

Geminin, a neuralizing molecule that demarcates the future neural plate at the onset of gastrulation

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SUMMARY

In an expression cloning screen in *Xenopus* embryos, we identified a gene that when overexpressed expanded the neural plate at the expense of adjacent neural crest and epidermis. This gene, which we named *geminin*, had no sequence similarity to known gene families. We later discovered that *geminin*'s neuralizing domain was part of a bifunctional protein whose C-terminal coiled-coil domain may play a role in regulating DNA replication. We report here on the neuralizing function of *geminin*. The localization, effect of misexpression and activity of a dominant negative *geminin* suggest that the product of this gene has an essential early role in specifying neural cell fate in vertebrates. Maternal *geminin* mRNA is found throughout the animal hemisphere from oocyte through late blastula. At the early gastrula, however, expression is restricted to a dorsal ectodermal territory that prefigures the neural plate. Misexpression of *geminin* in gastrula

ectoderm suppresses BMP4 expression and converts prospective epidermis into neural tissue. In ectodermal explants, *geminin* induces expression of the early proneural gene *neurogenin-related 1* although not itself being induced by that gene. Later, embryos expressing *geminin* have an expanded dorsal neural territory and ventral ectoderm is converted to neurons. A putative dominant negative *geminin* lacking the neuralizing domain suppresses neural differentiation and, when misexpressed dorsally, produces islands of epidermal gene expression within the neuroectodermal territory, effects that are rescued by coexpression of the full-length molecule. Taken together, these data indicate that *geminin* plays an early role in establishing a neural domain during gastrulation.

Key words: Neurogenesis, BMP4, Gastrulation, Patterning, *geminin*, *neurogenin-related 1*, *Xenopus*

INTRODUCTION

The phenomenon of neural induction was discovered in 1924 when dorsal mesoderm from an early gastrula stage amphibian embryo was shown to induce a secondary body axis and nervous system after transplantation to the ventral side of another early gastrula embryo (Spemann and Mangold, 1924). Recently, several key aspects of this phenomenon have been elucidated at the molecular level. Signaling by bone morphogenetic proteins (BMPs) promotes epidermal differentiation and blocks neural differentiation in ectoderm of both vertebrate and invertebrate organisms. Neural induction is now seen as a local suppression of this BMP signaling by factors released by the Spemann organizer in dorsal mesoderm that bind directly to BMPs to inhibit their function (reviewed in Harland, 1997; Sasai and De Robertis, 1997; Weinstein and Hemmati-Brivanlou, 1997). Neuralization pathways can be activated experimentally by disrupting signaling between ectodermal cells (Grunz and Tacke, 1989; Godsave and Slack, 1989), and epidermalization is restored by adding BMP4 protein to the dissociated ectoderm (Wilson and Hemmati-Brivanlou, 1995). In vivo, two secreted proteins expressed in gastrula organizer mesoderm, chordin (the vertebrate homolog of the *Drosophila* gene *short gastrulation*) and noggin, can

directly bind BMPs to prevent receptor binding (Piccolo et al., 1996; Zimmerman et al., 1996). Other neural-inducing molecules secreted by organizer cells (Xnr3, follistatin) also appear to act by antagonizing BMP signaling (Hansen et al., 1997; Yamashita et al., 1995; Harland and Gerhart, 1997). As this inhibition of BMP signaling neuralizes ectodermal explants, it is likely to initiate neural induction in vivo.

The cell signaling and transcriptional events occurring between mesodermal signaling and the earliest expression of neural differentiation markers in ectoderm are relatively obscure in vertebrate embryos. In *Drosophila*, patterning of the neurogenic region responds to a complex range of proneural and neurogenic gene activities, each of which is induced by earlier dorsal-ventral signal transduction cascades and regional specification by homeobox genes. In vertebrates, several transcription factors, mostly members of the basic helix-loop-helix family, have been identified that can act as proneural factors, converting ventral ectoderm to neural tissue or expanding the size of the neural plate when misexpressed (reviewed in Lee, 1997; Chitnis and Kintner, 1995). The earliest ectodermal expression of any of these genes in vivo is that of *neurogenin related-1* (*X-ngnr-1*; Ma et al., 1996), beginning at stage 10.5 along the lateral edges of the presumptive neural tissue plate. Later expression of these genes

localizes to stripes of primary neurons, consistent with the likely function of these genes in determination or differentiation of neurons.

We have attempted to identify molecules involved in early inductive events by injecting RNA made from relatively small pools of cDNA clones into early *Xenopus* embryos and assaying for changes in embryonic morphology. Previous studies of this kind, using cDNAs made from dorsalized gastrulae (Smith and Harland, 1991, 1992; Lemaire et al., 1995; Lustig et al., 1996b), have identified more than 10 genes involved in dorsal signaling. Here, an early embryonic library, made from stage 6-7 blastulae, was used in the hope of identifying new early patterning factors. mRNA pools from this library were injected into either ventral or dorsal blastomeres of early *Xenopus* embryos to identify genes that, when overexpressed, perturbed the embryonic axis. One such activity, which we named *geminin* (see below), caused expansion of the neural plate.

Geminin RNA and protein are localized to the animal hemisphere during early cleavages. At the early gastrula stage (stage 10- to 10+), *geminin* becomes restricted to an ectodermal domain that strikingly prefigures the neural plate. *Geminin*'s ability to neuralize ectoderm, its induction by neural inducers, capacity to induce early proneural genes and the inhibition of neural patterning by a putative dominant negative domain all suggest that *geminin* plays a key early role in neurogenesis.

While this work was underway, an independent biochemical screen in our laboratory for mitotically degraded proteins (using a different cDNA library) identified a gene closely related in sequence. Its pattern of expression and functional properties appear to be identical to *geminin*. The activity of this gene suggests it is an inhibitor of DNA replication (T. McGarry and M. K., unpublished data). These replication functions have been localized to a domain that is non-overlapping with the neuralizing domain. We have used the name *geminin*, after *gemi* meaning twins, to denote these two closely related genes and indicate their functional duality. These data suggest that *geminin* is a bifunctional protein that at the early gastrula stage marks and establishes neural cell fate in the dorsal ectoderm and that may, in other cellular contexts, also regulate progression through the cell cycle.

MATERIALS AND METHODS

Library construction and expression cloning

A directional cDNA library was constructed from early blastula (stage 6-7) embryos and cloned into pCS2+ (Turner and Weintraub, 1994). Capped RNA was synthesized from pools of 100-300 clones as described (Krieg and Melton, 1987) and approximately 2 ng of RNA was injected into one dorsal or ventral blastomere of a 4-cell embryo. Pools affecting embryonic morphology were selected as described (Lustig et al., 1996a) to isolate a single active cDNA. Single clones were sequenced on both strands on an automated DNA sequencer.

Xenopus embryos and explants

Xenopus embryos were obtained from previously unovulated *X. laevis* frogs (NASCO) by in vitro fertilization, dejellied and cultured at 16-18°C in 0.1× Marc's Modified Ringer's (MMR; Peng, 1991) containing 50 µg/ml gentamycin. Embryos were staged according to Nieuwkoop and Faber (1967). Embryos were injected with plasmids

or RNAs in 0.2× Marc's Modified Ringer's (MMR) containing 5% Ficoll and 50 µg/ml gentamycin and cultured in the same media at 16-18°C. For animal cap explants, both cells were injected superficially in the animal hemisphere at the 2-cell stage and explants were isolated at stage 8 and cultured in agarose-coated dishes containing 0.7× MMR with 50 µg/ml gentamycin until control embryos at the same temperature reached the desired stage.

Transgenic embryos were generated by restriction enzyme-mediated integration of linearized plasmid DNA into permeabilized sperm nuclei (Kroll and Amaya, 1996) with minor modifications (Kroll and Amaya, 1998).

Plasmids and RNAs

Plasmids encoding deletion mutants of *geminin* were produced by amplifying portions of the open reading frame with low-cycle number PCR using Pfu polymerase (Stratagene) and cloning as an *EcoRI-XbaI* fragment into pCS2+MT (Turner and Weintraub, 1994). Deletion mutants of *geminin* described in the text correspond to amino acids 38-90 (Ngem), 87-168 (Cdim), or 112-168 (Ccoil). cDNAs encoding mouse *geminin* were cloned as above using expressed sequence tags as template. For *geminin* dominant negative and rescue experiments, plasmids contained the CMV enhancer, promoter, polylinker and polyadenylation signal from pCS2+ and, in inverse orientation directly adjacent to the CMV enhancer, a minimal simian CMV promoter, second polylinker and polyadenylation signal (kindly provided by Robert Davis). The Cdim mutant was cloned into each polylinker to create two dominant negative constructs; full-length *geminin* was then cloned into both Cdim-containing plasmids. Cloning of Cdim and full-length fragments into each polylinker controlled for possible differences in transcription levels from the two promoters, but both dominant negative and both rescue constructs performed similarly. Plasmids were linearized with *NotI* for transgenesis.

Plasmids for producing capped RNA for BMP4 (Nishimatsu et al., 1992), X-ngnr-1 (Ma et al., 1996) and neuroD (Lee et al., 1995) have been described. To produce noggin (Smith and Harland, 1992) and chordin (Sasai et al., 1994) RNAs, the open reading frames were cloned into CS2+. RNAs were injected at the following doses for animal cap assays: 500 pg BMP4, 50 pg noggin, 250 pg chordin, 150 pg X-ngnr-1 and 500 pg neuroD. For most other experiments, 10-20 pg full-length *geminin* RNA, 50-500 pg Ngem RNA or 250 pg of pCS2+ plasmid encoding gem was injected into one blastomere at the 2-cell stage (see text for more details). For animal cap explants, embryos were injected with half this dose in each cell at the 2-cell stage.

In situ hybridization and immunostaining

For in situ hybridization (Harland, 1991), we used the modified double in situ hybridization protocol (Knecht et al., 1995). Antisense probe for *geminin* was produced by T3 transcription from *EcoRI*-digested pCMVgem. In situ hybridization to 7 µm sections of NIH Swiss mouse embryos (Novagen, Madison, WI) was performed after Wilkinson (1992) with NBT/BCIP as the alkaline phosphatase substrate. Probe was generated using the entire mouse *geminin* open reading frame. Myc-tagged *geminin* protein was detected with the 9E10 anti-myc antibody (Boehringer) and an HRP-conjugated secondary antibody. Endogenous *geminin* protein in embryos was detected using affinity-purified antibody against full-length *geminin* protein (Babco). N-CAM protein was detected with the 4D monoclonal antibody (Developmental Studies Hybridoma Bank). For both, an alkaline phosphatase-conjugated secondary antibody was detected using NBT/BCIP as the substrate.

Images were obtained using incident or transillumination on a Zeiss axiophot or Zeiss stereomicroscope and were captured by video using a 3-color video rate CCD camera controlled by Northern Exposure software (Phase 3 Imaging Systems).

Isolation of genomic clones

To produce haploid embryos, eggs were fertilized with UV-irradiated sperm: a thin layer of macerated testis was UV irradiated at a distance of 13 mm for 5 minutes using a Mineralight lamp (model UVGL-25 from UVP, Inc.). >95% of tadpoles from fertilizations with these sperm had the stunted, microcephalic phenotype characteristic of haploid embryos (Gurdon, 1960) and had a haploid karyotype. Genomic DNA was isolated from single haploid embryos and digested with *NotI*. Primers contained 3' sequences matching only one cDNA: gem L primers corresponding to our cDNA were 5'-agcaacatgaagcagagatc and 5'-aatcagatgtcaagcttcgc and primers to gem H were 5'-caacaagaagcagagatg and 5'-agcctagacagtatgtgc. Each primer set was demonstrated to be specific to one of the cDNAs and was then used to amplify three samples of haploid genomic DNA (25 cycles, 60°C annealing temperature). In all genomic samples, both gene-primer sets amplified bands of approximately 3 kb. These bands were isolated, sequenced and found to correspond to genomic copies of gem L and gem H, respectively.

RT-PCR

Gene expression in animal cap explants was analyzed by RT-PCR (Wilson and Melton, 1994). Primers for muscle actin, EF-1 α , otx 2 and Hox B9 have been described (Wilson and Melton, 1994; Rao, 1994). Other primers for RT-PCR were: BMP4 (5'-attgattgtggcactctct and 5'-ttgatctcagactcaacgg), X-ngnr-1 (5'-tacatctgggctcttagcga and 5'-caaatgaagcgtgctgg), geminin (5'-gctggacatgtaccagtaca and 5'-tcacctacataaaggctgg) and otx 2 (5'-ggatggattgttcaccagtc and 5'-cactctccgagctcattctc). In embryos injected with gem RNA, endogenous geminin was detected with the second (reverse primer) described above and the following forward primer (5'-gtggccgtaacattcga), as these sequences are within the 3' untranslated region not included in the injected RNA. For each primer set, we determined whether amplification was within a linear range by using dilutions of reverse-transcribed cDNA from embryonic samples as a PCR template and quantitating the band intensities with a Molecular Dynamics Phosphorimager.

RESULTS

Expression cloning of geminin assayed by its neuralizing effects

A cDNA library made from *Xenopus* blastulae (stage 6-7) was subdivided into pools of 100-300 clones each. To identify activities that could alter the embryonic axis, these pools, transcribed into RNAs, were injected into dorsal or ventral blastomeres of 4-cell *Xenopus* embryos. As about 2 ng of pool RNA was injected into each embryo, we selected for clones that could produce visible effects at doses of approximately 5-20 pg. One such clone caused expansion of the neural plate. We subdivided this positive pool, assaying smaller pools until we identified a single active clone.

To characterize further the activity of this gene product, which we named geminin, we injected RNA into one bilateral half of the embryo at the 2-cell stage and used in situ hybridization to detect perturbations of gene expression in the early nervous system. A neural-specific isoform of β -tubulin (N-tubulin; Richter et al., 1988; Oschwald et al., 1991) was used to detect primary neurons. At early neurula through tailbud stages, hypertrophy of neural tissue was visible on the injected side (Fig. 1). In some embryos, the density of primary neurons increased (Fig. 1D). In other embryos, more N-tubulin-expressing tissue formed laterally but this tissue appeared disorganized with discrete stripes of primary neurons

replaced by a large patch of neuroectoderm that expressed many neural-specific genes including N-tubulin (Fig. 1C,E), otx 2 (Fig. 2B; Lamb et al., 1993), Pax 6 (data not shown; Hirsch and Harris, 1997) and Delta (data not shown; Chitnis et al., 1995). Anterior structures such as the otic vesicle and eye anlagen were often replaced by a patch of N-tubulin-positive cells in these embryos. Extreme anterior structures such as the olfactory placode (Fig. 2A) and cement gland were sometimes also replaced by a patch of N-tubulin-positive cells. More often, they remained visible but were expanded in size. The earliest expression of N-tubulin in gem-injected embryos was often slightly delayed in the injected half relative to the control half (data not shown) and, when primary neurons initially formed, they were in a more lateral position than those on the uninjected side (Fig. 1A).

Dorsal hypertrophy of neural tissue was found in >85% of embryos injected with gem RNA or with a plasmid placing gem expression under control of the cytomegalovirus promoter to restrict expression to after the mid-blastula stage (pCMVgem). Ventral and lateral ectoderm was also converted to N-tubulin-expressing neural tissue by gem misexpression (Fig. 1B,F-H), although higher levels of misexpression were required than for obtaining dorsal hypertrophy. For example, geminin misexpression from injected mRNA or from pCMVgem plasmid induced N-tubulin relatively rarely in ventral cells (fewer than 5% of injected embryos in each

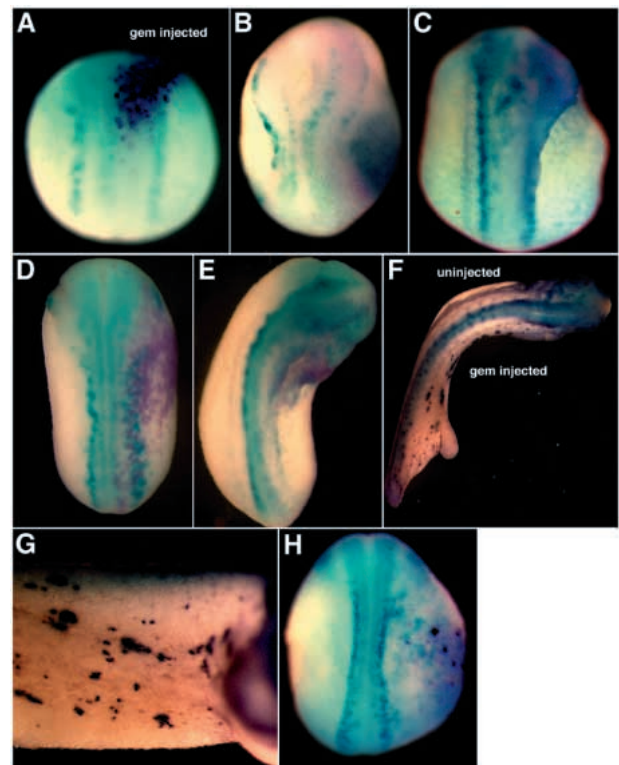


Fig. 1. Ectopic neurogenesis and neural plate expansion in embryonic ectoderm injected with geminin in one bilateral half (oriented to the right). In situ hybridization detects cells expressing N-tubulin (aqua in A-E, H; purple in F, G) and cells expressing injected geminin (dark pink in A-E; H) either from an expression plasmid (A, H) or from 15 pg injected full-length (F, G) or 250 pg N-gem (B-E) RNAs. Endogenous geminin RNA is not visible.

experiment). However, N-tubulin was frequently induced in ventral and lateral tissue (64% for 3 experiments; $n=388$) when a plasmid that elicits stronger transcription of geminin was used (pUASgem). In pUASgem, geminin transcription is under the control of GAL4 upstream activating sites with coinjected GAL4 RNA driving expression (Fig. 1H). We found that pUASgem drove substantially higher levels of geminin transcription than pCMVgem.

Ventral and lateral ectoderm injected with even very low doses of geminin failed to differentiate into epidermis, as measured by loss of expression of an epidermis-specific keratin (Fig. 2G,H; Jonas et al., 1985). This was consistent with conversion of this ectoderm to neural tissue. Formation of neural crest was sensitive to the dose of geminin injected. At geminin doses that effectively neuralized ectoderm, cells expressing the cranial neural crest marker twist (Hopwood et al., 1989) did not form on the injected side of the embryo (Fig. 2D,E). By contrast, a ten-fold lower dose of geminin expanded the twist-expressing domain (Fig. 2F). This is consistent with observations that minimal concentrations of several neural inducers sufficient to inhibit epidermal keratin upregulate

expression of a neural crest marker without inducing N-CAM, whereas N-CAM-inducing doses of the same molecules suppress neural crest (Morgan and Sargent, 1997). Neural crest is formed by cellular interactions occurring at neural plate borders where neural and non-neural ectoderm are juxtaposed (Moury and Jacobson, 1990; Selleck and Bronner, 1995) and subthreshold concentrations of neural-inducing activity may be present at these boundaries.

Geminin is a novel neuralizing molecule

The effects caused by ectopic expression of geminin in embryos were reminiscent of those elicited by several proneural genes that act to specify neural determination. In *Xenopus*, identified genes that have an overexpression phenotype resembling geminin include the *atonal* homolog *XATH-3* (Kim et al., 1997; Takebayashi et al., 1997), an *achaete-scute* homolog (*XASH-3*; Zimmerman et al., 1993; Ferreira et al., 1994; Turner and Weintraub, 1994), the neural differentiation gene *X-ngnr-1* (Ma et al., 1996) and the neural differentiation gene *neuroD* (Lee et al., 1995), all basic helix-loop-helix family genes. These effects also resembled misexpression of a POU-domain transcription factor, XIPOU2 (Witta et al., 1995) also the product of a likely proneural gene. However, geminin's sequence bears no resemblance to these or other known molecules.

Our *Xenopus* geminin cDNA encodes a highly charged protein of 216 amino acids. A second *Xenopus* geminin cDNA isolated by T. McGarry and M. K. (unpublished data) encodes a 219 amino acid protein (Fig. 3A). By low-stringency hybridization and sequence database searches, we detected genes related to *geminin* in other vertebrates (zebrafish, mouse and human) but not in yeast (*Saccharomyces cerevisiae*). The C-terminal domain of geminin contains heptad amino acid repeats predicted by the Parcoils (Berger et al., 1995) and Lupas algorithms (COILS program, version 2.1; Lupas et al., 1991) to form a coiled-coil domain commonly used in protein dimerization. To determine the intracellular localization of geminin protein, we used an antibody against geminin to detect the protein in blastula stage embryos. The endogenous geminin protein appeared to be predominantly nuclear-localized (Fig. 3B,C). We also injected plasmid encoding a myc epitope-tagged geminin protein into embryos and immunostained to detect the myc epitope. Most of this gem protein also translocated to the nucleus (data not shown).

By sequence similarity, the two *Xenopus* gem cDNAs are 85% identical within the open reading frame, although the 5' and 3' untranslated regions and sequences of the introns are much less highly conserved. Since the predicted proteins encoded by these cDNAs vary by three amino acids in size, we use the designations L (light) and H (heavy) to denote the cDNA reported here and that reported by T. McGarry and M. K. (unpublished data), respectively. To determine whether these cDNAs represented two separate genes or rather two alleles of the same gene, we assayed haploid genomes for the presence of each gene. In three haploid genomic backgrounds tested, we found that both genes were present and isolated genomic copies corresponding to both geminin cDNAs (see Materials and Methods; data not shown).

We defined two functional domains of geminin in embryos by testing a series of geminin deletion mutants for activity. An N-terminal domain (amino acids 38-90; Ngem) was sufficient to

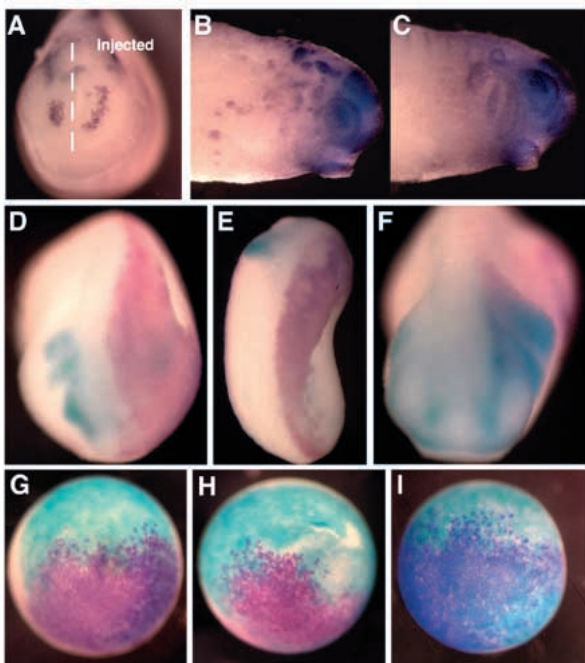


Fig. 2. Geminin suppresses epidermal and expands neural gene expression. (A) Synaptobrevin (Knecht et al., 1995; Richter et al., 1988) expression in the olfactory placode is expanded on the geminin-injected side of the embryo (15 pg full-length RNA injected). (B,C) *otx 2* expression on geminin-injected (B) or uninjected (C) sides of the same embryo. In B, *otx 2* expression is expanded posteriorly and the otic vesicle and branchial arches fail to form normally. (D-F) In situ hybridization for twist (aqua) and injected geminin (pink). Injection of 250 pg of Ngem RNA eliminates twist staining (D,E), whereas injection of 25 pg of Ngem expands twist expression on the injected (right) side relative to the control half (F). (G-I) Animal hemisphere views of late gastrulae stained to detect epidermal keratin (aqua) and either 250 pg injected Ngem (G,H, pink stain) or 250 pg injected GFP (I, pink stain). In G and H, epidermal keratin staining is lost in injected cells; in I, overlap of GFP and keratin produces a dark purple stain.

A

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MNSNPKQSRSDVENPSSMSIKNYIVDKTNEALAPRRRTLKVIQQSASGCLVGR Xenopus L
MNTNKKQRLDMEKPTMSIKNYFVDKTNESLAPRRRTLKVIQPSASGCLVGR Xenopus H
MNLSPMKQKQEGAQ-----ENVKNSPVPRRRTLKMVIQPSADGSLVGR mouse
MNPSPMKQKQE EIK-----ENIKNSSVPRRRTLKMVIQPSASGSLVGR human

TKEPAKNS TKRKLWNDQLTSKKAKVEVAVDPEHQENKDCPS--EAYDLM Xenopus L
TKEPVKNS TKRKLWNDQLTSKKAKVEVAVDPEHR ENKDCSS--EAYDLM Xenopus H
ENELPKGLFKRKLWD DQLASQTSSCG-----PEANENKDVGD L T Q E A F D L I mouse
ENEL SAGLS KRRKH RNDHLTS TTSSPGVI V - P E S S E N K N L G G V T Q E S F D L M human

VKETPTCLYWKDVAAEERRKALYEALQENEKHLHQEIELKDEE IARLKQEND Xenopus L
VKETPTCLYWK E VAAEERRKALYEALQENEKHLK E I E L K D E E I A R L K Q E N D Xenopus H
SKENPSSQYWK E VAEQR R K A L Y E A L K E N E K L H K E I E Q K D S E I A R L R K E N K mouse
IKENPSSQYWK E VAEK R R K A L Y E A L K E N E K L H K E I E Q K D N E I A R L K K E N K human

ELMELAGHVQYMANMIERL TGNAPQSLEDLKNLDLEEARFEDEA--ESR Xenopus L
ELMELAGHVQYMANMIERL TGNAPRSLEDLKDLDLEEARFEDEADMAEAR Xenopus H
DLAEVAE H V Q Y M A E V I E R L S N - - - E P L D N F E S P D S Q E - - F D S E E E A V E Y S mouse
ELAEVAE H V Q Y M A E L I E R L N G - - - E P L D N F E S L D N Q E - - F D S E E E T V E D S human

I E D E T D M T Q P S S S D Q N M D K Q T - - - V . Xenopus L
I E D E T D M A R P S N S D Q N M D A H T - - - V . Xenopus H
E L E D S G A G T C A E E T V S S S T D A R P C T . mouse
L V E D S E I G T C A E G T V S S S T D A K P C I . human

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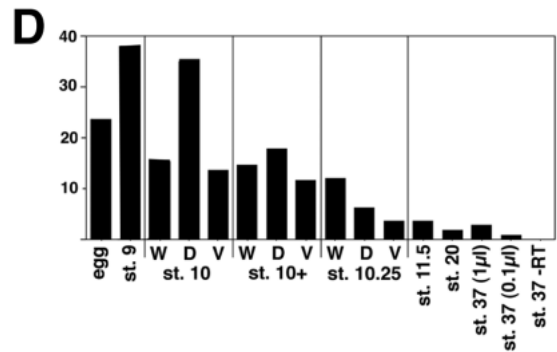
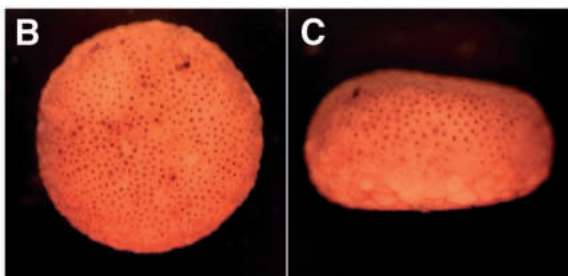


Fig. 3. Structure, intracellular localization and developmental profile of geminin expression in embryos. (A) Protein sequences of geminin. The N-terminal domain sufficient for neuralization is overscored in red and the C-terminal coiled-coil domain is overscored in blue. Accession numbers for geminin are AF068781 (gem L), AF068786 (gem H), AF068780 (mouse gem), and AF068785 (human gem). (B,C) Animal pole (B) and side (C) views of a blastula embryo immunostained with a geminin antibody. Most endogenous geminin is localized to the nucleus. (D) RT-PCR analysis of geminin RNA levels in the embryo during development. Between stage 10 and 10.25, embryos were analyzed whole (W) or subdivided into dorsal (D) or ventral (V) halves. New (zygotic) transcription of geminin is evident at stage 9 and in the dorsal half of the embryo at stage 10.

evoke neural hypertrophy and ectopic neurogenesis when injected at RNA levels slightly higher than those required for the full-length molecule. A C-terminal domain comprised of the coiled coils of the molecule (amino acids 112-168; Ccoil) was cytotoxic when expressed in early embryos. This domain has DNA replication inhibition activity and can fully account geminin's ability to regulate DNA replication in vitro (T. McGarry and M. K., unpublished data). By contrast, the N-

terminal domain has no effect on DNA replication or cell cycle progression (T. McGarry and M. K., unpublished data), is not cytotoxic in embryos at doses up to 2 ng and can fully account for geminin's neuralizing activity. These cDNAs also contain a destruction box that is near but not contained in the N-terminal neuralizing fragment (T. McGarry and M. K., unpublished data).

The neuralizing and cell cycle regulatory activities of geminin are physically separated into non-overlapping and

independently acting domains. However, alteration of the cell cycle state could conceivably be a precondition for neural determination or differentiation such that physical association of these domains would be advantageous. To test whether blocking the cell cycle elicited ectopic neurogenesis, we treated embryos with hydroxyurea and aphidicolin (HUA) from the early gastrula stage (data not shown). This HUA treatment, as described by Harris and Hartenstein (1991), blocks virtually all cell division and did not elicit neural hypertrophy in previous studies (Turner and Weintraub, 1994; Takebayashi et al., 1997). Likewise, we found that HUA treatment alone had no effect on neural plate formation or expression of neural genes. We also conversely found that neural hypertrophy elicited by geminin was not attributable to increased cell division as it occurred in HUA-treated embryos previously injected with geminin mRNA. Therefore, the neuralizing effects of geminin were neither sensitive to nor caused by perturbation of the cell cycle.

Geminin demarcates the future neural plate in early gastrulae

Geminin is expressed broadly in the early embryo with most prominent accumulation of mRNA (Fig. 4A) and protein (Fig. 3B,C) in the animal hemisphere during early cleavages. However, at the onset of gastrulation, geminin mRNA becomes restricted to a dorsal ectodermal territory (stage 10– to 10). At this time, geminin transcript levels increase dorsally relative to levels found in an equivalent volume of egg cytoplasm (Fig. 3D). This indicates that the dorsal enrichment of geminin mRNA seen by in situ hybridization must be due, at least in part, to new dorsal transcription. Ventrally, transcript levels are lower than levels found in the egg, although we can not determine from this whether the rate of gem mRNA turnover differs in dorsal versus ventral tissue.

Early in gastrulation, geminin transcripts are cleared from both ventral ectoderm and from the marginal zone, such that the domain of gem expression does not overlap with brachyury, which is expressed in presumptive posterior mesoderm (Fig. 4C,D). BMP4 is expressed throughout the animal hemisphere and marginal zone in early gastrulae and the same cells thus express both geminin and BMP4 at these stages (data not shown). By mid-gastrulation, however, expression of geminin and BMP4 is largely complementary and non-overlapping (Fig. 4F). BMP4 mRNA is restricted to ventral ectoderm and mesoderm, while geminin mRNA is restricted to dorsal ectoderm.

During neural plate stages, geminin is expressed in a wide anterior ectodermal territory that contributes to placodes, neural crest and neural plate. In trunk neuroectoderm, gem mRNA levels are slightly higher at the midline (notoplate) and at the lateral edges. Anteriorly, the lateral edge of the geminin expression domain is marked by the trigeminal ganglia (Fig. 4H). After neural tube closure, gem is expressed in both the floorplate and roofplate of the neural tube and more anteriorly throughout the brain, in the eye

vesicle, nose, otic vesicle, in cephalic neural crest (Fig. 4I,J) and in a dorsal region of the tailbud contiguous with the dorsal neural tube (Fig. 4K).

We also used in situ hybridization to mouse embryo tissue sections to visualize the expression pattern of the mouse gene. Mouse geminin becomes enriched in the nervous system by about day e8 to e9, although some staining outside the neural tube is also visible. At later stages (e15), expression localizes to cranial and trunk nerves, the trunk neural tube and much of the brain (Fig. 4L).

Geminin suppresses BMP4 expression and epidermal fate at gastrulation

While we identified geminin by its effect on neural plate boundaries, we found gem had much earlier effects on ectodermal cells. Injection of gem RNA into lateral or ventral ectoderm caused a loss of BMP4 mRNA that was visible during gastrulation in these cells (Fig. 5B,C). A like decrease in BMP4 transcript levels occurred in isolated ectoderm injected with geminin (Fig. 5D). These effects were elicited by low doses of full-length or Ngem RNA (10–100 pg) or injection of pCMVgem plasmid. At the onset of gastrulation, geminin expression is upregulated in dorsal ectoderm prior to the loss of BMP4 mRNA from these cells (Figs 4B–D, 6A; Hemmati-Brivanlou and Thomsen, 1995). By contrast, BMP4 transcripts persist throughout gastrulation in lateral and ventral ectoderm not expressing geminin (Fig. 4F). Thus, both geminin's action and its pattern and timing of expression suggest a role in downregulating BMP4 expression in dorsal ectoderm during gastrulation.

Does geminin's antagonism of BMP4 mRNA persistence in dorsal ectoderm account for its neuralizing properties? To address this question, we first compared the expression of

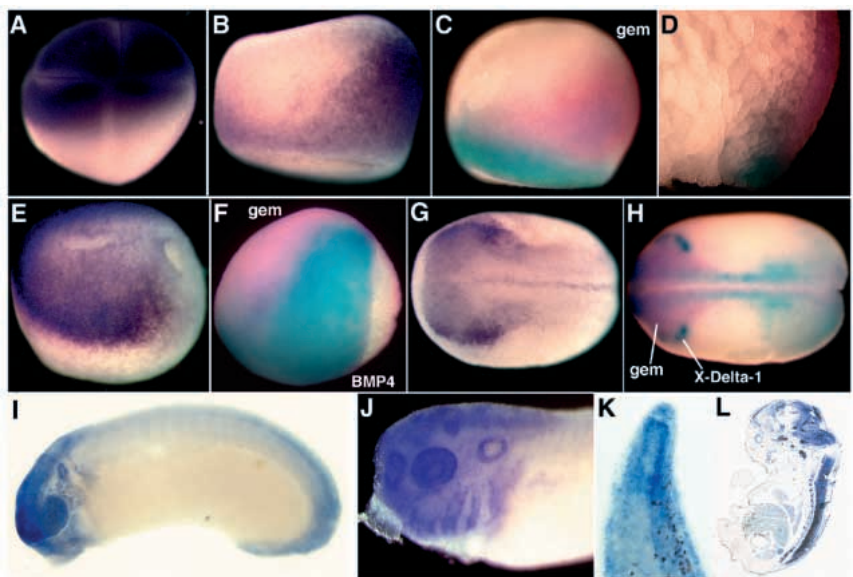


Fig. 4. Spatial distribution of geminin transcripts in the early embryo identified by in situ hybridization. (A,B,E,G,I–K) Geminin staining is purple in singly stained embryos. (C,D,F,H) Geminin expression is pink in doubly stained embryos. The expression patterns stained in aqua are brachyury (C,D), BMP4 (F), or X-Delta-1 (H). (L) Geminin expression detected in a section of an e15 mouse embryo. Dorsal is oriented to the right (B–D), top (E,F) or facing out (G,H). *Xenopus* embryos are stages 4 (A), 10 (B), 10.25 (C, D), 12 (F), 12.5 (E), 13.5 (G), 20 (H), 28 (I) and 38 (J,K).

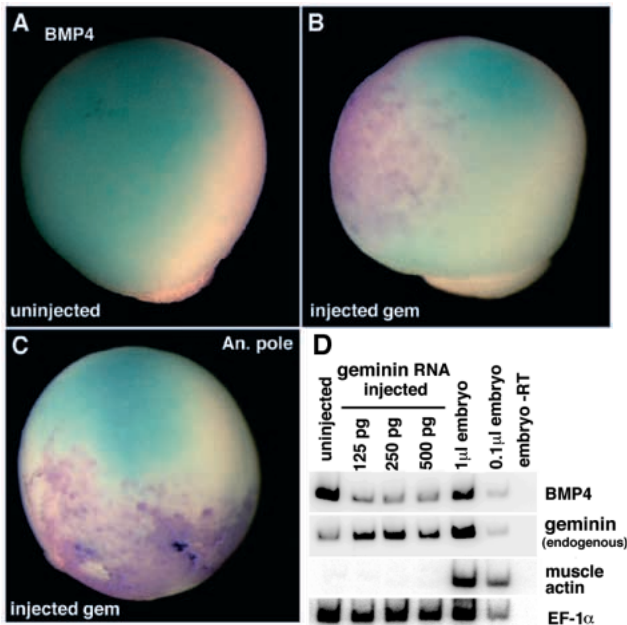


Fig. 5. Geminin inhibits expression of the epidermalizing growth factor BMP4 in ventral and lateral ectoderm during gastrulation. BMP4 expression (in aqua) occurs in lateral and ventral ectoderm of uninjected embryos (A) but is eliminated by injection of 250 pg Ngem RNA (pink staining B,C). A and B are lateral views; C is an animal hemisphere view; embryos are stage 12 and dorsal is to the right. (D) BMP4 expression is downregulated in ectoderm injected with Ngem RNA. Expression of endogenous geminin is induced by injected Ngem (primers recognize the 3' untranslated region not included in the injected RNA). Muscle actin is not induced by geminin.

geminin with that of epidermal keratin between stages 9 and 10.5. At the onset of gastrulation (stage 10– to 10), geminin expression is upregulated in dorsal ectoderm and transcripts are rapidly turned over in lateral and ventral cells, restricting geminin mRNA to dorsal ectoderm (Fig. 6A). Epidermal keratin is not present in these embryos at levels detectable by in situ hybridization. Shortly thereafter (stage 10+ to 10.25), levels of epidermal keratin rise in lateral and ventral cells outside of the geminin-expressing domain while keratin never appears in dorsal cells (Fig. 6B,C). At later stages, expression continues to localize to non-overlapping ectodermal domains (Fig. 6D).

Geminin expression precedes restriction of BMP4 and epidermal keratin expression to ventrolateral cells. When misexpressed, geminin causes both BMP4 and epidermal keratin mRNAs to be lost from cells fated to form epidermis. These observations are consistent with a mechanism where downregulation of BMP4 RNA levels is the primary means by which geminin neuralizes ectoderm. If this is so, coinjection of BMP4 mRNA with geminin should rescue epidermalization in coinjected cells. We found this indeed to be the case. Ventral cells coinjected with BMP4 mRNA and geminin in a 1:1 ratio formed epidermis (Fig. 6E), whereas ventral cells injected the same dose of geminin and mRNA for green fluorescent protein failed to express epidermal keratin, as expected from geminin's activity (Fig. 6F). Therefore, many

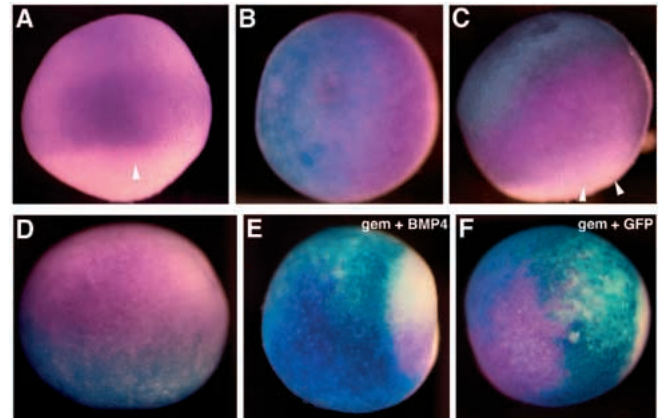


Fig. 6. BMP4 can relieve geminin's suppression of epidermal development. (A–F) Double in situ hybridizations with epidermal keratin (aqua) and geminin (pink) probes at stages 10– (A), 10+ (B, C), 12.5 (D) or 12 (E, F). A and C are dorsal views (arrowheads mark the dorsal lip); B, E and F are animal hemisphere views. (E) Coinjection of BMP4 and geminin; dark-purple tissue expresses both epidermal keratin and injected geminin. (F) Coinjection of geminin and GFP. Epidermal keratin expression is suppressed in regions expressing injected geminin.

of geminin's neuralizing properties could be attributed to its ability to lower intracellular BMP4 mRNA levels in dorsal ectoderm during gastrulation.

Geminin is induced by organizer molecules and induces neural genes

To determine how induction of geminin expression in dorsal ectoderm might occur in the embryo, we tested several neural inducers and proneural genes for effects on geminin mRNA levels in isolated ectoderm (Fig. 7A). Both *noggin* and *chordin* strongly induced geminin expression. By contrast, the proneural gene *X-ngnr-1*, which shows the earliest onset of neural expression in the embryo, failed to induce geminin. These data are consistent with the observation that dorsal upregulation of geminin transcription occurs at stage 10– to 10, after the onset of organizer gene expression (stage 9) but prior to expression of proneural genes including *X-ngnr-1* (stage 10.5–11). We also found that geminin could induce its own expression (Fig. 5D). BMP4 had neither a stimulatory nor inhibitory effect on geminin transcription (Fig. 7A).

Consistent with its ability to repress BMP4 and epidermal keratin expression and induce neural gene expression in ventral cells of the embryo, we found that geminin could induce neural gene expression in isolated ectoderm. The proneural gene *X-ngnr-1* was strongly induced in the absence of *muscle actin* gene expression, indicating that geminin's neuralizing activity is direct and not mediated by the formation of mesoderm with neural-inducing properties (Fig. 7B). *N-CAM* expression was also activated in this ectoderm (Fig. 7C) and in ventral tissue of injected embryos (Fig. 7E,F). However, by comparison with *X-ngnr-1* or *neuroD*, the level of *N-CAM* induction in ectodermal explants was relatively weak. *N-CAM* was induced in explants injected with a plasmid that drives robust transcription of geminin (pUASgem) but not by 10–20 pg of gem RNA. This was consistent with results of gem RNA or plasmid injections into the ventral ectoderm of embryos (Fig. 1). By comparison,

suppression of BMP4 mRNA levels was sensitive to injection of low doses of full-length or Ngem RNAs. The posterior neural marker Hox B9 was induced by geminin, while expression of the anterior marker *otx 2* (also expressed in non-neural ectoderm) was slightly suppressed. This was a somewhat surprising result, given that neural inducers that are thought to act strictly by antagonizing BMP receptor signaling (such as noggin, chordin, or dominant-negative receptors) induce neural tissue of anterior character. In this respect, geminin's neural-inducing activity resembles that of FGF, which has also been shown to induce posterior neural tissue in animal cap assays (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). We speculate that this induction represents an effect of geminin on neural gene transcription that is separate from its effects on BMP4 transcription, but the mechanism by which this occurs or its relevance *in vivo* remain unclear.

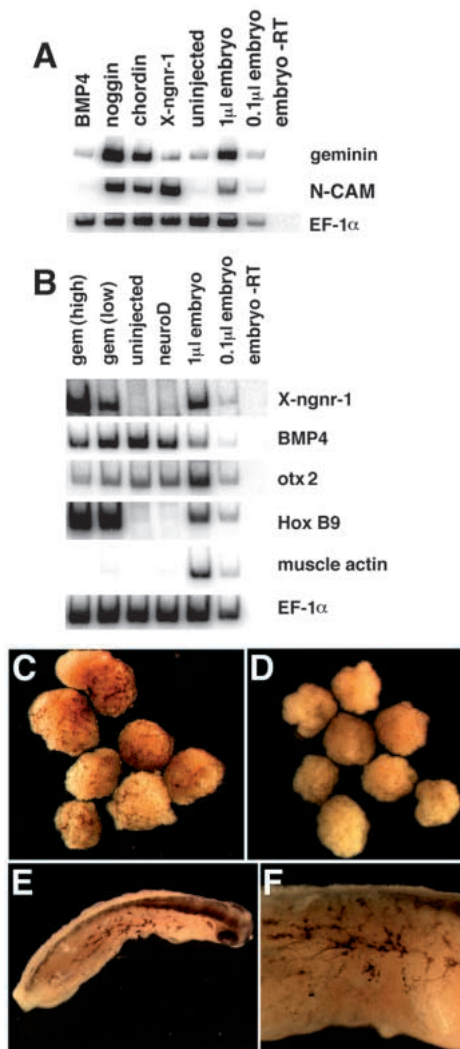


Fig. 7. Geminin is induced by organizer signals and activates neural gene expression in isolated ectoderm. (A) Geminin expression in response to inducing molecules, (B) induction of neural gene expression by geminin, (C-F) immunostaining for N-CAM in animal caps injected with pUASgeminin (C), uninjected animal caps (D) or embryos injected in one bilateral half with pUASgeminin (E,F).

Neurogenesis is blocked by overexpression of the C-terminal domain

While testing deletion mutants of geminin for activity, we found that a C-terminal domain containing the coiled-coil protein dimerization motif (Cdim) antagonized neural development by contrast with effects of the full-length or N terminus of geminin. Dorsal cells injected with a plasmid containing Cdim driven by the cytomegalovirus promoter (pCdim) expressed epidermal keratin ectopically and were suppressed from expressing N-tubulin. This effect was rescued by coexpressing full-length geminin.

We analyzed epidermal keratin expression in gastrula stage embryos injected dorsally with either pCdim or with a rescue plasmid containing cassettes for expression of both Cdim and full-length geminin, each from a separate CMV promoter (pCdim-gem). Epidermal keratin was expressed ectopically in 93% of embryos injected dorsally with pCdim ($n=137$; Fig. 8A,B). Injection of pCdim-gem fully suppressed ectopic epidermal keratin expression in some embryos while, in others, ectopic keratin was partially suppressed (data not shown). Complete suppression of ectopic dorsal keratin expression in most embryos was obtained by coinjection of full-length geminin (pCMVgem) with pCdim-gem (100 pg each; 91%, $n=216$; Fig. 8C,D).

We also analyzed the effects of this dominant negative

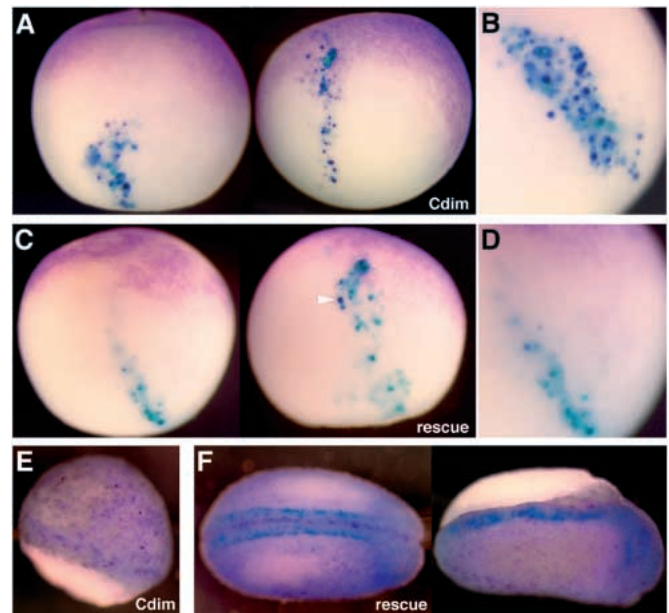


Fig. 8. A dominant negative geminin induces epidermis and suppresses neural development in dorsal cells. (A-D) *In situ* hybridization to detect epidermal keratin (pink) and injected geminin (aqua; endogenous not visible); overlap produces a dark blue-purple stain. Embryos are stage 12. (A,B) Dorsal views of embryos injected with the geminin C-terminal domain expression plasmid (pCdim). (C,D) Embryos injected with a plasmid encoding both Cdim and full-length geminin (pCdim-gem; 100 pg) and with a plasmid encoding full-length geminin (pCMVgem; 100 pg). A remaining keratin-expressing dorsal cell is indicated by an arrowhead. (E,F) *In situ* hybridization to detect N-tubulin (dark blue in F) and ectopically expressed geminin (pink) in transgenic embryos expressing either pCdim (E) or pCdim-gem (F). Embryos in E, F are stage 23/24.

domain of geminin on the formation of endogenous neural tissue, detected by N-tubulin staining. Initially, we found that small regions of the neural tube expressing injected pCdim failed to express N-tubulin (data not shown). We focused further analysis on transgenic embryos, as injected embryos were highly mosaic in the nervous system. Transgenic embryos expressing pCdim began gastrulation normally but cell movements appeared to arrest during gastrulation and later embryos were severely disturbed with no visible axial structures (Fig. 8E). Most of these embryos (86%; $n=76$ for two experiments) completely lacked N-tubulin staining while the others showed a partial loss of N-tubulin staining. Expression of pCdim-gem restored full N-tubulin staining to 89% of the embryos ($n=95$ for two experiments), although it did not rescue normal morphology in many embryos (Fig. 8F). The ability of this rescuable C-terminal domain construct to cause ectopic epidermis formation and to suppress neural tissue formation in dorsal ectoderm indicates that geminin could be required for some aspect of neural cell determination.

DISCUSSION

The role of geminin in neurogenesis

Geminin was cloned based on its potent activities in the embryo, which resemble those of the proneural genes (Ferreiro et al., 1994; Kim et al., 1997; Lee et al., 1995; Ma et al., 1996; Takebayashi et al., 1997; Turner and Weintraub, 1994; Witta et al., 1995; Zimmerman et al., 1993). Geminin can expand the neural plate and induce ectopic neurogenesis in lateral and ventral ectoderm in the absence of induced mesoderm and when expressed only at the time of neural induction. At even low doses, misexpressed geminin suppresses BMP4 mRNA expression or persistence in ventral ectoderm and blocks epidermal keratin expression. Geminin's expression pattern is consistent with a role in controlling BMP4 transcript levels in dorsal ectoderm as geminin defines a future neural territory in early gastrula ectoderm from which BMP4 transcripts become excluded and in which epidermal keratin expression never occurs. Epidermis can also be rescued in ventral ectoderm misexpressing geminin if BMP4 transcripts are coinjected to replace those suppressed by geminin, further suggesting that some aspects of geminin's neuralizing activities are mediated by regulation of BMP4 transcript levels in dorsal ectodermal cells.

Most research on the early stages of neuralization has focused on secreted molecules produced by dorsal mesoderm. Geminin bridges a gap between these extracellular signals and later establishment of proneural gene expression within dorsal ectodermal cells. Geminin's expression demonstrates that a future neural territory is established in the ectoderm by stage 10, while the earliest expression of proneural genes occurs only later within a more restricted domain by stage 10.5 (*X-ngnr-1*; Ma et al., 1996) or by stage 11 (*XIPOU2*; Witta et al., 1995). In addition to BMP4 and epidermal keratin, several homeobox genes begin to be expressed in or are restricted ventrally by stage 10.5 to 11. Ventral upregulation of some of these genes (*msx-1*, Suzuki et al., 1997 and *Xom*, Ladher et al., 1996) is an immediate, direct response to BMP signaling. Geminin's dorsal localization in future neurectoderm precedes these ventral restrictions of expression and indicates that ectoderm

is regionalized into dorsal and ventral domains earlier than was previously appreciated (by stage 10 to 10+). Geminin's expression and activities are also consistent with roles in controlling both downregulation of epidermal inducers and later upregulation of proneural genes such as *X-ngnr-1*.

Noggin and chordin inactivate extracellular BMPs by direct protein binding. However, ectodermal cells would be expected to continue to express and secrete active BMP4 protein dorsally in the absence of some intracellular response to this extracellular attenuation of BMP receptor signaling. Geminin may represent such an early response, downregulating BMP4 transcription and thus fully abolishing signaling by this BMP ligand dorsally during the period of early organizer signaling. Geminin's expression and dorsal accumulation could be stimulated by an initial attenuation of BMP signaling, as noggin and chordin induce geminin expression. Other dorsal signals may also be involved, such as those of the wnt pathway. Once present at high levels within dorsal cells, geminin can stimulate its own transcription, producing a positive autoregulatory circuit to reinforce dorsal fate. At the same time, geminin could mediate the complete loss of BMP4 mRNA and thus active BMP4 protein from the dorsal side of the embryo, establishing neural fate within the dorsal ectodermal cells. In *Drosophila*, BMP4 has been found to stimulate its own transcription, forming a positive autoregulatory loop that maintains and reinforces the epidermal state of cells in regions with high levels of extracellular BMP4 protein (Yu et al., 1996). There is some evidence such autoactivation may also occur in vertebrates (Schmidt et al., 1996; Jones et al., 1992), thus autoactivation circuits mediated by geminin and BMP4 on the dorsal and ventral sides of the embryo, respectively, might maintain well-defined neural and epidermal domains within the embryo and stimulate the expression of the appropriate downstream genes.

A secondary, intracellular system for controlling BMP4 levels might seem unnecessary if BMP4 antagonists are produced by the organizer at levels sufficient to block all protein that is produced. However, our experiments with expressed C-terminal domains indicate that geminin performs a function essential for establishing neural cell fate. In these experiments, epidermis was induced in dorsal ectoderm in immediate proximity to unperturbed, organizer mesoderm. Neural fate was rescued in these cells by expressing full-length geminin. Interpretation of these results is complicated by potential non-neuralizing functions of the C-terminal domain. This domain could inhibit other cellular functions, such as DNA replication. If the presence of excess C-terminal domain in the cell blocks geminin's interaction with another partner or the formation of active homodimers, expression of excess full-length protein could overcome this inhibition. Hence the experiment can be best seen as an indication that one pathway involving geminin plays a direct or indirect role in neural specification.

Geminin as a prepattern gene in ectodermal regionalization

Both geminin's activities and expression pattern distinguish it from proneural genes that have been identified in vertebrates. Geminin is expressed in an earlier or broader pattern than any known proneural gene, demarcating the future neural plate by early gastrulation. This expression establishes a dorsal

ectodermal domain in which cells will downregulate BMP4 expression, fail to express epidermal keratin, and later express proneural genes, all cellular functions that can be performed by *geminin*. Unlike the proneural genes, *geminin*'s expression never localizes specifically to primary neural clusters.

In keeping with its early expression pattern, *geminin* is more effective at inducing early neural markers than late. Doses of *geminin* that effectively suppress epidermal keratin and BMP4 are 5- to 10-fold lower than those needed to induce neural tissue. While *geminin* can induce neurogenesis in lateral and ventral ectoderm (unlike the proneural gene *XASH-3*; Turner and Weintraub, 1994), *geminin* expression results in fewer ectopic neurons than is seen in embryos injected with other proneural genes such as *X-ngnr-1* (Ma et al., 1996) or *neuroD* (Lee et al., 1995). This may reflect *geminin*'s in vivo role, as many cells expressing *geminin* during gastrulation do not later become neurons. *Geminin* may activate downstream processes that further select sites of primary neuron formation within the dorsal ectodermal cell field. In many respects, therefore, *geminin* appears to act earlier and more generally to subdivide ectoderm into neural and non-neural territories, with activities of downstream genes later determining neuronal fate within individual dorsal cells.

In *Drosophila*, genes play a similar prepatterning role in domains of the wing imaginal disc where proneural gene activity later occurs. Formation of the sensory organs is controlled by activation of the proneural genes *achaete* (*ac*) and *scute* (*sc*) in small clusters of cells. While factors controlling *ac-sc* expression are still poorly understood, two recently identified homeodomain genes are expressed in broader regions of the wing imaginal disc than the *ac-sc*-expressing clusters and can directly activate *ac-sc* transcription. These genes, named *arauacan* and *caupolican*, are designated 'pre-pattern' genes because their expression pattern and activities fill a gap between genes such as *engrailed* and *hedgehog* that broadly subdivide the imaginal discs and expression of *ac-sc* in small clusters of cells (Gomez-Skarmeta et al., 1996). In a similar manner, *geminin* responds to early dorsal-ventral patterning cues to demarcate a dorsal ectodermal domain within which proneural genes later act to establish neural cell fate.

Geminin is a bifunctional molecule

We found that *geminin*'s neuralizing activity localized to an N-terminal domain. A non-overlapping C-terminal domain composed of the predicted coiled-coil region had no neuralizing activity and was toxic when expressed at high levels in cleaving embryos (from injected RNA). However, this C-terminal domain was not deleterious to embryonic development if expression was restricted to after the mid-blastula transition in transgenic or plasmid-injected embryos, at levels that may be much lower. Furthermore, this C-terminal domain antagonized the function of the entire molecule and interfered with neurogenesis. T. McGarry and M. K. (unpublished data) have found that the coiled-coil domain of *geminin* inhibits the initiation of DNA replication, which may correspond to the early toxicity that we observed. As shown previously, inhibition of replication after the early gastrula stage has little effect on neural specification (Harris and Hartenstein, 1991).

Since the C-terminal domain of *geminin* may regulate cell

cycle progression, an intriguing possibility was that physical connection of neuralizing and DNA replication inhibition domains within *geminin* could coordinate a switch from proliferation to differentiation occurring after the neural cell fate decision. Thus far, however, we have found no simple link between the two processes during early neural specification. Inhibition of DNA replication did not alter neural patterning in embryos, indicating that this was not sufficient to stimulate the effects on the nervous system that we have observed. This is supported by similar studies with proneural genes (Turner and Weintraub, 1994; Takebayashi et al., 1997). Likewise, the domains sufficient to neuralize in vivo or block DNA replication in vitro are non-overlapping within the molecule, so neither function can be explained as a consequence of the other. Thus we cannot yet determine whether different cofactors control these two functions or speculate as to whether there are biological contexts, such as promoting the terminal differentiation of neurons, that might be more likely to use both functions coordinately. Studies to address these issues are underway.

Geminin's mechanism of action

Geminin is a novel molecule that can coordinately alter transcription in ectodermal cells. While *geminin* is nuclear, we have as of yet no evidence that this protein binds to DNA directly, although this would certainly account for its ability to affect transcription. Another possibility is that *geminin* interacts with transcription factors or other nuclear or cytoplasmic proteins to alter their activity; *geminin* may either interfere with the activity of epidermalizing molecules or may activate molecules in the neuralization pathway. In yeast, full-length *geminin* contains some transcriptional activation activity, detected when it is fused to a GAL4 DNA-binding domain (data not shown). It will be of interest to identify the binding partners of *geminin*.

The activities of the organizer in neural induction surprisingly are biochemically simple, direct inhibitors of BMP protein activity. Yet these inhibitors lead to the demarcation of the neural plate and ultimately the patterning of the nervous system. We might expect that other patterning processes are involved in building up complexity and delineating more accurately the domains of neural function. Such activities would come as a response to mesodermal signaling in the neural plate. *Geminin* potentially represents such an activity. Although it is not obvious how it functions biochemically, it seems clear that *geminin* mediates an important patterning event early in neural specification.

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