-conjugated anti-rabbit antibody, followed by DAB staining.

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Correspondence and requests for materials should be addressed to J.W. (Jochen.Wittbrodt@EMBL.de). The geminin sequence has been submitted to the EMBL database (accession number AJ608707); whole-mount expression data have been submitted to MEPD (Ol_emx1, Ol_geminin, Ol_rx2, Ol_six3, Ol_vax1; http://www.embl-heidelberg.de/mepd/).

The cell-cycle regulator geminin inhibits Hox function through direct and polycomb-mediated interactions

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Embryonic development is tightly controlled. The clustered genes of the *Hox* family of homeobox proteins play an important part in regulating this development and also proliferation. They specify embryonic structures along the body axis, and are associated with normal and malignant cell growth[1–4]. The cell-cycle regulator geminin controls replication by binding to the licensing factor Cdt1, and is involved in neural differentiation[5–7]. Here, we show that murine geminin associates transiently with members of the *Hox*-repressing polycomb complex, with the chromatin of *Hox* regulatory DNA elements and with Hox proteins. Gain- and loss-of-function experiments in the chick neural tube demonstrate that geminin modulates the anterior boundary of *Hoxb9* transcription, which suggests a polycomb-like activity for geminin. The interaction between geminin and Hox proteins prevents Hox proteins from binding to DNA, inhibits Hox-dependent transcriptional activation of reporter and endogenous downstream target genes, and displaces Cdt1 from its complex with geminin. By establishing competitive regulation, geminin functions as a coordinator of developmental and proliferative control.

To identify proteins that interact with geminin during embryogenesis, we performed a two-hybrid screen using a complementary DNA library prepared from 8.5 days post coitum (d.p.c.) mouse embryos. Eight positive yeast clones were shown to synthesize geminin-binding proteins (Fig. 1a). Three independent cDNAs each encoded parts of the homeodomain proteins Hoxd10 and Hoxa11. One clone encoded the sex comb on midleg homologue 1

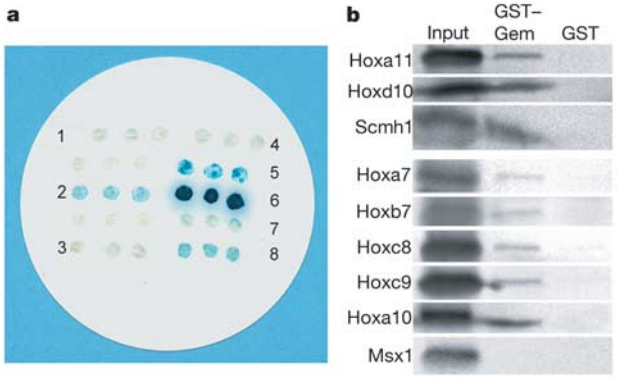

Figure 1 Isolation of Hox and Scmh1 as geminin-binding proteins. **a**, Yeast clones selected by two-hybrid analysis. Eight positive clones were shown to encode geminin-binding proteins, as visualized by β-galactosidase activity. Clones 1, 2, 8 encode Hoxa11, and clones 3, 4, 7 encode Hoxd10. Clone 6 encodes Scmh1. **b**, Pull-down assays. All the full-length *in-vitro*-transcription/translation products of the indicated genes except Msx1 were specifically bound by a GST–geminin fusion protein but not by GST alone.

(Scmh1) protein, which is the mouse homologue of *Drosophila* Scm, a member of the polycomb multiprotein complex[8,9]. Full-length Hox, mesenchymal transcription factor Msx1 and Scmh1 proteins were synthesized *in vitro* in the presence of [^{35}S]-methionine. These radiolabelled proteins were tested for their ability to bind to a recombinant glutathione *S*-transferase (GST)–geminin fusion protein and to GST alone, which acted as the control (Fig. 1b). In such pull-down assays, all the Hox proteins (Hoxa11, Hoxd10, Hoxa7, Hoxb7, Hoxc8, Hoxc9 and Hoxa10) and Scmh1, but not full-length Msx1, tested bound directly to GST–geminin. They exhibited no appreciable binding to GST alone. Together, our results identify homeodomain proteins of the Hox family, as well as the polycomb group member Scmh1, as binding partners of the cell-cycle regulator geminin.

To test whether geminin associates with other polycomb complex members, we analysed its *in vivo* co-localizations with Rae28 (also known as Mph1)[10] and Mel18 (ref. 11) proteins. Double immunofluorescence staining of U2-OS cells was performed with anti-geminin antibodies and antibodies against Rae28 or Mel18 (Supplementary Fig. 1). Endogenous geminin co-localized extensively with Rae28 and Mel18 in the nuclei of the cultured cells. The two polycomb proteins were detected in the nuclei of all cells; however, several nuclei did not contain geminin. A close inspection of these cells revealed that the presence of geminin depended on the phase of the cell cycle (Fig. 2a). Geminin was not detectable during interphase; it accumulated in the nucleus and persisted throughout mitosis until the anaphase–telophase transition. By contrast, Rae28 and Mel18 were continuously expressed throughout the cell cycle. Direct evidence for an *in vivo* association between geminin and a polycomb protein was obtained by immunoprecipitation from 11.5-d.p.c. mouse embryonic extracts using anti-geminin antibodies. A pre-immune rabbit serum was used as a negative control. An immunoprecipitated band with a mobility of 120 kDa was recognized by anti-Rae28 antibodies[9] (Fig. 2b), which indicates that the protein complex isolated from mouse embryos containing geminin also contained Rae28. These data demonstrate a cell-cycle-dependent association of geminin and the polycomb complex protein Rae28.

A fraction of geminin is chromatin associated[12]. To investigate whether geminin associates with *Hox* regulatory elements *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays to isolate DNA fragments that were bound to geminin-including protein complexes in primary cultured mouse embryonic fibroblasts (MEFs). Four *Hox* regulatory elements within the *Hoxd11* gene bind to promyelocytic leukaemia zinc finger (Plzf), a protein that associates with polycomb complex members and mediates transcriptional repression of *Hox* genes[13]. Three of these Plzf-binding sites, located within the *Hoxd11* intron or 3′ untranslated region (UTR), were co-precipitated by geminin antibodies but not by pre-immune serum (Fig. 2c). These results demonstrate that geminin associates *in vivo* with the *Hox* regulatory DNA elements that anchor Plzf.

To analyse the effect of ectopically expressed geminin on *Hox* gene transcription, we overexpressed geminin in the neural tube of chick embryos using *in ovo* electroporation. *CMV–EGFP–Gem* plus *CMV–Gem* or the control vector *CMV–EGFP* were injected into the neural tube of HH9–11-stage chick embryos, that is, at an embryonic time when *Abd-B*-related *Hox* genes such as *Hoxb9* become activated in the posterior body region. The plasmids were electroporated into the right side of the neural tube, where green fluorescent protein (GFP) expression was confirmed after 24 h of incubation and recorded (Fig. 3a, b). The embryos were then incubated for another 24 h, fixed at stage HH18–19, and examined using whole-mount *in situ* hybridization. The *Hoxb9* anterior transcription boundary was posteriorly shifted by the length of one to two somites on the electroporated, right side (Fig. 3c). By contrast, there was no posterior shift of the endogenous *Hoxb9* transcription domain in control embryos with only *CMV–EGFP*

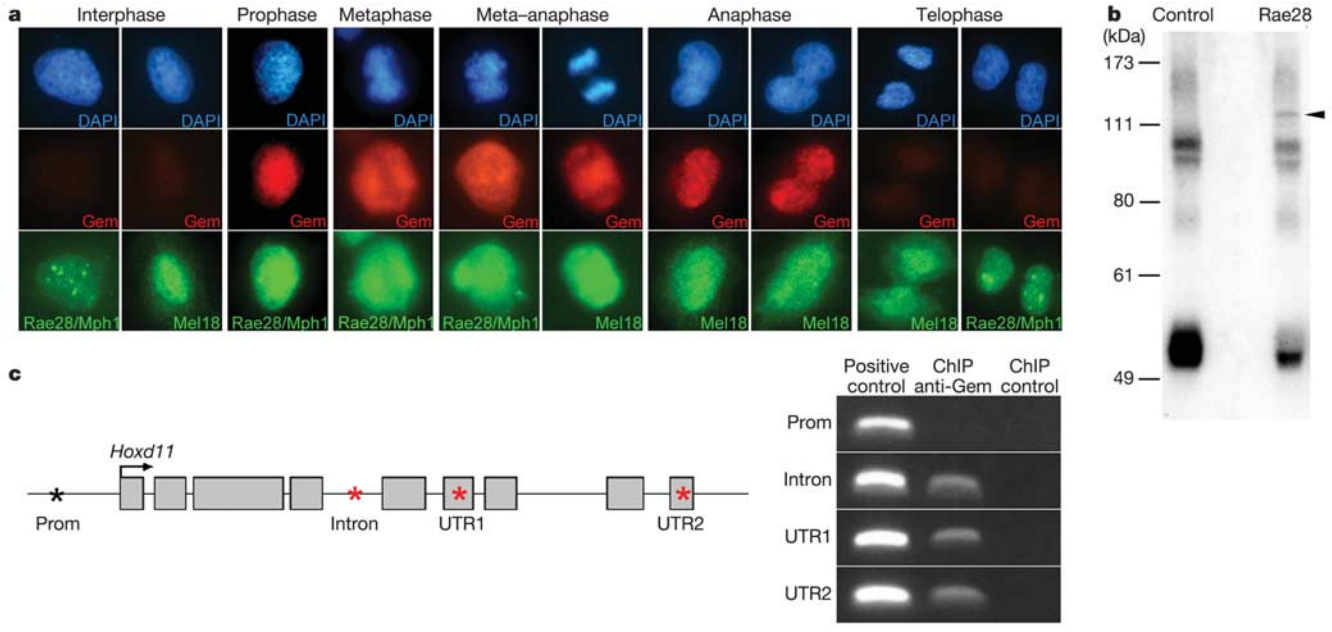

Figure 2 Geminin associates with the polycomb complex and *Hox* regulatory DNA elements *in vivo*. **a**, Subcellular co-localization of geminin and Rae28 or Mel18 during different phases of mitosis. Note the absence of geminin during interphase and telophase. DAPI, 4,6-diamidino-2-phenylindole. **b**, Co-immunoprecipitation of geminin and Rae28 from 11.5-d.p.c. mouse embryonic extracts. The arrowhead indicates that Rae28 was specifically co-precipitated by anti-geminin antibodies. **c**, Three Plzf-binding *Hox* regulatory elements within the *Hoxd11* intron and 3′ UTR (red asterisks) were identified by ChIP analysis. The Hox regulatory element in the promoter region (black asterisk) was not detected.

electroporated (Fig. 3d). This result shows that overexpressed geminin inhibits *Hox* gene activation.

To better characterize the role of geminin in the polycomb complex, we determined the geminin-binding domains of Scmh1. Binding of recombinant, histidine-tagged geminin (His–geminin) to an array of Scmh1 peptides revealed the domain of Scmh1 that was rich in basic amino acids to be the geminin-binding region, which lies outside the SPM domain (a conserved domain shared by Scm and polyhomeotic proteins), through which Scmh1 associates with other polycomb members (Fig. 3e)[9]. Using this information, a dominant-negative form of Scmh1 (amino acids 508–585, dnScmh1), which included the geminin-binding domain but not the SPM domain, was designed. The binding of dnScmh1 to geminin was confirmed using a pull-down assay (Fig. 3f). *In ovo* co-electroporations of a *dnScmh1* expression vector and *CMV–EGFP* to the right side of the neural tube and whole-mount *in situ* hybridization to *Hoxb9* (Fig. 3g–j) were performed as described above. We observed a derepression of *Hoxb9* transcription, one somite length anterior of the normal expression boundary (Fig. 3i). Direct elimination of geminin was carried out by the co-electroporation of small interfering RNA (siRNA) against the chick geminin gene (siGem) and *CMV–EGFP*. siRNA against luciferase was used as the control (siLuc; Fig. 3k–n)[14]. A pronounced derepression of *Hoxb9* by the length of one-and-a-half to two somites was observed (Fig. 3m). These findings show that the inhibition of *Hox* gene transcription by geminin is due to a geminin–polycomb interaction, that is, that geminin behaves like a polycomb protein *in vivo*.

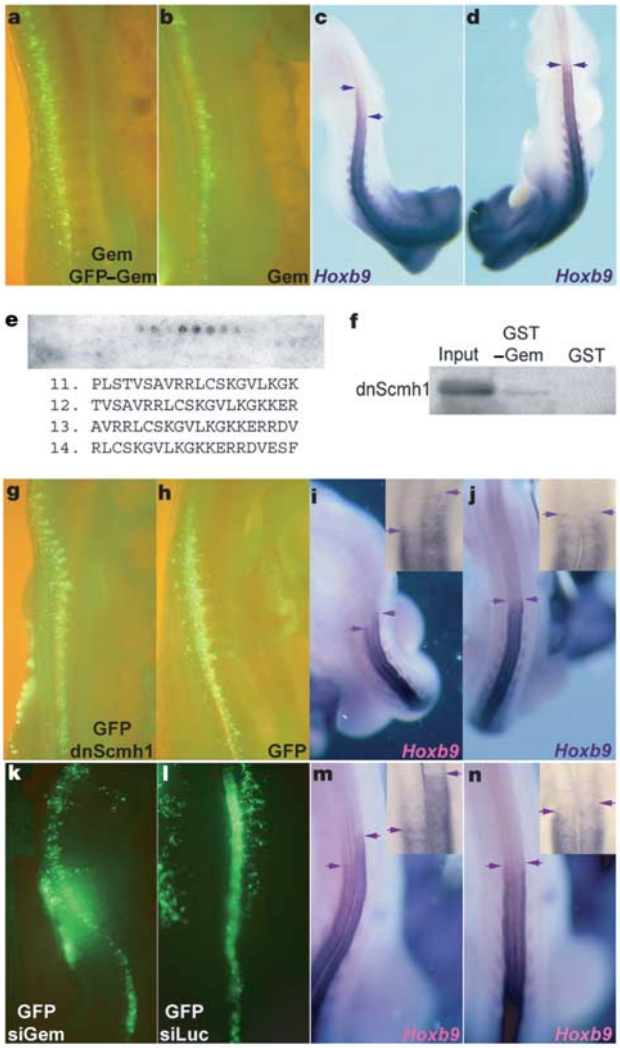

Figure 3 Geminin modulates the anterior boundary of endogenous *Hoxb9* transcription in the avian neural tube. **a**–**d**, The *Hoxb9* anterior transcription boundary was posteriorly shifted by ectopically expressed geminin. **e**, Binding of His-tagged geminin to a Scmh1 peptide array. Below, His–geminin-bound peptides are listed (numered 11 to 14). Amino acids 540–568 of Scmh1 comprise the geminin-binding domain. **f**, Confirmation of dnScmh1–geminin binding through a GST–geminin pull-down assay. **g**–**j**, The *Hoxb9* anterior transcription boundary was anteriorly shifted by ectopically expressed dnScmh1. **k**–**n**, The *Hoxb9* anterior transcription boundary was anteriorly shifted by siRNA against geminin. Arrowheads indicate the anterior transcription boundary of *Hoxb9*.

We identified the homeodomains of both Hoxa11 (Fig. 4a, black frame) and Hoxa7 (data not shown) as geminin-binding regions, with two clusters of basic amino acids as the core binding sequences, through the application of peptide arrays. The Hoxa11 interaction domain of geminin overlaps partly with the coiled-coil domain, which binds to Cdt1 (data not shown)[5,15]. These finding suggest that geminin is a specific antagonist of DNA binding by the Hox homeodomains. We performed electrophoretic mobility shift assays (EMSA), applying *in-vitro*-transcribed/translated Hoxd10, Hoxa11, Hoxb7 and Msx1 proteins, double-stranded oligonucleotides (including their respective consensus-binding sequences) and the recombinant His–geminin protein. The translated Hox proteins produced prominent shifts in the size of oligonucleotide bands during electrophoresis (Fig. 4b, lanes 2, 4, 6 and 9). Pre-incubations of geminin with Hox proteins resulted in the release of free probe, hence the significant reduction of the shifted bands (Fig. 4b, lanes 3, 5, 7 and 10). By contrast, the binding of Msx1 to its target sequence was not attenuated by geminin (Fig. 4b, lanes 11 and 12). In summary, geminin inhibits the binding of Hox proteins to their target DNA sequences as a result of interacting with, and thus blocking, their homeodomains. The amino terminus of Hoxa11 had a small positive influence on geminin binding, whereas N-terminal Msx1 sequences inhibited the interaction of geminin with the Msx1 homeodomain, as indicated by LacZ activities in two-hybrid and binding studies with truncated proteins (data not shown). From these results we conclude that the N terminus influences the binding of geminin to a homeodomain protein. In an accompanying paper, Wittbrodt and colleagues show that the entire Six3 homeoprotein is required for an interaction with geminin, and that geminin supershifts, but not attenuates, a Six3–DNA complex[16].

To examine whether geminin inhibits the transcriptional activation promoted by Hox *in vivo*, we designed a luciferase-reporter construct with a triple Hoxa11-binding sequence. Expression of luciferase was enhanced tenfold by the overexpression of Hoxa11. This increased level was reduced by 60% if, in addition to Hoxa11, geminin was overexpressed (Supplementary Fig. 2). Similarly, when a 500-base-pair fibroblast growth factor 2 (FGF2) promoter fragment including a Hoxb7-binding site[17] was applied, luciferase activity was increased markedly by overexpressed Hoxb7 in HeLa cells. This increase was reduced by 40% by geminin (Supplementary Fig. 2). Together, these data show that the geminin–Hox interaction interferes with the role of Hox proteins as transcriptional activators.

FGF2 is a downstream target gene of Hoxb7 in the melanoma cell line A375 (ref. 17). We used this well defined system to study the influence of geminin on the function of Hox proteins. *CMV–Gem*, a siRNA targeting human geminin messenger RNA (sihGem), or siLuc were transfected into cultured A375 cells. Subsequently, geminin, FGF2 or vimentin levels were detected by western blotting (Fig. 4c). We observed a decrease in the level of FGF2 in parallel with an increase in the level of geminin, and an increase in FGF2 in parallel with the suppression of geminin caused by specific siRNA. These results suggest that geminin modulates the function of the Hoxb7 protein *in vivo*, as detected here by measuring the product of its direct target FGF2. In addition, the FGF2 promoter region could

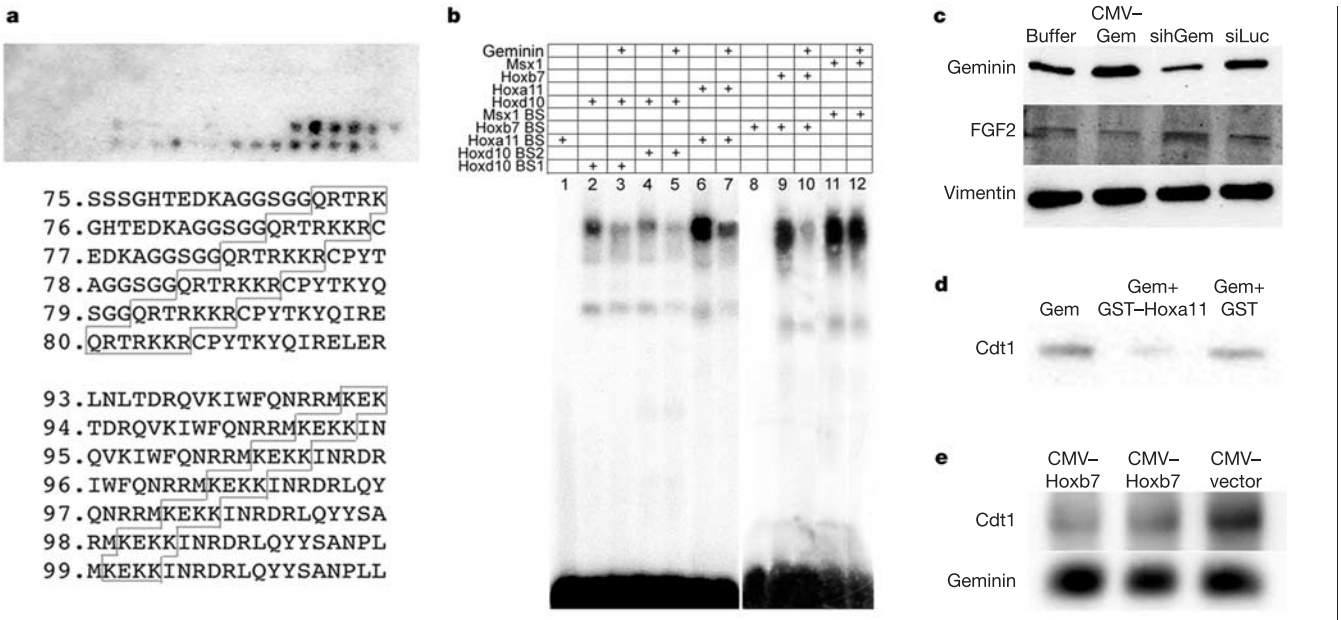

Figure 4 The geminin–Hox interaction inhibits DNA binding and competes with Cdt1–geminin complex formation. **a**, Binding of His–geminin to a Hoxa11 peptide array. Bound peptides are listed, and the common core sequences are framed in black. **b**, Geminin interferes with the binding of Hox proteins to specific double-stranded DNAs. Five different binding sites (BS) were tested in EMSAs. **c**, Geminin inhibits expression of FGF2, the downstream target of Hoxb7, in A375 cells. Note that overexpression or siRNA downregulation of geminin leads to reciprocal changes in FGF2 levels. Vimentin served as an internal control. **d**, Competition for geminin binding between Hoxa11 and Cdt1 *in vitro*. Note that the binding of endogeneous Cdt1 was significantly reduced when GST–Hoxa11, but not GST alone, was pre-bound to immobilized geminin. **e**, Competition for geminin binding between Hox proteins and Cdt1 *in vivo*. Note that the co-precipitated endogenous Cdt1 was significantly reduced when Hoxb7 or Hoxa11 was overexpressed in the cells, whereas the level of geminin itself was not affected.

not be detected when a ChIP assay was performed using an A375 cell extract and geminin antibodies (data not shown), which indicates that geminin is not recruited with Hoxb7 to its DNA target.

As both Hox and Cdt1 proteins bind to the coiled-coil domain of geminin, we further investigated whether Hox proteins compete for the binding of Cdt1 to geminin. The Cdt1 protein in the 11.5-d.p.c. embryonic extracts was pulled-down using geminin-coupled beads. The pull-down of Cdt1 was significantly decreased by pre-incubation of the geminin-coupled beads with GST–Hoxa11 recombinant protein but not with GST alone (Fig. 4d). This result was supported *in vivo* by the following results from primary cultured MEF cells. When Hoxb7 or Hoxa11 was overexpressed, the amount of Cdt1 co-precipitated by geminin antibodies was significantly reduced, in contrast to the control cells transfected with empty vector. The level of geminin itself was not affected (Fig. 4e). Together, these data indicate that Hox proteins can displace geminin from the Cdt1–geminin complex.

Geminin mRNA is widely expressed during vertebrate embryogenesis, and is expressed at slightly higher levels in neural tissues, the tail and the limb bud regions (ref. 7, and data not shown). Our study suggests that geminin is involved in two processes controlled by multiprotein complexes. One is the replication initiation of DNA during the cell cycle, which is controlled by the origin of replication complex, the minichromosome maintenance complex, as well as Cdt1 (refs 5, 6). The other is the specification of cellular identity during embryogenesis, which is controlled by Hox proteins and the polycomb complex, including Scmh1 (refs 1–3). Geminin can associate with components of both multiprotein machineries, remove them from their context, and inhibit their function, for example, the licensing of replication in the case of Cdt1 or the maintenance of Hox repression by the polycomb complex. The level as well as the timing during the cell cycle of geminin compared with proteins such as Cdt1, Hox or Scmh1 would influence the availability of a component. This conclusion is in line with the evidence for multiple roles of Hox as well as polycomb proteins not only in embryogenesis but also in proliferation, cell-cycle control and transformation[4,18–23]. By establishing competitive regulation, geminin functions as a coordinator of developmental and proliferative control. □

Methods

Yeast two-hybrid and GST pull-down assays

The yeast two-hybrid screen was performed using the ProQuest two-hybrid system (GIBCO BRL) according to the manufacturer's instructions. A total of 177 independent colonies were selected on plates lacking leucine, tryptophan and histidine, and supplemented with 50 mM 3-aminotriazole. All these clones were assayed for β-galactosidase activity, and eight positive cDNA clones were shown to encode geminin-binding proteins. The full-length cDNAs were subcloned, and GST pull-down assays were performed as described previously[24].

Cells, immunostaining, immunoprecipitation and ChIP assays

U2-OS cells were cultured using the protocol described by the supplier (ATCC). The cells were fixed, permeabilized, stained for immunofluorescence and analysed as described previously[25].

11.5-d.p.c. mouse embryonic extracts were prepared by solubilizing the embryos in lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 1% Triton X-100, 1 mM β-mecaptoethanol). One milligram of anti-geminin antibodies were pre-bound and crosslinked to 40 mg protein A Sepharose CL-4B (Amersham Pharmacia). These antibody-coupled beads were incubated with 1.5 ml mouse embryonic extracts (40 mg proteins ml^{-1}) for 2 h at 4 °C, then washed three times with washing buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM β-mecaptoethanol, 1 mM PMSF, 0.1% Triton X-100). The precipitated materials were eluted, analysed on a 10% SDS–polyacrylamide gel, and detected with anti-Rae28 antibodies[9] by western blotting. For the ChIP assay, 150 μg of geminin antibodies, 2×10^6 of primary cultured MEFs and four pairs of primers (Prom: 5′-CACGAGATTGCTCAGGGCTTAG-3′, 5′-CAATACTCAGCCAGCGTGGAAAC-3′; Intron: 5′-TTCAGAGCCTGCCTTGCCATC-3′, 5′-CACTCTGGCCACTGAGCTAG-3′; UTR1: 5′-CCACTACAGCCTGAGGAAGAG-3′, 5′-GACAGTGACTCATGCCCAAAG-3′; UTR2: 5′-CATAAGATGCACAGCAGCTCATGC-3′, 5′-GTGGGTCTGGATGTATGAGCCTG-3′) were applied using the ChIP assay kit (Upstate), and genomic PCR and pre-immune serum were used as controls.

In ovo electroporation

In ovo plasmid and siRNA electroporations of HH9–11-stage chick embryos and the analysis of endogenous *Hoxb9* transcription by whole-mount *in situ* hybridization were carried out as described previously[14,24,26].

Peptide array, EMSA and cell-transfection assays

Peptide-array membranes (Jerini) were incubated overnight with 1 μg ml^{-1} His–geminin recombinant protein in a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20 at 4 °C. Then, the His–geminin binding peptides were detected with anti-His antibodies (Novagen) using the manufacturer's protocol.

Sense strands of complementary oligonucleotides containing binding sites for Hoxa11, Hoxd10, Hoxb7 or Msx1 (refs 17, 27, 28) were end-labelled, purified and annealed with their respective antisense oligonucleotides to generate double-stranded DNA probes. *In-vitro*-transcribed/translated proteins were incubated on ice with 20,000 c.p.m. DNA probes in the retardation buffer (20 mM HEPES, pH 7.6, 4% Ficoll, 5 mM $MgCl_2$, 40 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.2 μg μl^{-1} poly[dI-dC]) for 1 h, with or without preincubation with 5 μg His–geminin recombinant protein. The reaction mixtures were applied to a 10% polyacrylamide gel and visualized by exposure to a BioMax film (Kodak) overnight.

Transfection of A375 cells with *CMV–Gem* or siRNAs and detections with antibodies against geminin, FGF2 (Chemicon) or vimentin were performed as described previously[29].

Competition assays

A total of 200 μg His–geminin recombinant protein was N-terminally coupled and crosslinked to 100 μl beads using AminoLink Plus Coupling Gel (Pierce) using the manufacturer's protocol. These geminin-coupled beads were then pre-incubated with 500 μg GST–Hoxa11 or pure GST in 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM β-mecaptoethanol, 0.1% NP-40 for 90 min at 4 °C, followed by washing twice with the same buffer. These beads, with or without pre-incubation, were sequentially incubated with 1 ml 11.5-d.p.c. mouse embryonic extracts for 2 h at 4 °C, washed twice with the same buffer, eluted and loaded on a 10% SDS–polyacrylamide gel. The materials pulled-down by the geminin-coupled beads were detected with anti-Cdt1 antibodies[5] by western blotting. Transfections of *CMV–Hoxb7* or *CMV–Hoxa11* into MEFs and immunoprecipitation were performed as described above.

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An eIF4AIII-containing complex required for mRNA localization and nonsense-mediated mRNA decay

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The specification of both the germ line and abdomen in *Drosophila* depends on the localization of *oskar* messenger RNA to the posterior of the oocyte[1,2]. This localization requires several *trans*-acting factors, including Barentsz and the Mago–Y14 heterodimer, which assemble with *oskar* mRNA into ribonucleoprotein particles (RNPs) and localize with it at the posterior pole[3–7]. Although Barentsz localization in the germ line depends on Mago–Y14, no direct interaction between these proteins has been detected[5]. Here, we demonstrate that the translation initiation factor eIF4AIII interacts with Barentsz and is a component of the *oskar* messenger RNP localization complex. Moreover, eIF4AIII interacts with Mago–Y14 and thus provides a molecular link between Barentsz and the heterodimer. The mammalian Mago (also known as Magoh)–Y14 heterodimer is a component of the exon junction complex[8–11]. The exon junction complex is deposited on spliced mRNAs and functions in nonsense-mediated mRNA decay (NMD)[9,11–14], a surveillance mechanism that degrades mRNAs with premature translation-termination codons. We show that both Barentsz and eIF4AIII are essential for NMD in human cells. Thus, we have identified eIF4AIII and Barentsz as components of a conserved protein complex that is essential for mRNA localization in flies and NMD in mammals.

To understand further how Barentsz (Btz) is recruited to *oskar* mRNPs, we screened a *Drosophila* two-hybrid complementary DNA library[15] with the amino-terminal conserved 399 amino acids of Btz as a bait. This screen identified a protein sharing 82% identity with