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The Role of Glycogen Synthase Kinase 3 in
Early *Xenopus* Development

by

Sarah B. Pierce

A dissertation submitted in partial fulfillment
of the requirements for the degree of

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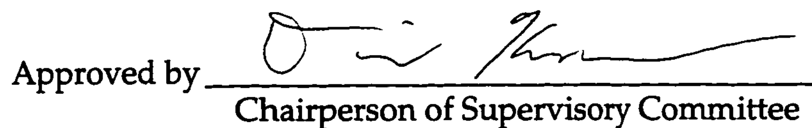

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University of Washington

Abstract

The Role of Glycogen Synthase Kinase 3 in Early *Xenopus* Development

by Sarah B. Pierce

Chairperson of the supervisory committee: Associate Professor David Kimelman,
Department of Biochemistry

Experiments early in this century indicated that dorsoventral axis formation in the *Xenopus* embryo requires the activity of the Spemann organizer, or gastrula organizer. Cell and cytoplasmic transplantation studies subsequently defined the earlier acting blastula organizer, or Nieuwkoop center, which induces formation of the Spemann organizer. Several secreted factors, including members of the Wnt family, were found to mimic the Nieuwkoop center when ectopically expressed, but none of these were shown to be endogenously required for dorsoventral axis formation.

Here I describe the isolation and characterization of *Xenopus* glycogen synthase kinase 3 (Xgsk-3), a serine/threonine kinase which is homologous to a component of the intracellular signaling pathway utilized by the *Drosophila* Wnt homolog Wg. Using a dominant-negative mutant of Xgsk-3, I show that Xgsk-3 is a negative regulator of the dorsal fate. This indicates that Wnt and Wg signal through homologous pathways and that the Wnt pathway is necessary for endogenous axis formation. In addition, dominant-negative Xgsk-3 acts non-cell-autonomously, indicating that a subsequent intercellular signal is required for Spemann organizer induction.

The isolation and characterization of, GBP, a protein that binds Xgsk-3, is also described. GBP induces the formation of a secondary axis, suggesting that

GBP interferes with the action of Xgsk-3, but it does not inhibit the enzymatic activity of Xgsk-3. However, GBP joins a complex with Xgsk-3, APC and β-catenin. Xgsk-3 regulates dorsoventral axis formation by negatively regulating the stability of β-catenin and APC is thought to participate in this process. I present a model in which GBP induces axis formation by disrupting the function of this complex.

It was found that overexpression of Xgsk-3 in the prospective ectoderm resulted in the an expansion of the cement gland and other non-neural ectodermal tissues at the exprense of lateral ectoderm, without disrupting the borders of the neural plate. This resulted from a greater responsiveness of the ectoderm to cement gland-inducing signals, which could be mimicked by the secreted factor noggin. A model for the role of Xgsk-3 in ectodermal patterning is presented which considers the expression of Wnt family members that could regulate Xgsk-3 during the time of cement gland induction and the endogenous role of noggin as an inhibitor of BMP-4 signaling.

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Introduction

Overview

My thesis research has been aimed at understanding the process by which the basic vertebrate body plan is established as an organism develops from a radially symmetrical egg. The model system I have chosen to work with is the frog *Xenopus laevis*, because the relatively large size of the embryo makes it amenable to both phenotypic and biochemical analysis and manipulation. I have focused on the question of how the dorsoventral axis is initially established in the embryo. By way of introduction, I will briefly review early *Xenopus* development and the history of embryological experiments aimed at understanding dorsoventral axis formation. I will then discuss more recent experiments that have provided candidates for the molecules involved in this process, particularly members of the *Wnt* family of secreted proteins. Finally, I will review the genetic and biochemical evidence leading to the hypothesis that glycogen synthase kinase 3, which is the focus of this thesis, could play an important role in the process by which the dorsoventral axis is established.

Early *Xenopus* development

Within the female, oocytes are arrested at prophase of meiosis I. A hormonal signal triggers maturation to metaphase of meiosis II, the stage at which oocytes can be fertilized. These steps can be induced in the laboratory by injection of the females with gonadotropins. Fertilization and embryogenesis take place entirely outside of the mother. The sperm enters the egg within the pigmented animal hemisphere, triggering a rotation of the egg cytoplasm toward the sperm entry point (SEP). This rotation of the egg cytoplasm relative to the

cortex, referred to as cortical rotation, usually results in the dorsal side of the embryo arising opposite the SEP (Gerhart *et al.*, 1989). Cell division begins approximately 90 minutes after fertilization and continues approximately every 30 minutes for twelve synchronous rounds. The intercellular signaling events that establish the future mesoderm at the equator of the embryo occur during these early cleavages (Jones and Woodland, 1987). The end of these synchronous cell divisions is known as the midblastula transition (MBT), when the embryo has about 4000 cells and zygotic transcription increases dramatically (Newport and Kirschner, 1982). At this time, cells of the future ectoderm lie in the animal hemisphere, future endodermal cells are in the vegetal hemisphere, and the future mesoderm lies at the equator of the embryo, or marginal zone (Fig. 1). At about twelve hours after fertilization at room temperature, gastrulation begins with the thinning and spreading of the blastula animal hemisphere. Near the equator, cells intercalate in the medio-lateral direction, resulting in the extension of the group of cells at the dorsal midline (Keller and Tibbetts, 1989). This extension drives first the dorsal marginal zone, and eventually the entire marginal zone, around the blastopore lip and into the blastocoel. Prospective mesodermal and endodermal cells of the involuting marginal zone migrate upward along the inner surface of the blastocoel, forming the archenteron roof, while the prospective ectoderm continues to spread toward the vegetal pole until in covers the embryo. Thus, gastrulation results in the proper positioning of the three germ layers (ectoderm, mesoderm and endoderm) relative to one another. During the process of gastrulation, opposing signals from the dorsal and ventral mesoderm act to pattern the more lateral mesoderm (Dale and Slack, 1987b) (Graff, 1997) and to induce neural tissue in the overlying ectoderm. Just after gastrulation, at about twenty hours, the neural folds appear on the dorsal surface

and roll up to form the neural tube. By about thirty hours neurulation is complete and the tailbud begins to extend. During the next two days the brain and organs develop so that by three days the embryo has become a swimming tadpole. Their large size and rapid development outside the mother make *Xenopus* and other amphibians excellent systems for studying the very early events of vertebrate embryogenesis. The ability to overexpress specific genes by mRNA microincjection, followed by phenotypic and biochemical analysis, make *Xenopus* a particularly good system for analyzing the intercellular signaling mechanisms that control early development.

Classical embryological experiments

Amphibians have been used to study vertebrate embryology for over a century. In 1924, Hilde Mangold, working with Hans Spemann, performed an experiment that set the course for 20th century research aimed at understanding the formation of the vertebrate body plan. Working with newt embryos, she transplanted tissue from just above the dorsal blastopore lip of an early gastrula to the ventral side of another embryo, which then developed into a tadpole with a duplicated axis and two heads (Spemann and Mangold, 1924). By using differentially pigmented donor and host embryos, it was possible to determine that although the secondary notochord derived from the donor, much of the tissue in the new axis came from the host. This result indicated that the transplanted dorsal tissue had the ability to divert host tissue from its normal ventral fate to a dorsal fate, prompting Spemann to name this dorsal region the "organizer". More recently, these results have been confirmed in *Xenopus* embryos using lineage tracers to mark the donor and host tissue (Smith and Slack, 1983).

In another classical experiment, Nieuwkoop demonstrated that mesoderm does not develop autonomously but is instead an induced tissue. He found that when presumptive endodermal tissue was explanted from the vegetal pole of an axolotl embryo and cultured in contact with explanted animal pole tissue (the "animal cap"), the animal pole tissue was diverted from its normal ectodermal fate to a mesodermal fate (Nieuwkoop, 1969a). Blastula-stage fate maps show that mesoderm normally arises from the equatorial region of the embryo, or marginal zone, where the animal and vegetal hemispheres come together (Dale and Slack, 1987a; Moody, 1987). Together these results indicate that animal hemisphere cells of the marginal zone are induced to become mesoderm by the underlying vegetal cells and that this juxtaposition of animal and vegetal cells at the equator results in the correct placement of the future mesoderm.

In addition, Nieuwkoop found that the mesoderm induced in conjugates of presumptive endoderm and ectoderm possessed a dorsoventral polarity that reflected the polarity of the embryo from which the explants were taken. By rotating the animal and vegetal tissue by 180° with respect to one another, he was able to show that the dorsoventral polarity of the induced mesoderm was determined by the vegetal tissue (Nieuwkoop, 1969b). To further explore the regional differences within the vegetal tissue, vegetal explants were cut into dorsal, ventral, and lateral quadrants and combined with animal tissue. Boterenbrood and Nieuwkoop (Boterenbrood and Nieuwkoop, 1973) found that the dorsal quadrant most strongly induced the most dorsal mesoderm, such as notochord, and did not induce the most ventral mesoderm such as blood. The reverse was true for the ventral quadrant while the lateral quadrants were somewhat able to induce all types of mesoderm. These results indicate that prior to the time when organizer tissue can be isolated from the region of the dorsal

blastopore lip, the dorsoventral polarity of the embryo has been established by the concentration of dorsal mesoderm inducing activity on one side of the embryo.

Embryological approaches to understanding dorsoventral axis formation

In subsequent decades the results of Spemann and Nieuwkoop have been refined to provide a more precise picture of the source of dorsal mesoderm inducing activity within the early embryo. Gimlich and Gerhart (1984) transplanted vegetal blastomeres from 64-cell embryos into host embryos of the same stage and found that only the most dorsal vegetal cells could induce dorsal axis formation. In addition, using a fluorescent lineage tracer, they showed that the transplanted cells remained below the dorsal blastopore lip and never contributed to the ectopic axis. These experiments thus defined an organizer activity that was distinct from the Spemann organizer by its presence in the early blastula and by the fact that cells which themselves never contributed to the axis induced the formation of an axis by other cells. This activity in the dorsal vegetal cells was defined as the Nieuwkoop center, or early blastula organizer, which is necessary for the formation of the Spemann organizer or gastrula organizer (Fig. 1) (Gerhart *et al.*, 1991). Additional cell transplantation experiments have shown that this early dorsalizing activity is present at the 16-cell stage in a broad region on the dorsal side, with the highest activity in the most dorsal subequatorial cells (Kageura, 1990). It was also shown that cytoplasm from the same dorsal cells or from the vegetal pole of an unfertilized egg could induce axis formation when injected into a ventral cell of a 16-cell host (Fujisue *et al.*, 1993; Holowacz and Elinson, 1993) Furthermore, in the unfertilized egg the activity is concentrated in the cortical cytoplasm of the vegetal pole and decreases during the first cell cycle with a concomitant appearance of activity on the future dorsal side of the embryo

(Fujisue *et al.*, 1993). The activity on the dorsal side continues to increase from the two to sixteen cell stage. These results suggest that the initial establishment of the dorsoventral axis depends on the asymmetric localization of a dorsalizing activity to the future dorsal side of the embryo as a result of its displacement from the vegetal pole.

The indication that there is movement of a dorsalizing activity in the *Xenopus* embryo during the first cell cycle is consistent with the results of many experiments looking at movements within the egg after fertilization. In many amphibians a region known as the gray crescent appears above the equator of the fertilized egg opposite the SEP partway through the first cell cycle. Early experiments by Ancel and Vintemberger (1948) indicated that the gray crescent marks the future dorsal side of the embryo and that it arises as a result of a rotation of the thin outer cortex of the egg relative to the inner cytoplasm. This work has been confirmed and extended to show that in general the dorsal midline of the embryo arises within a region 120° to 180° from the SEP (Gerhart *et al.*, 1981), which is the location of the gray crescent. At the time when the gray crescent appears, the inner cytoplasm rotates relative to the cortex by approximately 30° (Vincent *et al.*, 1986). In general, the cytoplasm rotates toward the meridian defined by the SEP, or the future ventral side. When eggs are freely floating, the dense cytoplasm remains fixed while the egg cortex rotates around it. If, however, the cortex is fixed by embedding the egg in gelatin, the cytoplasm rotates within the cortex against the force of gravity. This suggested that the generation of force was necessary for cortical rotation and led to an examination of the microtubule networks within the fertilized egg. Elinson and Rowning (1988) found that midway through the first cell cycle a dense parallel array of microtubules appears in an orientation parallel to the axis defined by the SEP.

The microtubule array is assembled within a matter of minutes and disassembles by the first cleavage, consistent with its being involved in cortical rotation. In addition, all treatments that interfere with the formation of the microtubule array, such as drugs that depolymerize microtubules or ultraviolet (UV) radiation, also prevent cortical rotation (Malacinski *et al.*, 1977; Elinson and Rowning, 1988).

The results discussed above are consistent with a network of microtubules being responsible for cortical rotation. It has also been demonstrated that cortical rotation is necessary for the movement of the dorsalizing activity from the vegetal pole to the dorsal side and for the development of an embryo with a normal dorsoventral axis. Prior to the identification of the microtubule-based mechanism of cortical rotation, it had been shown that UV irradiation of the vegetal pole of fertilized eggs blocks cortical rotation and produces radially symmetric embryos completely lacking dorsal structures (Malacinski *et al.*, 1977). UV irradiation also prevents the movement of the dorsalizing activity from the vegetal pole to the dorsal side of the embryo (Fujisue *et al.*, 1993), providing an explanation for the effect of UV on *Xenopus* development. It was also shown that axis formation in embryos from UV irradiated eggs could be rescued by tilting the eggs by 90° for forty minutes (Scharf and Gerhart, 1980). This suggested that UV irradiation blocked a movement of material within the egg that was necessary for dorsal axis formation and that this movement could be induced by gravity in tilted eggs. In addition, tilting untreated eggs can override the bias for dorsal formation opposite the SEP, such that dorsal structures form on the side which was facing up (Gerhart *et al.*, 1981), and centrifugation of fertilized eggs in particular orientations results in duplication of the axis (Gerhart *et al.*, 1981; Black and Gerhart, 1986). These data support a model in which fertilization results in

the formation of a microtubule network that rotates the egg cytoplasm and cortex relative to one another, relocating a dorsalizing activity from the vegetal pole to the dorsal side. It is thought that the microtubules of the sperm aster serve to bias the orientation of the microtubule array such that the location of the SEP generally correlates with the future ventral side (Elinson and Rowning, 1988).

The molecular nature of the dorsalizing activity is not known, although possibilities will be discussed below. It should be noted however, that there is evidence that the chemical nature of the activity may change during oogenesis. Irradiation of oocytes, followed by maturation and fertilization also results in the development of embryos without dorsal structures (Holwill *et al.*, 1987). However, in contrast to embryos from UV irradiated eggs, axis formation in embryos from eggs that had been UV irradiated as oocytes is not rescued by tilting (Elinson and Pasceri, 1989), and vegetal cortical cytoplasm from these eggs has no dorsalizing activity (Holowacz and Elinson, 1993). Further characterization has shown that the transition of the vegetal cytoplasm from UV-sensitive to UV-insensitive occurs during the process of oocyte maturation (Holowacz and Elinson, 1995). This suggests that in immature oocytes the dorsalizing activity itself may be destroyed by UV irradiation while after fertilization only the mechanism of movement is damaged, indicating that the nature of the activity changes during oocyte maturation. One possibility is that the activity is translated from RNA to protein during maturation. Alternatively, a protein could undergo post-translational modification during this time with the modification process being sensitive to UV.

Mesoderm induction

Prior to a discussion of molecular candidates for the dorsalizing activity identified in the *Xenopus* embryo, it is necessary to discuss what is known about

the molecular nature of mesoderm induction, since dorsalization is first manifest as a dorsalization of mesoderm. The experiments of Nieuwkoop, demonstrating an induction of mesoderm by prospective endoderm, led to a search for molecules that could mimic this activity. It was found that members of the fibroblast growth factor (FGF) (Kimelman *et al.*, 1988; Slack *et al.*, 1988; Isaacs *et al.*, 1992) and transforming growth factor β (TGF-β) (Rosa *et al.*, 1988; Smith *et al.*, 1990; Thomsen *et al.*, 1990; van den Eijnden Van Raaij *et al.*, 1990) families could both induce animal cap prospective ectoderm to form mesoderm. Furthermore, it was found that while FGF's could induce ventral and lateral mesoderm (Green *et al.*, 1990; Isaacs *et al.*, 1992), certain TGF-β family members, such as activin, could induce the full range of mesodermal tissues, including the most dorsal (Green and Smith, 1990; Green *et al.*, 1992; Thomsen and Melton, 1993). These activities are consistent with the previously proposed three-signal model, in which it was postulated that one signal was responsible for inducing dorsal mesoderm (the organizer), a second signal induced ventral and lateral mesoderm, and that subsequently a third signal from the dorsal mesoderm spread laterally to pattern the lateral mesoderm (Dale and Slack, 1987b). The results of subsequent experiments have led to a modification of this model.

The necessity for both FGF and activin-like activities for normal mesoderm induction was demonstrated by expressing dominant-negative forms of the FGF and activin receptors. Embryos expressing the dominant-negative FGF receptor are lacking all trunk mesoderm, although head formation is relatively normal (Amaya *et al.*, 1991). Expression of the dominant-negative activin receptor results in embryos that lack all mesoderm. Although these results demonstrate that both FGF and activin-like signals are required for mesoderm induction, they fail to define clearly separable roles for the two types

of signals as both FGF and activin-like signals are apparently required for trunk mesoderm formation. An exploration of these results led to the finding that FGF signaling is necessary for the induction of many activin responsive genes (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). This complex relationship between FGF and activin-like signaling is not consistent with a model in which FGF induces ventral-lateral mesoderm and activin-like signals induce dorsal mesoderm, suggesting that the three-signal model is too simplistic.

In addition, another group of signaling molecules has been identified which do not themselves induce mesoderm but can dorsalize the mesoderm induced by FGF or activin. These factors will be discussed below but their existence suggests that, in the embryo, mesoderm may be regionally patterned by combinations of overlapping signals rather than by single factors acting within specific regions (Fig. 1) (Kimelman *et al.*, 1992). Consistent with this model, it has been shown that the dorsalizing activity present in the vegetal cortical cytoplasm of the fertilized egg does not induce mesoderm itself but will dorsalize the mesoderm induced by FGF (Holowacz and Elinson, 1995).

Candidate dorsalizing molecules

By their ability to induce a dorsal axis when ectopically expressed in vegetal cells, several molecules have been identified as candidates for the endogenous dorsalizing activity. These secreted factors include members of the *Wnt* family, noggin, and Vg1, a vegetally localized TGF-β family member. Although I will briefly describe the other factors, I will focus on the *Wnt* family since this thesis and other work in recent years has implicated the Wnt signal transduction pathway in endogenous axis formation. *Noggin* is ubiquitously expressed at low levels maternally. After MBT the levels of *noggin* transcript increase dramatically with localization to the region of the Spemann organizer

and later the neural tube and future head mesoderm. Ectopic expression of *noggin* RNA prior to MBT induces full axis formation, as would be expected for the early dorsalizing activity (Smith and Harland, 1992). However, noggin expressed after MBT also has axis inducing activity and soluble noggin can dorsalize mesoderm explanted from the ventral marginal zone of a gastrula stage embryo, properties that would be expected of signal emitted from the Spemann organizer itself (Smith *et al.*, 1993). In addition, noggin can directly induce ectoderm to form neural tissue, in the absence of any mesoderm (Lamb *et al.*, 1993). Considering the activities of noggin and its high level of expression in the dorsal mesoderm, it is likely that noggin accounts at least partly for the activities of the Spemann organizer rather than the early blastula organizer. Vg1 transcripts are localized to the vegetal cortex until oocyte maturation, resulting in the vegetal localization of Vg1 protein in the early embryo (Weeks and Melton, 1987). However, none of the Vg1 protein detected in the embryo is subjected to necessary proteolytic processing (Massagué, 1987; Dale *et al.*, 1989; Tannahill and Melton, 1989). By fusing Vg1 to precursor sequences from related proteins that were known to be processed, it was demonstrated that Vg1 is a potent mesoderm inducer and can induce axis formation when expressed in vegetal cells (Dale *et al.*, 1993; Thomsen and Melton, 1993). It has been suggested that localized processing of Vg1, initiated by cortical rotation, could produce the early dorsalizing signal in the embryo (Thomsen and Melton, 1993).

The founding members of the *Wnt* family, the mouse proto-oncogene *int-1* (now called *Wnt-1*) and the *Drosophila* segment polarity gene *wingless* (*wg*), have since been joined by a large family of vertebrate *Wnts* (reviewed in (McMahon, 1992; Nusse and Varmus, 1992)). The *Xenopus Wnt* (*Xwnt*) family includes at least a dozen members (Ku and Melton, 1993; Moon, 1993). Evidence that *Wnts* might

be important in *Xenopus* development came from the finding that ectopic expression of mouse Wnt-1 induces a secondary axis in *Xenopus* embryos (McMahon and Moon, 1989). Several *Xwnts*, including Xwnt-8, were subsequently identified that had the same activity (Smith and Harland, 1991; Sokol *et al.*, 1991). As would be expected for a factor involved in the initial dorsalization of the embryo, Xwnt-8 can induce axis formation when it is expressed in vegetal cells that will not contribute to the axis (Smith and Harland, 1991). In addition, it was shown that Xwnt-8 is not able to induce mesoderm when it is ectopically expressed in presumptive ectoderm. However, the mesoderm formed when Xwnt-8 expressing ectoderm is exposed to FGF is of a much more dorsal type than that induced by FGF in ectoderm from uninjected embryos (Christian *et al.*, 1992). Although these experiments suggest that a member of the *Xwnt* family would be a good candidate for the endogenous early dorsalizing activity, no *Xwnt* with the activity of Xwnt-8 has been identified that is expressed at the right time and place to act as the endogenous signal. *Xwnt-8* itself is not maternally expressed. *Xwnt-8* is zygotically expressed on the ventral side of the embryo and ectopic expression of Xwnt-8 after MBT causes anterior defects which are quite distinct from the dorsalizing effects of early ectopic expression (Christian *et al.*, 1991a; Christian and Moon, 1993). Of the two maternally expressed *Xwnts* with dorsalizing activity, *Xwnt-8b* is expressed more animally than vegetally (Massagué, 1987; Dale *et al.*, 1989), and Xwnt-11 is unable to induce a complete axis (Ku and Melton, 1993). Thus the question remains whether the endogenous dorsalizing activity is encoded by a maternal *Xwnt* that has not yet been identified, or whether ectopically expressed Xwnts simply mimic an unrelated endogenous signal.

Perturbation of signaling pathways to study endogenous functions

As described above, the ability of multiple factors to accomplish the same task, namely the initial establishment of the dorsoventral axis in *Xenopus*, makes it difficult to determine which factors act endogenously. Just because Xwnt-8, noggin and Vg1 can induce axis formation does not mean that they do so in the embryo. In order to show that a factor is necessary for a particular developmental process, it is necessary to remove the factor from the organism or perturb its function in some way. Ideally this is done genetically, by screening for or targeted mutation of genes encoding the secreted factor or a component of the relevant signal transduction pathway. However, the long generation time and partially duplicated genome of *Xenopus* make it unsuitable for genetic manipulation. As an alternative approach, the ectopic expression of dominantly interfering mutant proteins (dominant-negative mutants) has been quite successful. As described above, dominant-negative mutants of the FGF and activin receptors were used to show that both FGF and activin-like signals are required for mesoderm induction (Amaya *et al.*, 1991; Hemmati-Brivanlou and Melton, 1992). This approach took advantage of the fact that these transmembrane receptors normally function as dimers. Ectopically expressed mutant receptors which lack the intracellular kinase domain are thought to dimerize with endogenous wild-type receptors, creating non-functional receptor dimers. Intracellular kinases, which act as components of receptor-mediated signal transduction pathways, can be inactivated by mutation of a conserved lysine residue in the ATP-binding region of the kinase domain and in several cases this has resulted in mutant proteins with dominant negative activity. This approach was used to show that Raf-1 is necessary for FGF-mediated mesoderm induction in *Xenopus* (MacNicol *et al.*, 1993). Further studies identified a residue

in Raf-1 that is essential for binding by the upstream activator Ras and demonstrated that the dominant-negative effect of the kinase-inactive Raf-1 is dependent on this binding, suggesting that the dominant-negative mutant acts by titrating Ras (Fabian *et al.*, 1994). Dominant-negative mutants of Src and Fyn kinases were also shown to act by blocking access to an upstream activator (Twamley-Stein *et al.*, 1993). Thus, if a suitable target can be identified, analysis of the effects of a dominant-negative mutant can be very useful in the determination of whether the target is required for a particular process.

Wg pathway

At the time this project was undertaken, the mechanism of vertebrate Wnt signaling was unknown. However, the *Drosophila* gene *wg* encodes a member of the *Wnt* family which has axis-inducing properties when expressed in the *Xenopus* embryo (Chakrabarti *et al.*, 1992). Several genes have been identified that potentially act downstream of *wg*, including *zeste-white 3/shaggy* (*zw3/sgg*), *armadillo* (*arm*), and *dishevelled* (*dsh*) (Siegfried *et al.*, 1992; Noordermeer *et al.*, 1994; Siegfried *et al.*, 1994) (Fig. 2). *Zw3/sgg* encodes a cytoplasmic Ser/Thr kinase whose effects are antagonized by *wg* signaling (Bourouis *et al.*, 1990; Siegfried *et al.*, 1992). *Drosophila* genetic studies have indicated that Wingless signaling acts via the novel protein Dsh (Noordermeer *et al.*, 1994; Siegfried *et al.*, 1994) to antagonize Zw3/Sgg, which in turn acts to oppose Arm (Noordermeer *et al.*, 1994). Thus Wg signals by inactivating a negative regulator, Zw3/Sgg. Wg signaling acts via Arm at several points during *Drosophila* development to control cell fate decisions (reviewed in Klingensmith and Nusse, 1994)It was not known at this time whether the vertebrate Wnts would signal through a pathway composed of homologs of the proteins thought to act in Wg signaling but two of these components were known to have vertebrate homologs. The vertebrate

homolog of Arm, β-catenin, had been identified as a component of adherens junctions and was thought to be important for cell adhesion (McCrea *et al.*, 1991). In addition, rather surprisingly, antibodies to β-catenin produced a duplicated axis when injected into the ventral side of a *Xenopus* embryo (McCrea *et al.*, 1993). *Zw3/sgg* was found to encode a homolog of the mammalian glycogen synthase kinase 3 (GSK-3) (Siegfried *et al.*, 1992).

GSK-3

GSK-3 was originally identified as a serine/threonine kinase involved in glycogen metabolism (Cohen, 1985). The activity of GSK-3 is inhibited by insulin signaling in mammalian tissue culture cells (Sutherland *et al.*, 1993). In addition to the metabolic enzymes glycogen synthase (Cohen, 1985) and ATP-citrate lyase (Hughes *et al.*, 1992), an enormous array of GSK-3 substrates has been identified, including the transcription factors c-jun (Boyle *et al.*, 1991; de-Groot *et al.*, 1993; Nikolakaki *et al.*, 1993), c-myc (Saksela *et al.*, 1992), c-myb (Woodgett, 1991), and CREB (Fiol *et al.*, 1994), translational initiation factor eIF-2B (Welsh *et al.*, 1996), the regulatory subunit of Mg-ATP-dependent protein phosphatase-1 (Vandenheede *et al.*, 1980; Hemmings *et al.*, 1981), and the microtubule associated protein tau (Hanger *et al.*, 1992; Mandelkow *et al.*, 1992). A member of the proline-directed class of kinases, GSK-3 often phosphorylates sequential sites on substrates with the consensus sequence SXXXS (Fiol *et al.*, 1987). In general, phosphorylation by GSK-3 appears to inhibit the function of substrates. In addition to two mammalian GSK-3 isoforms, GSK-3α and β, and *Drosophila zw3/sgg*, GSK-3 homologs have also been identified in yeast and plants (Bianchi *et al.*, 1993; Pay *et al.*, 1993; Bianchi *et al.*, 1994; Puziss *et al.*, 1994). The wide variety of GSK-3 substrates suggests that GSK-3 may be involved in multiple biological processes. The conservation of *zw3/sgg*/GSK-3 among species and its regulation

by Wg and other growth factors suggest that it could be involved in intracellular signaling in *Xenopus*, perhaps during dorsoventral axis determination.

Wnt activities in other aspects of *Xenopus* development

Although I have focused on the activity of Xwnts during the initial specification of the dorsoventral axis, most *Xwnts* are expressed zygotically rather than maternally and are thus likely to be involved in other aspects of development. As discussed above, endogenous *Xwnt-8* is expressed in ventral mesoderm and its overexpression causes head defects, suggesting that it may act as a ventralizing agent during gastrulation (Christian and Moon, 1993). Several *Xwnts* have restricted expression patterns in the nervous system, suggesting a role for Xwnts in neural development (reviewed in Moon, 1993). This hypothesis is supported by the fact that deletion of the *Wnt-1* gene in mice results in the loss of particular structures in the brain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Thus, if a *Xenopus* GSK-3 homolog were to act during dorsoventral axis determination as part of the signaling pathway used by Xwnts, it is likely that it would also play a role in Xwnt signaling later in development. In addition, a second class of *Xwnts*, as typified by *Xwnt-5a*, lacks dorsalizing activity and instead causes head and tail defects when expressed in dorsal blastomeres and reduces the morphogenetic movements associated with dorsal mesoderm (Moon *et al.*, 1993). It appears that the *Xwnt-8* and *Xwnt-5a* classes operate through distinct signal transduction pathways (Slusarski *et al.*, 1997).

Summary

Although an activity has been shown to reside in the dorsal equatorial cells of cleavage stage embryos, the molecular nature of this activity has not been determined. Molecules of the *Wnt* family have been implicated in this process,

but it is not known whether a maternal *Wnt* is involved in endogenous axis formation. It is also not known whether molecules shown to be involved in signaling by the *Drosophila Wnt* homolog *wg*,, such as *zw3/sgg*,, are involved in *Wnt* signaling in *Xenopus* during dorsoventral axis formation or other developmental processes. These questions are the subject of this dissertation.

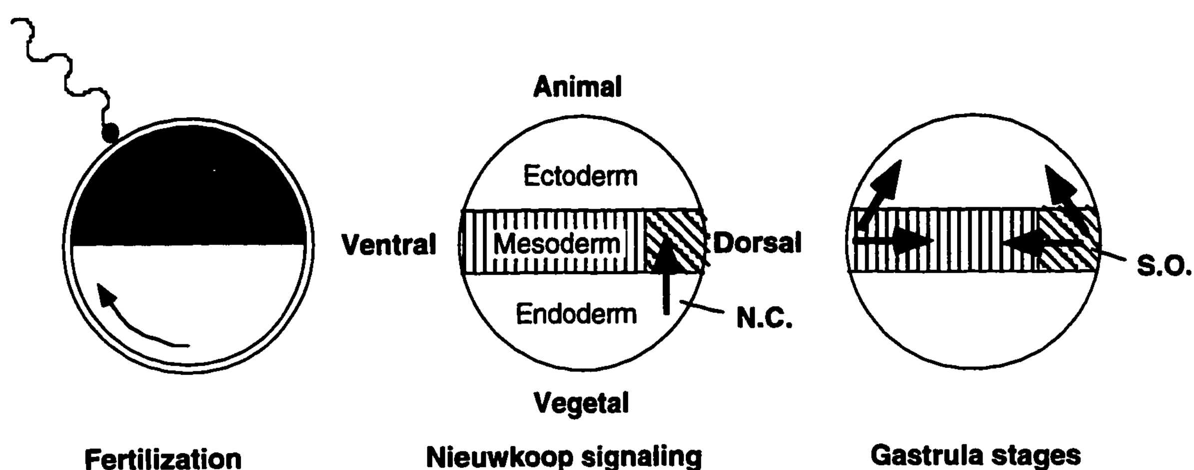

Figure 1: Establishment of the *Xenopus* body plan. Fertilization generates a rotation of the egg cytoplasm relative to the cortex (cortical rotation) which establishes the Nieuwkoop signaling center in the vegetal cells of the future dorsal side (N.C.). Intercellular signaling from cells of the future endoderm to cells in the middle of the embryo position the mesoderm at the equator (signals not shown). Signals from the N.C. establish the most dorsal mesoderm as an additional signaling center, the Spemann organizer (S.O.). During the gastrula stages, signals from the S.O. and the ventral mesoderm further pattern the mesoderm and ectoderm.

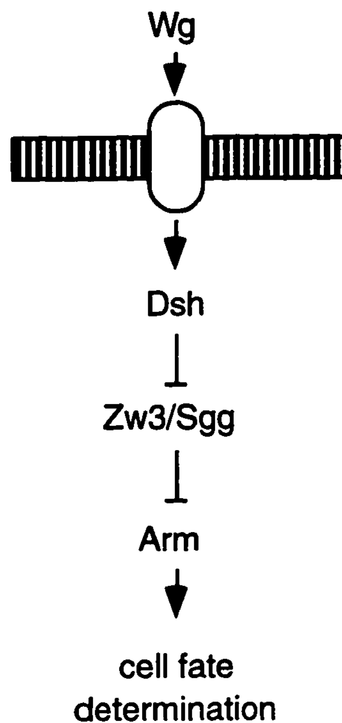

Figure 2: The *Drosophila* Wingless pathway. The secreted factor Wingless (Wg) influences cell fate through an intracellular pathway that includes Dishevelled (Dsh), Zeste white-3/Shaggy (Zw3/Sgg), and Armadillo (Arm). Dsh opposes the activity of Zw3/Sgg, which in turn opposes Arm. Thus, Wg activity relieves the repression of Arm by Zw3/Sgg.

Chapter 1: Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3

Introduction

When this study began it was known that several factors could induce the formation of a secondary axis when ectopically expressed on the ventral side of a *Xenopus* embryo. These included certain members of the *Wnt* family, noggin, and the constitutively processed form of Vg1. Although these factors had been shown to have axis-inducing activity, none of them had been shown to be necessary for endogenous axis formation. It was possible that they were simply mimicking the endogenous dorsalizing signal. To demonstrate an endogenous role for a factor, it would be necessary to show that perturbation of the intracellular signaling pathway used by the factor disrupted axis formation. Because little was known about the signaling mechanisms of noggin and Vg1, and because of the likely involvement of Vg1 in mesoderm induction, the role of these factors in dorsal axis formation was not amenable to testing by this method. The *Wnt* family, however, is homologous to the product of the *Drosophila wg* gene and the intracellular signaling pathway used by Wg had been well characterized genetically. It was not known whether the vertebrate Wnts would signal through a homologous pathway. In order to test this and as a tool to attempt to perturb dorsal axis formation, I decided to clone and study the *Xenopus* homolog of the *Drosophila zw3/sgg* gene, which encodes a Ser/Thr kinase that acts as a negative regulator of Wg signaling.

Results

Using a PCR-based approach, I isolated a cDNA encoding the *Xenopus* homolog of *zw3/shaggy* and mammalian GSK-3, which I named *Xgsk-3*. RNA

analysis using the RNase protection assay demonstrated that *Xgsk-3* is present as a maternal transcript (Appendix B, Fig. 2A) and is not localized within the embryo during the early or late blastula stages (Appendix B, Fig. 2B).

To study the function of Xgsk-3, I attempted to create a dominant inhibitory mutant, which I hoped would interfere with the intracellular pathway utilized by Xgsk-3. This approach has been successfully used to demonstrate a role for Ras and Raf in mesoderm induction during *Xenopus* development (Whitman and Melton, 1992; MacNicol *et al.*, 1993). Dominant-negative mutants of some kinases have been produced by converting an invariant lysine in the ATP-binding site to an arginine residue (MacNicol *et al.*, 1993), a mutation that eliminates the activity of the kinase. When I injected RNA encoding the kinase-inactive mutant of Xgsk-3, Xgsk-3K->R, into the ventral side of two to four-cell stage *Xenopus* embryos, 86% of tadpoles developed with secondary axes (Appendix B, Fig. 3). Injection of *Xgsk-3K->R* RNA into the dorsal side of embryos did not produce an observable change in embryonic development. In addition, ectopic expression of Xgsk-3K->R rescued dorsal axis formation in embryos ventralized by UV irradiation. Importantly, the dorsalizing effect of *Xgsk-3K->R* RNA injection could be eliminated by co-injecting a two-fold excess of RNA encoding the wild-type Xgsk-3, demonstrating that the dorsalizing effect was specifically caused by Xgsk-3K->R and supporting the proposal that Xgsk-3K->R acts as a dominant-negative mutant. In contrast, injection of wild-type *Xgsk-3* RNA on the dorsal side interferes with normal axis formation. These embryos develop with a range of ventralized phenotypes from reduced eyes to severe head truncations. However, I was unable to cause complete ventralization, such as results from UV irradiation, by expression of Xgsk-3 on the dorsal side. Using whole-mount in situ hybridization, I showed that injection of *Xgsk-3K->R*

RNA on the ventral side or *Xgsk-3* RNA on the dorsal side results in ectopic expression or decreased expression, respectively, of the *Xnot* and *goosecoid* genes (Appendix B, Fig. 5). These genes are early markers for the Spemann organizer.

Using the *Xenopus* animal cap assay, I found that Xgsk-3K->R does not induce mesoderm on it own, although it can dorsalize the mesoderm induced by bFGF (Appendix B, Fig. 2C). In addition, I found that Xgsk-3K->R can induce dorsal axis formation when it is expressed in the deep vegetal cells, descendants of which do not themselves contribute to the axis (Appendix B, Fig. 6).

Discussion

Several important conclusions can be drawn from the fact that the inactive form of Xgsk-3, Xgsk-3K->R, can induce axis formation in the *Xenopus* embryo. Because this effect is identical to the effect of ectopic expression of certain Wnts (Smith *et al.*, 1991a; Sokol *et al.*, 1991), this result indicates that vertebrate Wnts signal through a pathway that is homologous to the *Drosophila wg* pathway. This conclusion is supported by the identification of *Xenopus* homologs of two other pathway components which are positive regulators of Wg signaling. Xdsh, the Dsh homolog, and β-catenin, the Arm homolog, can both induce formation of a secondary axis when ectopically expressed on the ventral side of embryos (Guger and Gumbiner, 1995; Sokol *et al.*, 1995). These results also indicate that Xgsk-3K->R acts as a dominant-negative mutant. By analogy to the action of Zw3/Sgg as a negative regulator of Wg signaling, I would predict that blocking the function of Xgsk-3 would mimic activation of the pathway, or mimic the effect of Wnt, which is exactly the result I obtained. Most importantly, this result suggests that the *Wnt* pathway is involved in endogenous axis formation and, more specifically, that blocking the action of Xgsk-3 is sufficient for axis formation. The fact that excess Xgsk-3 on the dorsal side interferes with axis

formation indicates that blocking the action of Xgsk-3 is also necessary for axis formation. In support of the conclusion that the *Wnt* pathway is necessary for axis formation, it has been shown that β-catenin, which is genetically downstream of Xgsk-3, is essential for this process. The embryos that developed from oocytes in which maternal β-catenin mRNA had been depleted with antisense oligonucleotides completely lacked axial structures (Heasman *et al.*, 1994).

Perhaps the most surprising result of these experiments is the finding that Xgsk-3K->R can act non-cell-autonomously to induce axis formation. Wnt also has this property (Smith and Harland, 1991), which is consistent with the hypothesis that a Wnt could represent the activity of the Nieuwkoop signaling center and act to induce formation of the Spemann organizer (Gimlich and Gerhart, 1984). In support of this, perturbation of Wnt signaling by ectopic expression of Xgsk-3 and Xgsk-3K->R perturbs the expression of early organizer genes, indicating that the initial induction of the organizer is being affected. However, because Wnts are secreted factors, they are expected to act on cells other than the ones in which they are expressed, whereas Xgsk-3 is expected to act in the cells receiving the Wnt signal, cells which will contribute to the organizer and ultimately to the dorsal axis. The ability of Xgsk-3K->R to act non-cell-autonomously indicates that the action of the Nieuwkoop signaling center involves an additional intercellular signal downstream of that mediated by the *Wnt* pathway. It also suggests that the effects of Wnt may generally be mediated by subsequent intercellular signaling. This is supported by experiments with the Drosophila leg imaginal disc, showing that clones of cells that have lost the *zw3/shaggy* function have all the properties of Wg-expressing cells, including the

ability to determine the fate of cells around them (Diaz-Benjumea and Cohen, 1994).

In summary, these experiments have demonstrated that the establishment of the *Xenopus* dorsoventral axis is mediated by the *Wnt* pathway and suggest a model in which the pathway is selectively activated on the dorsal side, resulting in a localized reduction of Xgsk-3 activity (Fig. 3). Many questions, however, remain to be answered. These experiments have not addressed the mechanism by which the *Wnt* pathway is activated during axis formation. The simplest model would be that it is activated by a maternal Wnt that has not yet been identified. Alternatively, or in addition, noggin or Vg1 could be involved in activating the pathway. It is also possible that the specialized process of cortical rotation activates the *Wnt* pathway by an intracellular mechanism, dispensing with the need for a secreted signal. This is an attractive possibility since the signaling that establishes the axis, which is triggered by cortical rotation prior to egg cleavage, is thought to take place during the very early cleavage stages when only a small number of cells are present to participate in intercellular signaling. Thus, the mechanism by which Xgsk-3 activity is inhibited or blocked also remains to be elucidated. In *Drosophila, dsh* has been genetically defined as upstream of *zw3/sgg* but *dsh* encodes a novel protein of unknown function and a direct interaction between the two proteins has not been demonstrated. In contrast, the mechanism by which Xgsk-3 regulates β-catenin has begun to be elucidated. In *Drosophila, arm* is genetically downstream of *zw3/sgg* and Arm protein is apparently activated as a result of Zw3/Sgg inactivation. It has now been shown that Wnt/Wg signaling regulates the stability of Arm/β-catenin such that in the presence of Wnt/Wg signaling or the dominant-negative Xgsk-3 mutant, Arm/β-catenin accumulates in the cytoplasm and nuclei of cells (Peifer *et al.*, 1994b; Yost

et al., 1996). In *Xenopus* it has been shown that the maintenance of a low level of cytoplasmic β-catenin in the absence of Wnt signaling is dependent on the presence of a GSK-3 consensus phosphorylation site at the N-terminus of β-catenin. β-catenin is an in vitro substrate for Xgsk-3 and the removal of the GSK-3 phosphorylation site generates a molecule that is more stable and hence more active in vivo (Yost *et al.*, 1996).

The recently identified homeobox-containing transcription factor *siamois* appears to be a downstream effector of the Wnt pathway. *Siamois* is expressed on the dorsal side just after the onset of zygotic transcription at the mid-blastula transition (MBT). Injection of a very small amount of ectopic RNA encoding siamois is sufficient to induce secondary axis formation on the ventral side in a non-cell-autonomous fashion, similar to the activity of components of the Wnt pathway (Lemaire *et al.*, 1995). The spatial expression pattern and activity of *siamois* make it a good candidate for an effector of Wnt signaling. However, the timing of *siamois* expression was a surprise and has modified our thinking about the sequence of events leading to Spemann organizer formation. Initially it was thought that a Wnt-like signal directly specified the fate of cells that would become part of the organizer and contribute to the embryonic axis. The non-cell-autonomous effect of Xgsk-3K->R challenged this notion and suggested that Wnt was triggering the release of an additional secreted signal that was acting on presumptive organizer cells. I expected, however, that this was taking place during the early cell divisions, prior to MBT, and that presumptive organizer cells were thus poised to carry out their fate when transcription began. The discovery of *siamois* suggests that presumptive organizer cells do not receive an inductive signal until after MBT when the transcription of *siamois* is required for the release of this signal (Fig 3). The nature of the downstream signal generated

by the *Wnt* pathway remains unknown, although its apparent action after the onset of zygotic transcription means that there are many potential candidates.

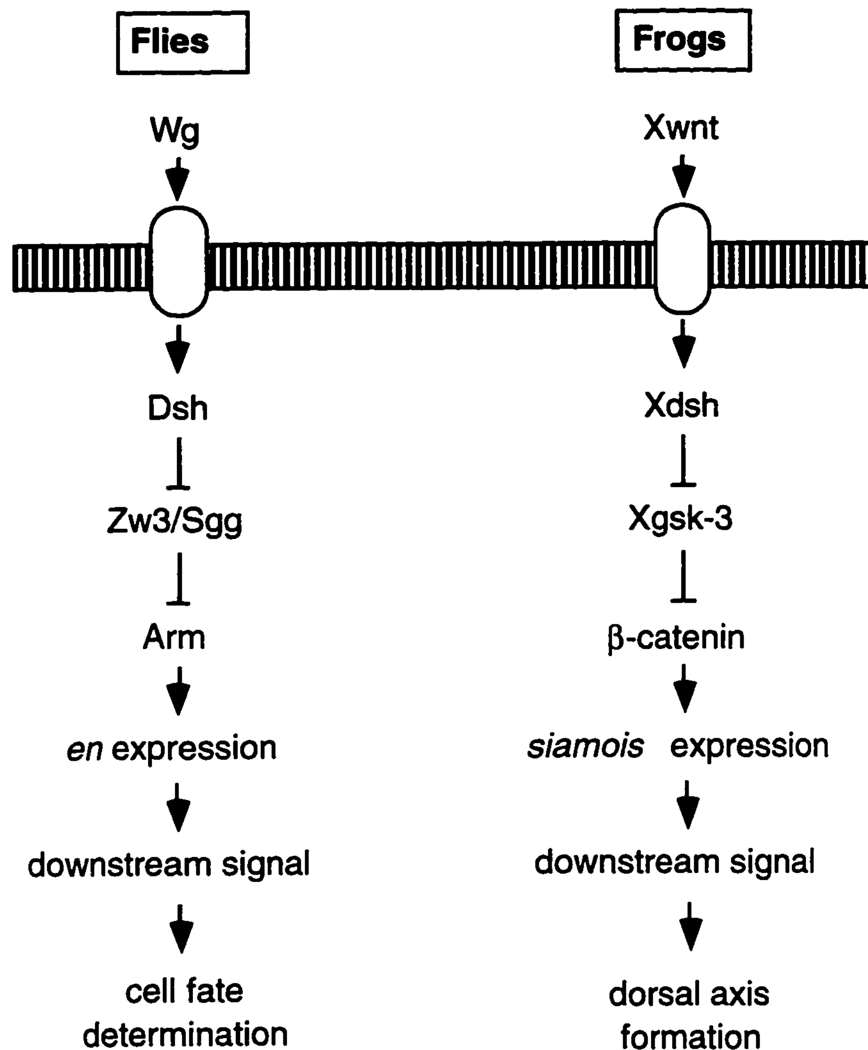

Figure 3: The Wg/Xwnt pathway. The *Xenopus* Wnt (Xwnt) pathway is homologous to the *Drosophila* Wg pathway and includes *Xenopus* dishevelled (Xdsh), Xgsk-3 and β-catenin. This pathway induces *siamois* transcription and acts via an unknown downstream signal to induce dorsal axis formation. During *Drosophila* development Wg signaling induces *en* transcription and acts via a downstream signal to influence cell fate.

Chapter 2: Regulation of Xgsk-3 by the novel GSK-3 binding protein GBP

Introduction

In the previous chapter I showed that Xgsk-3, the *Xenopus* homolog of *Drosophila* Zw3/Sgg and mammalian GSK-3, is a component of the Wnt signaling pathway and that this pathway is involved in dorsoventral axis determination in the *Xenopus* embryo. The regulatory events that take place downstream of Xgsk-3 have started to become clear with the discovery that Xgsk-3 regulates the stability of β-catenin and the identification of the downstream transcription factor *siamois*. What is still unknown is how the activity of Xgsk-3 is inhibited or opposed during axis formation. It is probable that Xgsk-3 can be regulated by Wnt, since overexpression of Xgsk-3 inhibits the axis-inducing activity of *Xwnt-8* (He *et al.*, 1995) and overexpression of a dominant-negative Xgsk-3 mutant entirely mimics the activity of Wnts [Appendix B; (Dominguez *et al.*, 1995; He *et al.*, 1995)] By comparison to *Drosophila*, it is likely that this is mediated by Xdsh, which has similar activity to Wnts (Sokol *et al.*, 1995). Dominant-negative mutants of *Xwnt-8* and *Xdsh* have recently been constructed that can block ectopic axis induction by *Xwnt-8* and *Xdsh*, respectively. However, these mutants have no effect on the initial establishment of the endogenous dorsoventral axis, although each has effects on later stages of development (Hoppler *et al.*, 1996; Sokol, 1996). This indicates that Wnt and Xdsh are not necessary for dorsoventral axis determination and suggests that Xgsk-3 is regulated by some other mechanism in response to cortical rotation, perhaps one that is entirely intracellular.

To investigate the regulation of Xgsk-3, a yeast two-hybrid approach was taken to identify proteins that physically interact with Xgsk-3. I initiated this

project by constructing a yeast two-hybrid library in collaboration with Michelle Chen in the laboratory of Jonathan Cooper. The rest of the project has been carried out in collaboration with Cynthia Yost and Hank Farr in the Kimelman laboratory. In this chapter I will describe the identification and characterization of a GSK-3 binding protein, GBP, and present our model for how GBP may regulate Xgsk-3 during establishment of the *Xenopus* dorsoventral axis.

Results

Isolation of GBP by yeast two-hybrid library screening: The yeast two-hybrid system allows the identification of novel proteins that physically interact with a protein of interest. Briefly, yeast are transformed with an expression plasmid encoding the protein of interest (the "bait") fused to a DNA binding domain that recognizes a specific DNA sequence. A library of cDNA sequences is ligated into an expression plasmid that encodes a library of proteins fused to a strong transcriptional activation domain and this library is coexpressed in yeast with the bait plasmid. If a protein encoded by a library plasmid interacts with the bait protein, a functional transcriptional activator is reconstituted, allowing transcription of a reporter gene positioned downstream of multiple binding sites for the DNA binding domain of the bait fusion. We used a modification of the original two-hybrid system (Chien *et al.*, 1991), designed by Hollenberg and coworkers (Hollenberg *et al.*, 1995). Fusion proteins containing the LexA DNA binding domain or the VP16 transcriptional activation domain are coexpressed in yeast strain L40 (Hollenberg *et al.*, 1995), containing *HIS3* and *lacZ* reporter genes under the control of multiple LexA operators. Construction of a random-primed cDNA library from *Xenopus* late stage oocyte and unfertilized egg mRNA was essentially as described (Hollenberg *et al.*, 1995). cDNA fragments of 500 to 1300 bp were selected from the original cDNA pool, amplified by PCR via added

linkers and the amplified cDNA was used to construct a library of 1.4×10^7 clones. The plasmid used to express the LexA-Xgsk-3 fusion protein, BTM116 (Bartel *et al.*, 1993), was modified to reduce the copy number per cell thus reducing the background in the screen. We screened 5×10^6 library transformants for the expression of proteins that interact with Xgsk-3 and obtained 41 positive clones that were capable of growing without histidine and were β-galactosidase positive. The specificity of the interactions was verified by the lack of interaction of the library clone with a heterologous fusion protein, LexA-Lamin. Of the 18 clones that interacted with LexA-Xgsk-3, but not with LexA-Lamin, we chose to focus on the 13 identical clones that interacted with Xgsk-3 more strongly than the others, as judged by their higher β-galactosidase activity. The interacting protein encoded by the library clones was designated GBP (GSK-3 binding protein). To obtain the full-length coding sequence for GBP, the 274 bp fragment isolated from the library plasmid was used as a probe to screen a *Xenopus* oocyte cDNA library (Rebagliati *et al.*, 1985) from which several clones were isolated. The GBP cDNA is predicted to encode a novel protein of 169 amino acids. The GBP cDNA fragment isolated in the two-hybrid screen encodes approximately the C-terminal half of the protein. Sequence database searching did not identify any full-length GBP homologs or any known protein motifs within GBP, but a human expressed sequence tag (EST) with homology to GBP was identified. An alignment of the predicted amino acid sequences of GBP and the human EST are shown in Figure 4.

GBP interacts with Xgsk-3 in *Xenopus* embryos: In order to determine whether the interaction between Xgsk-3 and GBP identified in the two-hybrid screen could also occur in *Xenopus* embryos, RNA expression constructs were made encoding Xgsk-3 containing the *myc* epitope tag (Xgsk-3-*myc*) and GBP

containing the FLAG epitope tag (GBP-FLAG). RNA encoding these constructs was injected, separately or in combination, into the animal pole of both cells at the two-cell stage. After approximately 4 hours, the embryos were lysed and proteins were immunoprecipitated with antibodies against the *myc* or FLAG epitope. Immunoprecipitated proteins were analyzed by polyacrylamide gel electrophoresis and immunoblotting with anti-*myc* or anti-FLAG antibodies. Figure 5 shows that Xgsk-3-*myc* can be detected in anti-FLAG immunoprecipitates and GBP-FLAG in anti-*myc* immunoprecipitates only when the two proteins are coexpressed (Fig. 5, lanes 6 and 14). When expressed alone, each protein is only immunoprecipitated by the antibody appropriate for its epitope tag (Fig. 5, compare lanes 1 and 5, and lanes 15 and 19). A small amount of Xgsk-3-*myc* is precipitated by the Protein G Sepharose beads in the absence of any antibody but this is significantly less that the amount that is coprecipitated with GBP-FLAG. These results confirm that GBP specifically interacts with Xgsk-3 and demonstrate that this interaction can occur in the *Xenopus* embryo.

GBP is a maternal gene whose product accumulates after oocyte maturation: In order to determine whether the GBP transcript is present maternally, as would be predicted for a gene that is involved in dorsoventral axis determination, RNA was isolated from *Xenopus* oocytes and early embryos and analyzed by the RNase protection assay. Using a probe derived from the 5' end of the GBP cDNA, we found that GBP was expressed in oocytes and early embryos (Fig. 6A). GBP transcript levels declined dramatically after the blastula stages and were detected at very low levels through the late tailbud stages. To determine whether GBP transcripts are localized in the early *Xenopus* embryo, RNA was isolated from dissected embryos and analyzed by the RNase protection assay. When 4 to 8-cell-stage embryos were dissected into dorsal and ventral

halves and 16 to 32-cell-stage embryos were dissected into animal and vegetal halves, GBP transcripts were found to be expressed at similar levels in all parts of the embryo (Fig. 6B).

In the course of our experiments, we found that when γ^{32}P-ATP was added to anti-*myc* immunoprecipitates from embryos expressing ectopic Xgsk-3-*myc* and untagged GBP or GBP-FLAG, a protein became phosphorylated which was the expected size for GBP (Fig. 7A, lane 2). Phosphorylation of this protein was dependent on the presence of active Xgsk-3 since it did not become phosphorylated when co-immunoprecipitated with dominant-negative Xgsk-3, although its presence could be demonstrated by Western blotting (data not shown). Protein kinase A (PKA) was also added to these phosphorylations because it somewhat enhanced the signal without dramatically affecting the pattern of phosphorylated proteins. In phosphorylated immunoprecipitates from embryos expressing ectopic Xgsk-3-*myc* and a frame-shift mutant of Xgsk-3 (Xgsk-3FS) (Pierce and Kimelman, 1996), instead of ectopic GBP, a protein was present that appeared to be the same size as the ectopically expressed GBP (Fig. 7A, lane 1). We hypothesized that this protein could be endogenous GBP that was immunoprecipitated by virtue of its interaction with the ectopically expressed Xgsk-3-*myc*. To test this, we compared the partial proteolysis patterns of ectopically expressed GBP and the putative endogenous GBP using the in-gel proteolysis method of Cleveland (1977). A portion of the samples shown in Figure 7A was run in separate lanes of a gel. The GBP bands were excised from the wet gel and loaded into the wells of a second gel in the presence or absence of *Staphylococcus aureus* V8 protease. As a negative control, a higher molecular weight band was excised from the lane containing the putative endogenous GBP. Figure 7B shows that ectopically expressed GBP and the putative endogenous

GBP have identical V8 protease digestion patterns which are clearly different from the digestion pattern for the higher molecular weight protein. This result was confirmed by digestion with another protease, chymotrypsin (data not shown). We conclude that endogenous GBP protein is present in early embryos and that it binds to ectopically expressed Xgsk-3.

We used the phosphorylation of GBP to determine whether GBP protein is present during oogenesis. Stage VI oocytes were isolated from female frogs and injected with Xgsk-3-*myc* RNA in combination with RNA encoding GBP or Xgsk-3FS. The oocytes were cultured for approximately 4 hours prior to overnight culture in the presence or absence of progesterone to induce maturation. The oocytes were lysed and the proteins were immunoprecipitated with anti-*myc* antibody and labeled by phosphorylation. As shown in Figure 7C, endogenous GBP protein was detected in oocytes expressing Xgsk-3-*myc* and Xgsk-3FS after maturation with progesterone but not before, suggesting that GBP accumulates in oocytes after maturation. Alternatively, prior to oocyte maturation, GBP may be unable to bind Xgsk-3 or unable to be phosphorylated.

Ectopic expression of GBP induces dorsal axis formation and stabilizes β-catenin: To explore the function of GBP, GBP was ectopically expressed in embryos. Injection of 0.25 or 0.75 ng per blastomere of RNA encoding GBP into two cells of the ventral marginal region at the 2- to 16-cell stage resulted in the development of tadpoles with split body axes, indicating duplication of anterior dorsal structures (Fig. 8A). Axis duplication was typically observed in greater than 80% of the embryos (Table 1). The higher dose of RNA induced more complete axis formation, as evidenced by the presence of eyes in the secondary axis. When GBP RNA was injected on the dorsal side, axis duplication was never observed. Because ectopic expression of the dominant-negative Xgsk-3 mutant

also induces a secondary axis, we hypothesized that GBP might be inducing axis formation by blocking the action of Xgsk-3.

Another property of the dominant-negative Xgsk-3 mutant is its ability to stabilize β-catenin. We tested whether GBP also had this ability by expressing *myc* epitope tagged β-catenin (β-catenin-*myc*) (Yost *et al.*, 1996) alone or in combination with GBP or dominant-negative Xgsk-3 and monitoring β-catenin-*myc* protein levels by Western blotting. Embryos were injected at the 4-cell stage with 50 pg of β-catenin-*myc* RNA alone or in combination with 2 ng of RNA encoding GBP or dominant-negative Xgsk-3. After 4 hours, the embryos were lysed and protein extracts were analyzed by immunoblotting with anti-*myc* antibody. Figure 8B shows that when β-catenin-*myc* was expressed alone, very low levels of the protein accumulated. In contrast, when β-catenin-*myc* was coexpressed with GBP or dominant-negative Xgsk-3, high levels of β-catenin-*myc* protein accumulated. This result indicates that, like dominant-negative Xgsk-3, GBP stabilizes β-catenin in the embryo.

Exposure of fertilized eggs to UV radiation prevents endogenous axis formation (Malacinski *et al.*, 1977). Normal axis formation can be rescued in UV-irradiated embryos by ectopic expression of various dorsalizing factors, including dominant-negative Xgsk-3 (Pierce and Kimelman, 1995). We tested whether ectopic expression of GBP could also rescue axis formation in UV-irradiated embryos. Fertilized eggs were irradiated for 60 seconds with UV light within 40 minutes after fertilization and, at the two to 4-cell stage, embryos were injected with 1 ng of GBP RNA. After 3 days, the results were quantitated by scoring the dorsoanterior index (DAI) of the embryos, in which 5 represents normal embryos and zero represents the most severely ventralized embryos (Kao and Elinson, 1988). Figure 9 shows that while UV-irradiated control embryos or

UV-irradiated embryos injected with 2 ng of *Xgsk-3* RNA are severely ventralized, injection of GBP RNA caused significant axis rescue. In contrast, co-injection of 1 ng of GBP RNA and 2 ng of *Xgsk-3* RNA completely blocked the rescuing activity of GBP. The ability of Xgsk-3 to overcome the rescuing activity of GBP indicates that GBP functions by interfering with the action of Xgsk-3.

GBP does not inhibit Xgsk-3 kinase activity: The simplest hypothesis for how GBP could induce axis formation by interfering with Xgsk-3 was that GBP was acting as an inhibitor of Xgsk-3 kinase activity. To test this, we measured Xgsk-3 activity in immunoprecipitates from embryos expressing ectopic Xgsk-3-*myc* with Xgsk-3FS or GBP-FLAG. Anti-*myc* antibody was used to isolate Xgsk-3-*myc* from embryos expressing Xgsk-3-*myc* and Xgsk-3FS and anti-FLAG antibody was used to isolate Xgsk-3-*myc* complexed to GBP-FLAG from embryos expressing Xgsk-3-*myc* and GBP-FLAG. Incorporation of γ-^{32}P-ATP into the prephosphorylated CREB peptide substrate (p-CREB) (Wang *et al.*, 1994) was used to measure the Xgsk-3 activity in the immunoprecipitates. P-CREB is a specific GSK-3 substrate while the non-phosphorylated peptide (CREB) is not. Figure 10 shows that a significant amount of Xgsk-3 activity is isolated from embryos expressing Xgsk-3-*myc* as compared to immunoprecipitates from uninjected embryos or purified bacterially expressed Xgsk-3. When GBP-FLAG is coexpressed with Xgsk-3-*myc* and the complexes are isolated via the GBP-FLAG epitope tag, the activity of Xgsk-3-*myc* is approximately the same as when it is coexpressed with Xgsk-3FS. This result suggests that GBP does not directly act as an inhibitor of Xgsk-3 enzyme activity and therefore must interfere with the action of Xgsk-3 in some other way. However, it is possible that, if GBP were a competitive inhibitor of Xgsk-3, inhibition would not be detected under the

above conditions in which the peptide substrate concentration is much higher than the GBP concentration.

GBP joins a complex with APC, β-catenin and Xgsk-3: It has been found in human colon cancer cell lines that GSK-3 and β-catenin form a complex with the APC protein, which is mutated in these cells (Rubinfeld *et al.*, 1996). These cells have abnormally high levels of cytoplasmic β-catenin which can be alleviated by the transfection of a gene encoding wild-type APC, suggesting that APC normally regulates β-catenin levels (Munemitsu *et al.*, 1995). We were interested in whether this complex could be detected in *Xenopus* embryos and whether it was affected in any way by GBP expression. Embryos were injected at the two to 4-cell stage with 1 ng each of RNA encoding β-catenin-*myc* and wild-type or dominant-negative Xgsk-3-*myc*, with or without GBP-*myc* RNA. After approximately 3 hours, proteins were extracted and isolated by immunoprecipitation with antibodies to APC. Figure 11 shows that β-catenin-*myc*, wild-type and dominant-negative Xgsk-3*myc*, and GBP-*myc* are immunoprecipitated with antibodies to APC. It appears that significantly more Xgsk-3-*myc* is isolated in the presence of GBP-*myc*. These results demonstrate that Xgsk-3, β-catenin, and APC can form a complex in the *Xenopus* embryo and are consistent with the hypothesis that Xgsk-3 and APC could cooperatively regulate β-catenin levels. The ability of GBP to join this complex suggests that it might act by binding to and disabling the APC complex, preventing the degradation of β-catenin.

Discussion

GBP, the isolation and characterization of which I have described in this chapter, represents a potential key component of the signaling system that translates cortical rotation into the formation of the dorsoventral axis. It is now

known that β-catenin, which can induce secondary axis formation when ectopically expressed on the ventral side of a *Xenopus* embryo, directly interacts with transcription factors of the HMG-box family, such as LEF-1 and the TCF's (Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.*, 1996), and that this complex acts to regulate the transcription of specific genes (Giese *et al.*, 1995; Riese *et al.*, 1997; van de Wetering *et al.*, 1997). Thus, the regulation of β-catenin levels is directly responsible for the regulation of dorsal-specific genes, such as *siamois* (Giese *et al.*, 1995; Brannon and Kimelman, 1996; Fagotto *et al.*, 1997; Riese *et al.*, 1997; van de Wetering *et al.*, 1997). In keeping with this, it has recently been shown that β-catenin accumulates on the dorsal side of the embryo as early as the two-cell stage (Larabell *et al.*, 1997; Rowning *et al.*, 1997) It now appears, therefore, that the early blastula or Nieuwkoop organizer consists of a group of cells in which cytoplasmic and nuclear levels of β-catenin are elevated. The stability of β-catenin appears to be regulated, at least in part, by phosphorylation by Xgsk-3, since the presence of dominant-negative Xgsk-3 or the removal of putative GSK-3 phosphorylation sites within β-catenin result in β-catenin stabilization (Yost *et al.*, 1996). This is supported by the finding that the β-catenin expressed by several human colon and melanoma cancer cell lines with elevated cytoplasmic β-catenin levels is mutated at one of the GSK-3 phosphorylation sites (Morin *et al.*, 1997; Rubinfeld *et al.*, 1997).

The high level of maternal GBP transcripts and the accumulation of GBP protein after oocyte maturation suggest that GBP plays an important role in the early embryo. The ability of GBP to induce an axis when ectopically expressed in ventral vegetal cells is consistent with the possibility that it acts in the establishment or function of the early blastula or Nieuwkoop organizer. Because GBP binds to Xgsk-3 and has the same effect as the dominant-negative Xgsk-3

when ectopically expressed, we hypothesize that GBP interferes in some way with the action of Xgsk-3. The simplest explanation for this would be that GBP directly inhibits the kinase activity of Xgsk-3. In our experiments this hypothesis turned out not to be correct. It is possible that a much higher ratio of GBP to Xgsk-3 is necessary to achieve Xgsk-3 kinase inhibition. It is also possible that GBP itself is not an inhibitor of Xgsk-3 but that it recruits an inhibitor that was not retained in the immunoprecipitates. We cannot rule out either of these possibilities but under our assay conditions, in which up to three times as much GBP as Xgsk-3 RNA was injected into the embryos, GBP was not able to inhibit Xgsk-3.

An alternative mechanism for GBP function is suggested by the finding that, as in human colon cancer cells, β-catenin and Xgsk-3 form a complex in the *Xenopus* embryo with the APC protein and that GBP can join this complex. In many of the colon cancer cell lines studied, the APC protein is truncated and cytoplasmic levels of β-catenin are high (Munemitsu *et al.*, 1995). Transfection of these cells with a gene encoding full-length APC results in a reduction of β-catenin levels, suggesting that APC normally functions to promote β-catenin degradation (Munemitsu *et al.*, 1995). In vitro experiments have suggested that phosphorylation of APC by GSK-3 promotes the binding of β-catenin to APC (Rubinfeld *et al.*, 1996). These data, together with data indicating that the stability of β-catenin is regulated by GSK-3 phosphorylation (Yost *et al.*, 1996; Morin *et al.*, 1997; Rubinfeld *et al.*, 1997), suggest a model in which GSK-3 and APC act together to regulate the stability of β-catenin. GBP could act to block this mechanism by interfering with necessary contacts between components of the complex by, for example, preventing phosphorylation of APC or β-catenin by Xgsk-3. The experiments we have done to identify this complex have utilized

ectopic expression of the components at levels and in proportions that may be quite different from those in vivo. Although the amount of Xgsk-3-*myc* in the complex appears to increase in the presence of GBP-*myc*, this could reflect an overall increase in the number of complexes or a specific increase in Xgsk-3-*myc*. Thus, we do not know whether the presence of GBP changes the configuration of the complex, such that other components are present at higher or lower levels, or whether the phosphorylation state of any of the components is affected. It is also not known whether GBP directly interacts with other components of the complex in addition to Xgsk-3.

The mechanism of cortical rotation, which is necessary for the establishment of the dorsoventral axis in *Xenopus*, is a specialized process of amphibians. The necessity for Xgsk-3 and β-catenin, but not for an Xwnt or Xdsh, for axis establishment suggests that there must be a novel mechanism for regulating Xgsk-3 which could be a specialized response to cortical rotation. The high maternal and very low zygotic expression of GBP suggest that it could play a role that is solely maternal and potentially specific to cortical rotation. However, the existence of homologs in other species suggests that this probably is not the case. A partial zebrafish cDNA with homology to GBP was recently isolated by PCR (J. Kiefer and D. Kimelman, personal communication). The ability of ectopic β-catenin to induce a secondary axis in zebrafish (Kelly *et al.*, 1995) suggests that zebrafish also use components of the *Wnt* pathway during establishment of the dorsoventral axis, although there is no cortical rotation in the zebrafish fertilized egg. The presence of a GBP homolog in zebrafish suggests that GBP is not specialized to cortical rotation. The existence of a mammalian GBP homolog, as suggested by the human EST, suggests that GBP may have a

more general role in the Wnt signal response or the β-catenin degradation process.

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                      .         .         .         .         .
GBP       51 LKPVSRAGPSCSCVRGRSTPYPVCTPRGAARHAQHHHHHSPRQQGTGGNK 100
                          ||||  || |    :|:  |     :  :  :    :
humEST     1 .............VRGRAAPYCVAEV.AAGPSALPGPCRRGWLRDAVTSR 36
                      .         .         .         .         .
GBP      101 RLCGRGWGRCNCRKHAGTEEEDDPHELLQELLLSGNLIKEAVRRLHMA.. 148
             || | |     |      ::|||| |||:|:|||||||||||||: |
humEST    37 RLQQRRWTQAGAR.....AGDDDPHRLLQQLVLSGNLIKEAVRRLQRAVA 81
                      .         .         .
GBP      149 ......GESPDPPGSRRVSECTETTVQ....... 169
                   :  |:| |:|  |:     :|
humEST    82 AVAATGPASAPGPGGGR..SGPDRIALQPSGSLL* 114
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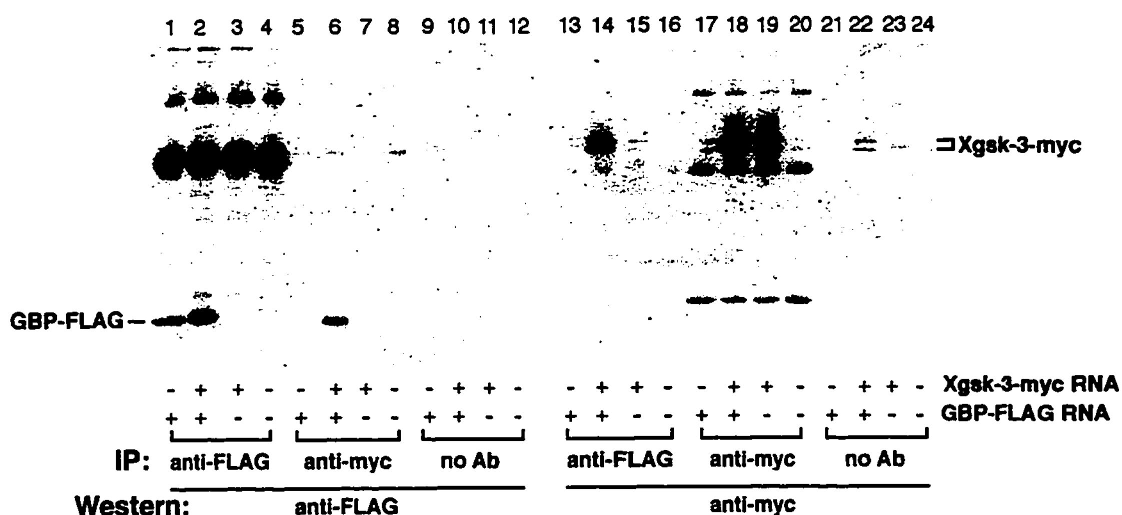
Figure 4: Amino acid sequence of GBP. The predicted amino acid sequences of GBP and a human expressed sequence tag (humEST) are compared. Solid lines indicate identical residues and semicolons indicate residues of similar character.



'**Figure 5: GBP associates with Xgsk-3 in the *Xenopus* embryo.** Embryos were injected in the animal pole at the 2-cell stage with 1.0 ng of RNA encoding Xgsk-3-*myc* or GBP-FLAG, as indicated. After 4 hours, proteins were isolated by immunoprecipitation with or without antibodies to the *myc* or FLAG epitope, as indicated. Xgsk-3-*myc* and GBP-FLAG were detected by immunoblotting with anti-FLAG (right side) and anti-*myc* (left side) antibodies. The locations of GBP-FLAG and Xgsk-3-*myc* are marked. Each lane represents ten embryos.

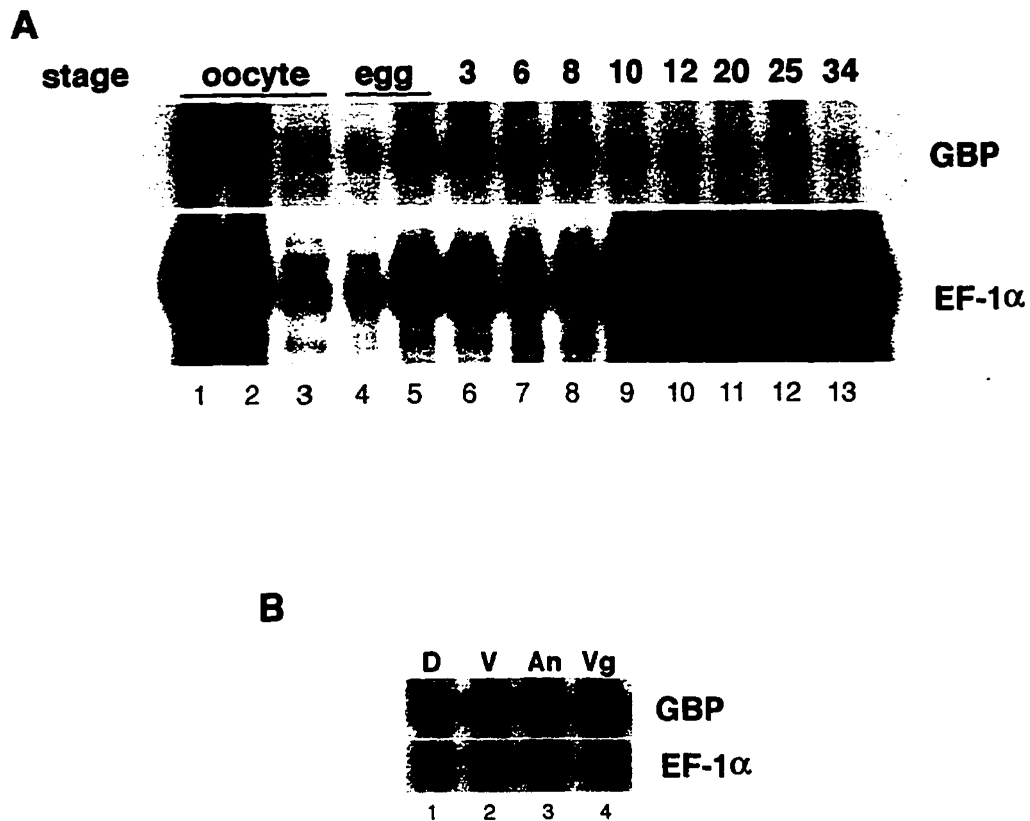


Figure 6: Temporal and spatial expression of GBP. RNA levels were determined with the RNase protection assay using a mixture of GBP and EF-1α probes. EF-1α is a ubiquitously expressed gene in the *Xenopus* embryo; EF-1α levels increase from the mid-blastula transition at stage 8 (Krieg *et al.*, 1989). **(A)** Analysis of 10 μg of total RNA from oocytes at stages I-III, IV-V, and VI (lanes 1, 2, and 3, respectively, unfertilized (lane 4) and fertilized (lane 5) eggs, and embryos at the following stages: 3 (4-cell), 6 (32-cell), 8 (mid-blastula), 10 (early gastrula), 12 (late gastrula), 20 (late neurula), 25 (tailbud), and 34 (tadpole). **(B)** Analysis of 10 μg of total RNA isolated from dissected embryos. Four to 8-cell stage embryos were dissected into dorsal and ventral halves (lanes 1 and 2). Sixteen to 32-cell stage embryos were dissected into animal and vegetal halves (lanes 3 and 4).

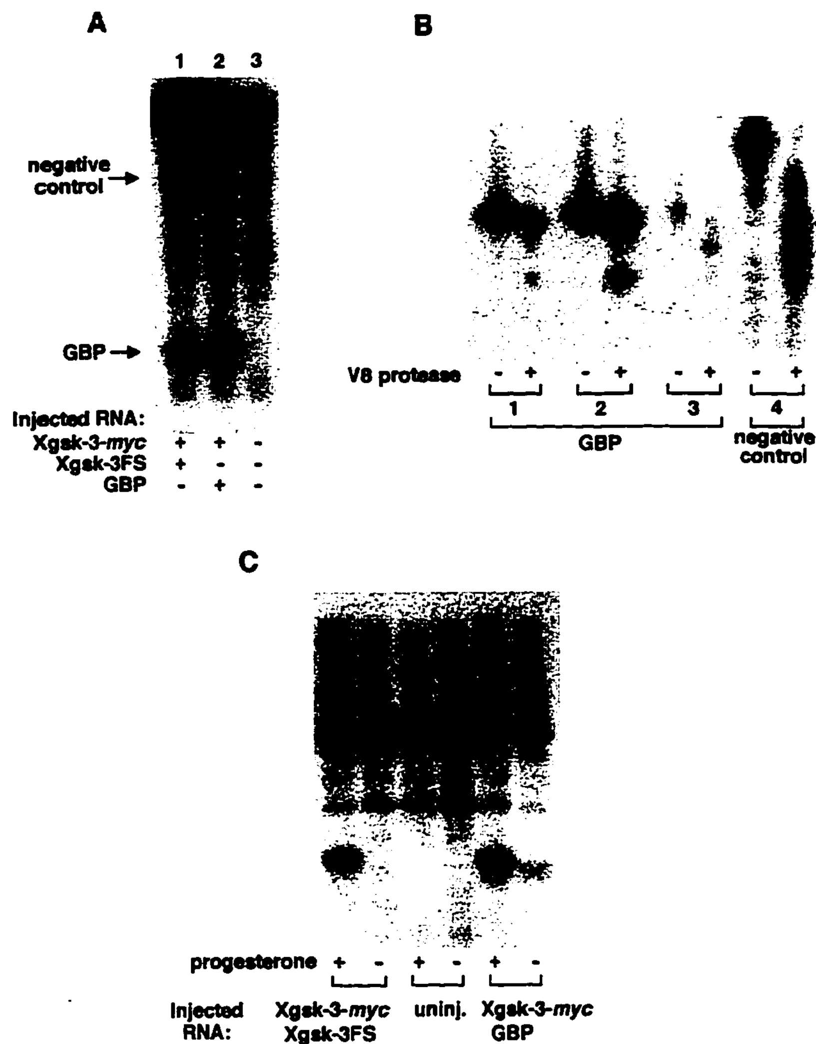


Figure 7: GBP protein is present in the *Xenopus* embryo and accumulates after oocyte maturation. (A) Proteins from uninjected embryos (lane 3) or embryos injected with 2 ng of *Xgsk-3-myc* RNA in combination with 0.5 ng of *Xgsk-3FS* (lane 1) or GBP (lane 2) RNA were immunoprecipitated 2.5 hours after injection with anti-*myc* antibodies. Samples were labeled with ^{32}P as described in Appendix A and 20% of the samples were analyzed by SDS-PAGE. The locations of the GBP and negative control bands analyzed in (B) are indicated. **(B)** 80% of the samples in (A) were separated by SDS-PAGE and protein from the GBP bands in lanes 1, 2, and 3, and the negative control band in lane 1, as indicated in (A), were run on a 16% gel in the absence or presence of V8 protease. There were 200 embryos in the sample in lane 1 and 75 in the samples in lanes 2 and 3. **(C)** Defolliculated stage VI oocytes were injected with RNA as in (A) and cultured for 22 hours in the presence or absence of 5 µg/ml of progesterone. Proteins were immunoprecipitated and analyzed by ^{32}P labeling as in (A).

Table 1: Axis Duplication By GBP

Injection		Secondary axes: Complete n (%)	Partial n (%)	None n (%)
Dorsal 0.5ng	Exp. 1	0	0	27 (100)
	Exp. 2	0	0	21 (100)
Dorsal 1.5 ng	Exp. 1	0	0	26 (100)
	Exp. 2	0	0	16 (100)
Ventral 0.5 ng	Exp. 1	1 (3)	12 (33)	23 (64)
	Exp. 2	0	14 (37)	24 (63)
Ventral 1.5 ng	Exp. 1	15 (44)	17 (50)	2 (6)
	Exp. 2	8 (27)	16 (53)	6 (20)

Synthetic RNA encoding GBP was injected at the indicated doses into two dorsal or ventral cells of early cleavage stage embryos (2- to 16-cell) in the vegetal marginal zone. Axis duplication was scored after uninjected controls had well-developed eyes, approximately 3 days. Complete secondary axes had one or two eyes. Partial axes had no eyes. Data from two experiments (Exp. 1 and 2) are shown.

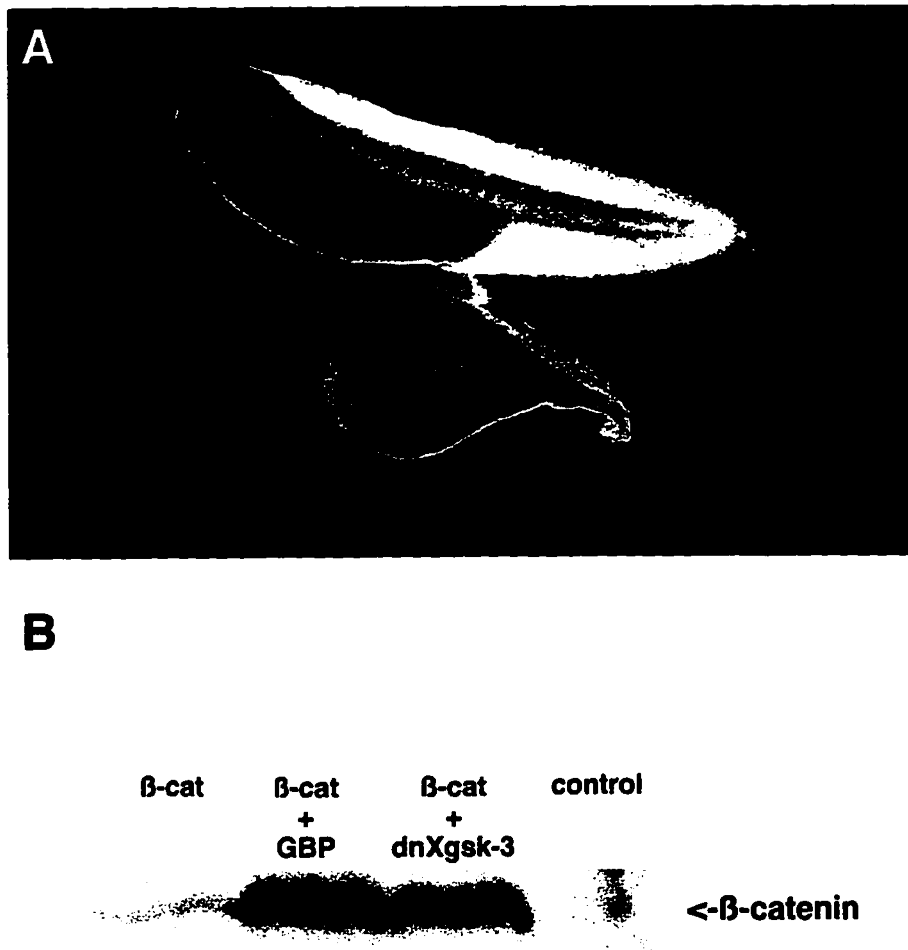


Figure 8: GBP causes dorsal axis duplication and stabilizes β-catenin. (A) Two ventral marginal cells at the 2- to 16-cell stage were injected with 0.75 μg per blastomere of GBP RNA and embryos were allowed to develop for 3 days. Representative uninjected (top embryo) and injected (bottom embryo) embryos are shown. **(B)** Embryos were injected in 2 cells at the 4-cell stage with a total of 50 pg of β-catenin-*myc* RNA alone or in combination with 2 ng of GBP or dominant-negative Xgsk-3 (dnXgsk-3) RNA. Control embryos were uninjected. After 4 hours, β-catenin levels were determined by immunoblotting of protein extracts with anti-*myc* antibodies.

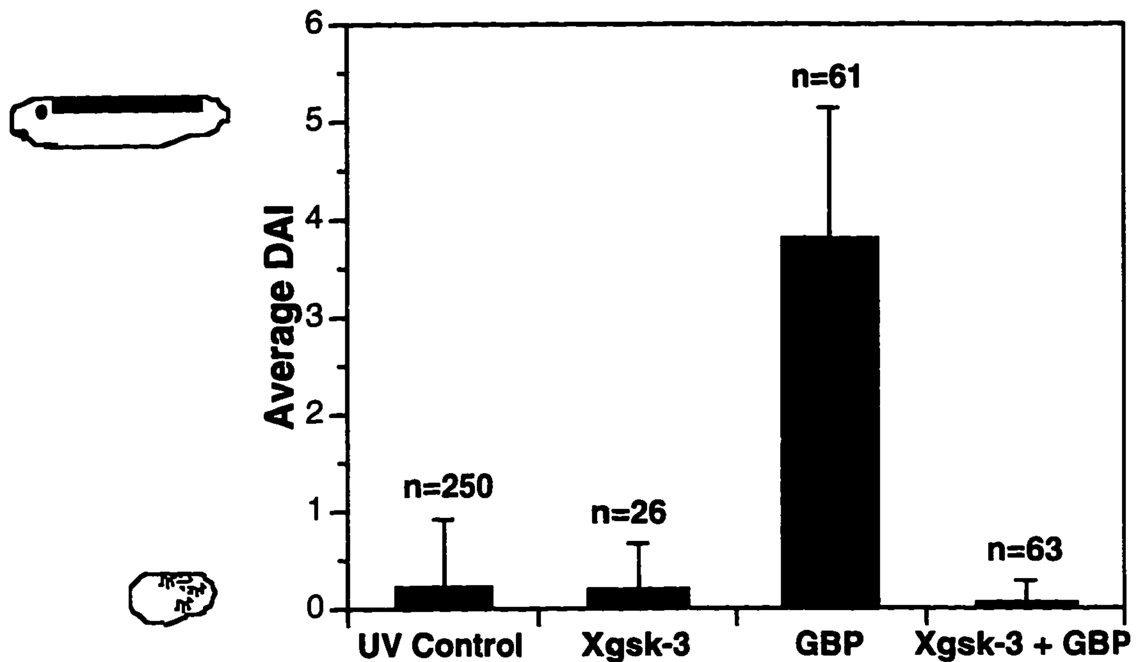


Figure 9: Xgsk-3 blocks the axis-inducing activity of GBP. Fertilized eggs were UV-irradiated within 40 minutes after fertilization. At the 2- to 4-cell stage, one cell was injected with 2 ng of Xgsk-3-*myc* RNA or 1 ng of GBP RNA, alone or in combination, as indicated. UV control embryos were uninjected. The dorsoanterior index (DAI) of the embryos was scored after 3 days and the average DAI for each sample is shown, with the sample size indicated above. Embryos with a DAI of 0 lack dorsal structures and those with a DAI of 5 are normal, as diagrammed on the left.

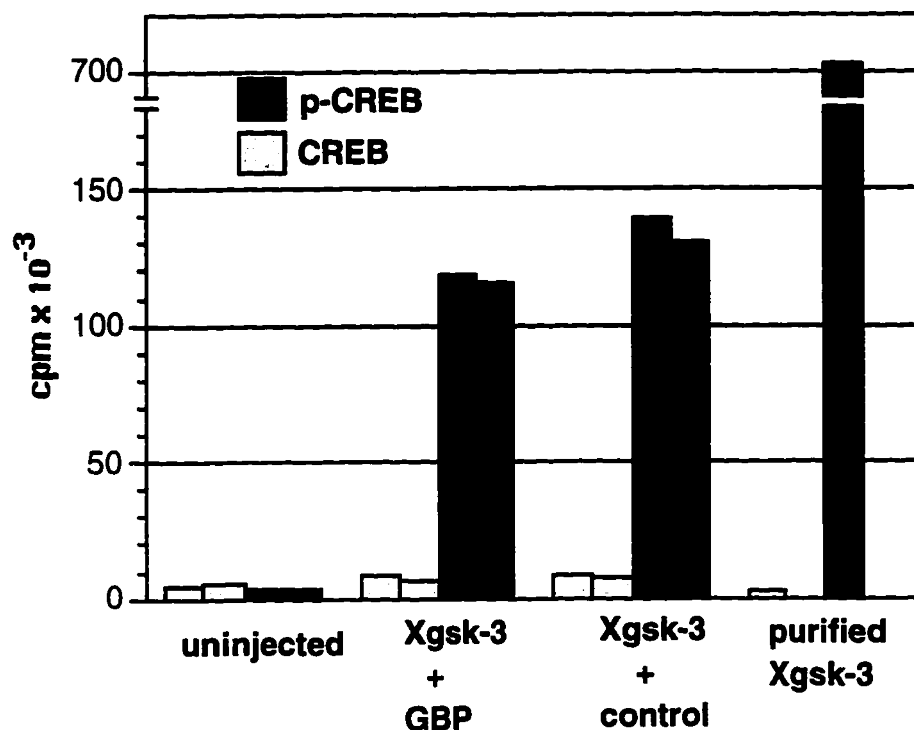


Figure 10: GBP does not inhibit Xgsk-3 kinase activity'. Samples of 60 embryos were left uninjected or injected in 2 animal pole cells at the 2- to 8-cell stage with 1 ng of Xgsk-3-*myc* RNA and 3 ng of GBP-FLAG or control Xgsk-3FS RNA. After 3 hours, proteins were extracted and immunoprecipitated with anti-FLAG (Xgsk-3 + GBP), anti-*myc* (Xgsk-3 + control), or both (uninjected) antibodies. The Xgsk-3 activity of the immune complexes and purified Xgsk-3 (Yost *et al.*, 1996) was determined by measuring ^{32}P incorporation into the prephosphorylated CREB peptide (p-CREB; dark bars), which is a specific GSK-3 substrate. The non-phosphorylated CREB peptide (CREB; light bars) is not a GSK-3 substrate. The activity of duplicate immune complex samples is shown.

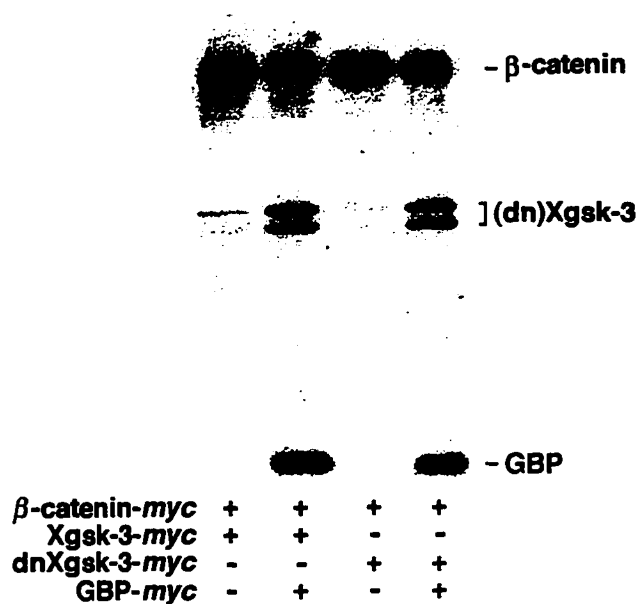


Figure 11: GBP joins a complex containing APC, β-catenin, and Xgsk-3. Embryos were injected with 1 ng each of the indicated RNAs and, after 3 hours, proteins were extracted and immunoprecipitated with antibodies against APC. β-catenin-*myc*, Xgsk-3-*myc*, dominant-negative Xgsk-3-*myc* (dnXgsk-3-*myc*) and GBP-*myc* were detected by immunoblotting with anti-*myc* antibodies. The positions of *myc*-tagged β-catenin, wild-type and dominant-negative Xgsk-3 [(dn)Xgsk-3], and GBP are indicated.

Chapter 3: Overexpression of Xgsk-3 disrupts anterior ectodermal patterning in Xenopus

Introduction

In the previous two chapters I presented evidence that Xgsk-3 is involved in dorsoventral axis determination and that it acts in a signaling pathway that can be activated by certain Wnts. Ectopic expression of Xgsk-3 in dorsal mesoderm or a dominant negative mutant of Xgsk-3 in ventral mesoderm disrupts normal dorsoventral axis formation. In the course of these experiments I found that ectopic expression of Xgsk-3 in the animal pole region, the future ectoderm, resulted in the formation of excess cement gland. The cement gland is a non-neural ectodermal structure at the most anterior point of the embryo (Sive *et al.*, 1989). Because the *Wnt* family includes several members that are involved in many developmental processes at different times in development (Moon, 1993), it is not surprising that Xgsk-3 would be involved in other aspects of development in addition to dorsoventral axis determination. Here I will present my analysis of the effect of Xgsk-3 on cement gland development.

Results

Ectopic expression of Xgsk-3 in the future ectoderm led to an expansion of the cement gland (Appendix C, Fig. 1), which became visible during the late neurula stages. By whole mount in situ hybridization to detect an early genetic marker for cement gland, *XAG-1*, I showed that this expansion occurred by the beginning of neurulation (Appendix C, Fig. 3A), soon after cement gland induction is thought to begin (Sive *et al.*, 1989). Using β-galactosidase as a lineage tracer, I found that although cement gland expansion was limited to cells in which Xgsk-3 was expressed, it was also limited to the anterior half of the

embryo, even when β-galactosidase was detected along the length of the embryo (Appendix C, Fig. 2).

Although cement gland and neural tissue are both of ectodermal origin, gastrulation results in progressively more anterior dorsal tissue becoming specified as cement gland until the cement gland is finally positioned in the most dorsal-anterior region of the embryo, anterior to the neural plate (Sive *et al.*, 1989). I hypothesized that the expansion of cement gland caused by Xgsk-3 might reflect the specification of a larger region of the embryo as non-neural ectoderm at the expense of neural ectoderm. However, when I examined embryos at the early neurula stage, I found that *XAG-1* expression always expanded laterally and ventrally, even when the use of β-galactosidase showed that Xgsk-3 was expressed in the neural plate (Appendix C, Fig. 3B). I also examined the expression of two other anterior markers, *Xotx2* (Blitz and Cho, 1995; Pannese *et al.*, 1995) and *XANF-2* (Mathers *et al.*, 1995), whic are expressed in and adjacent to the cement gland region, and an epidermal marker, *Xgbx-2* (von Bubnoff *et al.*, 1996). While, like *XAG-1*, *Xotx2* and *XANF-2* were expanded laterally (Appendix C, Fig. 3C-F), *Xgbx-2* expression was reduced or eliminated (Appendix C, Fig. 3G and H). Thus, the anterior non-neural region of the embryo appeared to be expanded as a result of Xgsk-3 overexpression at the expense of the epidermal region.

Since cement gland expansion is a specific effect of animal pole injection of *Xgsk-3* RNA, and is different from the effect of injection in the presumptive mesoderm (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995), I tested whether the effects of Xgsk-3 were due to a direct effect on the ectoderm. To examine this, and to determine whether Xgsk-3 could directly induce cement gland, I cultured isolated blastula stage ectoderm (animal caps) from embryos

injected with *Xgsk-3* RNA and evaluated *XAG-1* expression by whole-mount in situ hybridization. A very small amount of *XAG-1* expression was induced in these animal caps when cultured alone (Appendix C, Fig. 4B), but when caps were cultured in the presence of dorsal mesoderm from uninjected embryos, much more *XAG-1* expression was seen in caps expressing Xgsk-3 than in uninjected caps (Appendix C, Fig, 4D and E). This effect could be mimicked in isolated caps by co-injecting *noggin* RNA with the *Xgsk-3* RNA (Appendix C, Fig. 5) but not by co-injecting *Xenopus banded-hedgehog* (*X-bhh*) RNA. Both noggin and X-bhh are effective inducers of cement gland in isolated ectoderm (Lamb *et al.*, 1993; Ekker *et al.*, 1995; Lai *et al.*, 1995). Thus, Xgsk-3 does not directly induce cement gland to a significant extent but it enhances the response of ectoderm to endogenous cement gland-inducing signals from dorsal mesoderm (Sive *et al.*, 1989), and to the secreted factor noggin.

In addition to my study of the effects of Xgsk-3 expression on patterning of the anterior non-neural ectoderm, I examined the effects of Xgsk-3 on the expression of genes within the neural plate. Both *Xotx2* and *XANF-2*, have regions of expression in the anterior neural plate in addition to their regions of expression anterior to the neural plate (Appendix C, Fig. 3 C and E). While the non-neural region of expression of these markers was expanded in over 85% of embryos as a result of Xgsk-3 expression, their neural regions of expression were also often expanded (Appendix C, Fig. 3F), although less frequently or reproducibly. I also examined the expression of *Krox-20*, which is expressed in rhombomeres three and five of the developing hindbrain and *en-2*, which is expressed at the midbrain/hindbrain boundary. Because I found it difficult to reproducibly show the normal expression pattern of these genes, I was unable to consistently show a strong effect of Xgsk-3 their expression. However, the

general trend was the reduction or absence of *Krox-20* and *en-2* expression as a result of Xgsk-3 overexpression.

Discussion

The ability of ectopic Xgsk-3 to cause an expansion of the cement gland that is limited to the anterior half of the embryo suggests that ectopic Xgsk-3 may be enhancing the responsiveness of the ectoderm to endogenous cement gland-inducing signals originating in the anterior dorsal mesoderm. This enhanced responsiveness would be expected to result in cement gland induction in a broader region than normal, but the extent of this region would still be limited by the spread of the inducing signal. This hypothesis is supported by the fact that Xgsk-3 induces very little *XAG-1* expression in animal caps that are cultured alone but when animal caps are cultured with dorsal mesoderm, more cement gland is induced in those caps that are expressing ectopic Xgsk-3 than in those that are not. The finding that co-expression of Xgsk-3 with noggin but not X-bhh can mimic this effect suggests that Xgsk-3 is affecting a specific signaling pathway or pathways, rather than ubiquitously enhancing the response to all cement gland-inducing signals.

Sive and coworkers have shown that at least two mesoderm-derived signals are required for correct cement gland localization: an inducing signal that specifies cement gland formation from anterior ectoderm and a dominant inhibitory signal that could produce a stable border between neural and non-neural ectoderm (Sive *et al.*, 1989). Noggin is well positioned to act as the endogenous inducer of cement gland since *noggin* RNA is expressed in the gastrula stages in the dorsal midline (Smith and Harland, 1992). In addition, both of the anterior marker genes whose expression is expanded by Xgsk-3, *Xotx2* and *XANF-2*, can also be induced in animal caps by noggin (Lamb *et al.*, 1993; Blitz

and Cho, 1995; Lai *et al.*, 1995), and *Xotx2* can directly induce cement gland (Blitz and Cho, 1995; Pannese *et al.*, 1995). Because Xgsk-3 in prospective ectoderm causes a lateral and ventral expansion of cement gland, I propose that Xgsk-3 is enhancing the responsiveness of ectoderm to endogenous cement gland-inducing signals without affecting the inducing signal itself or the proposed inhibitory signal. Thus, cement gland induction would be limited posteriorly by the inhibitory signal forming the border of the neural plate and laterally and ventrally by the spread of noggin and the responsiveness of the ectoderm (Fig. 12.

My results suggest that Xgsk-3 could be a component of the ectodermal intracellular pathway that responds to endogenous anterior-inducing signals by activating the transduction of genes such as *XAG-1, Xotx2,* and *XANF-1*. In this role, Xgsk-3 could be constitutively active, thus providing a baseline responsiveness to inducing signals, or its activity may be directly regulated by factors such as members of the *Wnt* family. If a Wnt were acting to oppose the action of Xgsk-3, as would be predicted, the enhancement by Xgsk-3 of cement gland induction by noggin would suggest that noggin signaling is normally antagonized by Wnt signaling. Work from other labs provides a precedent for an antagonism between noggin and Wnt. Using a system in which ectodermal explants were cultured in contact with oocytes expressing Wnt-1 and/or noggin, it was shown that Wnt-1 significantly inhibits the ability of noggin to induce cement gland (Lustig and Kirschner, 1995). In addition, when noggin was coexpressed in ectodermal explants with Xwnt-3a or β-catenin, the ability of noggin to induce *XAG-1* expression was inhibited (McGrew *et al.*, 1995). This suggests that in the embryo, Xgsk-3 could function in a Wnt signaling pathway to spatially regulate the responsiveness to noggin or noggin-like signals.

When these experiments were completed, nothing was known about the signal transduction pathway used by noggin, making it difficult to speculate about the mechanism of the interaction between noggin and Wnt signaling. It has recently been shown that noggin acts, not by signaling through a receptor and intracellular signaling pathway, but by binding to BMP-4, another secreted factor, and preventing its interaction with the BMP-4 receptor (Holley *et al.*, 1996; Zimmerman *et al.*, 1996). BMP-4 is expressed in ventral and lateral mesoderm and experiments with overexpression of BMP-4 or a dominant-negative BMP receptor indicate that the regulation of BMP-4 signaling is critical for the proper establishment of the neural plate boundaries (Schmidt *et al.*, 1995). Noggin is considered to be a neural inducer because it can induce, in addition to cement gland, pan-neural and anterior neural genes in isolated animal caps (Lamb *et al.*, 1993; Knecht *et al.*, 1995). Thus, the boundaries of the neural plate and the anterior non-neural ectoderm that includes the cement gland are likely to be determined by the apposition of noggin and BMP-4. The fact that overexpression of Xgsk-3 results in an expansion of the anterior non-neural ectoderm suggests that a Wnt signaling pathway normally acts in concert with BMP-4 in the ventral and lateral regions of the embryo. This is likely since the expression of *Xwnt-8*, which is also expressed in ventral and lateral mesoderm (Christian *et al.*, 1991b), is dependent on BMP-4 signaling (Schmidt *et al.*, 1995). Thus, the synergism seen between Xgsk-3 and noggin in animal caps may result from particularly efficient inhibition of BMP-4 signaling that comes from preventing the interaction of BMP-4 with its receptor and inhibiting Wnt signaling that normally occurs in concert with of BMP-4.

To explain the effects of ectodermal overexpression of Xgsk-3 in the whole embryo and in animal caps I propose a model in which BMP-4, acting with a

Wnt, opposes the formation of neural and anterior non-neural tissues. The border of these regions is established by the action of noggin and/or perhaps other BMP/TGF-β binding proteins, such as chordin and follistatin, which prevent the activation of the BMP-4 receptor. Xgsk-3 synergizes with noggin by inhibiting Wnt signaling and further attenuating the effects of BMP-4, allowing an expansion of the anterior non-neural region.

One thing that is still unknown is how, if BMP-4 signaling establishes the borders of a region that includes both the neural plate and anterior non-neural tissues, Xgsk-3 overexpression results in an expansion of the anterior non-neural region without disrupting the borders of the neural plate. Patterning of the neural region is quite complex and it is likely that many as yet unidentified factors are involved in this process and may be involved in separating the anterior non-neural ectoderm from the neural plate. It is also possible that gradients or overlapping regions of BMP binding proteins result in regional differences in the extent of BMP-4 inhibition. These differences could allow the distinction between the anterior non-neural region and the neural plate and perhaps the distinction of regions within the neural plate.

Another question yet to be addressed is why does overexpression of Xgsk-3 also disrupt the expression of genes within the borders of the neural plate? This is likely to be due to the disruption of Wnt signaling within the neural plate since several *Wnt* family members are expressed within the developing nervous system (McGrew *et al.*, 1992; Wolda and Moon, 1992). In fact, the results of experiments by McGrew and coworkers with overexpression of Xwnt-3a and a dominant-negative Xwnt (dnXwnt-8) were quite consistent with my results. They found that in animal caps that have been neuralized with noggin, *XAG-1*, *Xotx2* and *XANF-2* are suppressed by Xwnt-3a while *en-2* and *Krox-20* are induced

(McGrew *et al.*, 1995). In contrast, in whole embryos and mesoderm/ectoderm explants dnXwnt-8 caused an expansion or elevation of expression of *XAG-1* and *XANF-2*, while *en-2* and *Krox-20* expression was decreased (L. McGrew, S. Hoppler, and R. Moon, personal communication). In contrast to my results, McGrew and coworkers found that *dnXwnt-8* had no effect on the expression of *Xotx2*. However, overall, their results are similar to the results of my experiments in which Xgsk-3 is overexpressed, suggesting that my results can be explained by a disruption of Wnt signaling within the neural plate.

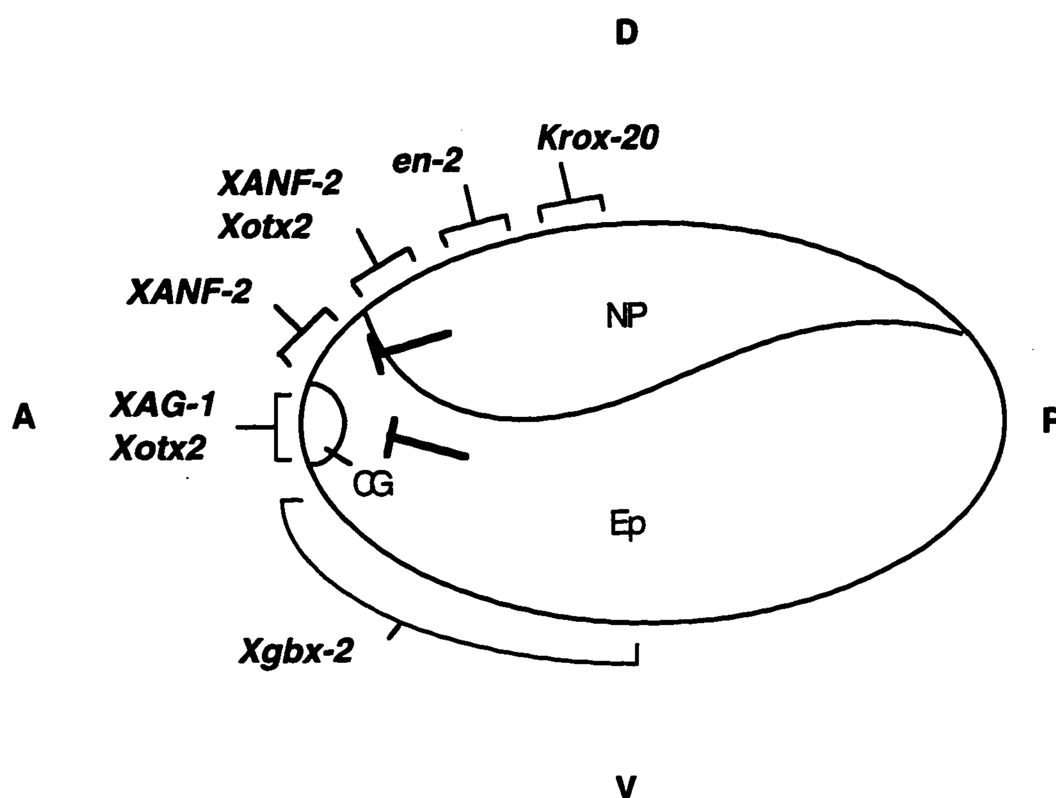


Figure 12: Ectodermal patterning in the *Xenopus* embryo. A *Xenopus* embryo at the early neurula stage is diagrammed. The orientation of the dorsal/ventral (D, V) and anterior/posterior (A, P) axes, and the regions of the neural plate (NP), cement gland (CG), and epidermis (Ep) are indicated. Brackets around the perimeter indicate the regions of expression of genes discussed in the text. The borders of the anterior non-neural region are likely to be determined, in part, by repressive signals from the neural plate and epidermal regions.

Chapter 4: Conclusions

The work presented in this thesis has demonstrated that the serine/threonine kinase Xgsk-3 plays a critical role in dorsoventral axis determination in *Xenopus* and may be involved in later patterning of the anterior non-neural ectoderm. I have also described the isolation and characterization of GBP, a protein that binds to and appears to negatively regulate the function of Xgsk-3. As a result of this work and additional work in the Kimelman laboratory and other laboratories, a model has emerged for the mechanism of dorsoventral axis determination. As described in the Introduction, formation of the *Xenopus* body plan is a complex process involving multiple overlapping intercellular signals acting throughout the first several hours of development. The first phase of this process, which establishes the dorsoventral axis, involves signals from a group of dorsal vegetal cells, known as the Nieuwkoop center, acting on the most dorsal mesoderm to establish the Spemann organizer. In this chapter, I will discuss the model which has emerged for how the dorsoventral axis is specified.

The radial symmetry of the egg is broken by fertilization, which triggers a rotation of the egg cortex relative to the inner cytoplasm. Cortical rotation causes the release or activation of a signal on the future dorsal side of the embryo. When I began my work, this activity had been shown to reside in the dorsal vegetal cells of the early blastula, which could non-cell-autonomously induce an ectopic axis when transplanted to the vegetal side of a host embryo (Gimlich and Gerhart, 1984). This dorsal vegetal signaling center is known as the Nieuwkoop center. Several molecular candidates for the Nieuwkoop signal, including members of the *Wnt* family, had been defined by their ability to induce an axis when ectopically expressed on the ventral side of an embryo but none of them had been demonstrated to play a role in endogenous axis formation. As a way of

attempting to understand the signal transduction pathways involved in dorsoventral axis determination in vivo, I chose to try to perturb the *Wnt* signaling pathway. Although nothing was known about the signal transduction mechanism of the vertebrate *Wnts*, I hypothesized that it would be similar to the signaling pathway used by the *Drosophila Wnt* homolog, *wg*, which had been well characterized genetically. I chose to clone and study the *Xenopus* homolog of *zw3/sgg*, which encodes an intracellular serine/threonine kinase that acts as a negative regulator of Wg signaling (Siegfried *et al.*, 1992; Noordermeer *et al.*, 1994).

I found that the action of Xgsk-3, the Zw3/Sgg homolog, must be opposed for normal dorsoventral axis determination to occur, supporting a role for *Wnts* in axis formation and the hypothesis that the *Wnt* pathway would be homologous to the *wg* pathway. This is shown by the ability of ectopically expressed Xgsk-3 to inhibit endogenous axis formation and the dominant-negative Xgsk-3 mutant to induce axis formation. In addition, I found that the dominant-negative Xgsk-3 mutant can induce axis formation when expressed in cells that will not contribute to the axis. This was a surprising result and suggested that rather than directly inducing the Spemann organizer, as expected, Wnt signaling induced a secondary downstream signal.

Additional components of the *wg* pathway were also shown to have *Xenopus* homologs that were likely to be involved in axis determination. These included β-catenin, the homolog of Arm, which could induce a secondary axis when ectopically expressed (Guger and Gumbiner, 1995), and which *Drosophila* genetic experiments suggested would function downstream of Xgsk-3. Xgsk-3 has been shown to phosphorylate β-catenin at several N-terminal sites, which apparently targets β-catenin for degradation (Yost *et al.*, 1996). In keeping with

this, mutation or deletion of the GSK-3 phosphorylation sites in β-catenin causes it to be much more stable than wild-type β-catenin and, as a result, a much more potent dorsalizing factor. Thus, blocking the activity of Xgsk-3 allows the accumulation of β-catenin protein. Within the embryo, accumulation of β-catenin on the dorsal side can be visualized by the two-cell stage (Larabell *et al.*, 1997; Rowning *et al.*, 1997).

An understanding of the role that β-catenin plays in *Wnt* signal reception has come from the finding that β-catenin can play a direct role in gene regulation. β-catenin binds to architectural transcription factors of the HMG-box family, such as LEF-1 and the TCF's, and this complex regulates the transcription of specific genes (Giese *et al.*, 1995; Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.*, 1996; Riese *et al.*, 1997; van de Wetering *et al.*, 1997). Thus, the accumulation of β-catenin on the dorsal side of the embryo results in the activation of dorsal-specific genes. One of these genes is the homeobox-containing transcription factor, *siamois*, which can be induced by β-catenin (Brannon and Kimelman, 1996; Fagotto *et al.*, 1997) and had been identified by its ability to induce a secondary axis when ectopically expressed on the ventral side of embryos (Lemaire *et al.*, 1995). *Siamois* has also been shown to induce an axis when expressed in vegetal cells which will not contribute to the axis, suggesting that *siamois* transcription is upstream of the unknown secondary signal generated by *Wnt*. I had expected that the signaling events that induce the Spemann organizer were entirely maternal, resulting in the specification of the organizer prior to MBT. These results suggest, however, that the inducing signal is not sent until after zygotic transcription begins and *siamois* is expressed. Thus, the Spemann organizer is established during the late blastula stages, not long before it is active as a signaling center during gastrulation.

Although the biochemical events that take place downstream of Xgsk-3 were becoming clear, the mechanism by which cortical rotation is translated into an opposition of Xgsk-3 was still unknown. Although *Wnt* and the homolog of the *Drosophila dsh* gene, *Xdsh*, were predicted to act upstream of *Xgsk-3*, dominant-negative mutants of Xwnt-8 and Xdsh have no effect on dorsoventral axis determination (Hoppler *et al.*, 1996; Sokol, 1996). This suggested that Xgsk-3 may be regulated by a novel mechanism in response to cortical rotation. To approach this question, a two-hybrid screen for Xgsk-3-interacting proteins was undertaken in the Kimelman laboratory. In this screen we isolated GBP, a protein which binds to Xgsk-3 and induces an axis when ectopically expressed. This effect, which could be blocked by the coexpression of Xgsk-3, suggested that GBP acts by interfering with the action of Xgsk-3. The simplest mechanism for this would be the inhibition of Xgsk-3 kinase activity by GBP. We found, however, that Xgsk-3 is just as active in a complex with GBP as it is when isolated, indicating that GBP interferes with Xgsk-3 in some other way.

An alternative model for the effect of GBP in embryos is suggested by the finding that in human colon cancer cells, the APC protein can promote the degradation of β-catenin and can form a complex with β-catenin and GSK-3 (Munemitsu *et al.*, 1995; Rubinfeld *et al.*, 1996). In vitro, phosphorylation of APC by GSK-3 promotes the binding of β-catenin to APC (Rubinfeld *et al.*, 1996). These results, together with data indicating that β-catenin stability is regulated by GSK-3 phosphorylation (Yost *et al.*, 1996; Rubinfeld *et al.*, 1997), suggest that GSK-3 and APC may act together within a complex to regulate the stability of β-catenin. Our finding that β-catenin and Xgsk-3 associate with APC in *Xenopus*, suggests that this mechanism could be active in the embryo. We also found that GBP associates with this complex, suggesting that GBP could act by interfering with

its function. Perhaps, for example, GBP can block the phosphorylation of a large protein substrate, like β-catenin, by Xgsk-3, even though it does not prevent phosphorylation of the p-CREB peptide substrate in vitro (Fig. 13). In addition, GBP may directly bind other components of the complex, such as APC or β-catenin, and disrupt the β-catenin degradation via these interactions

Although many of the biochemical events leading to the specification of the dorsoventral axis have been identified, much about this process is still unknown. In the following chapter, I will suggest possible approaches to several unanswered questions about the roles of Xgsk-3 and GBP in dorsoventral axis formation and Xgsk-3 in ectodermal patterning.

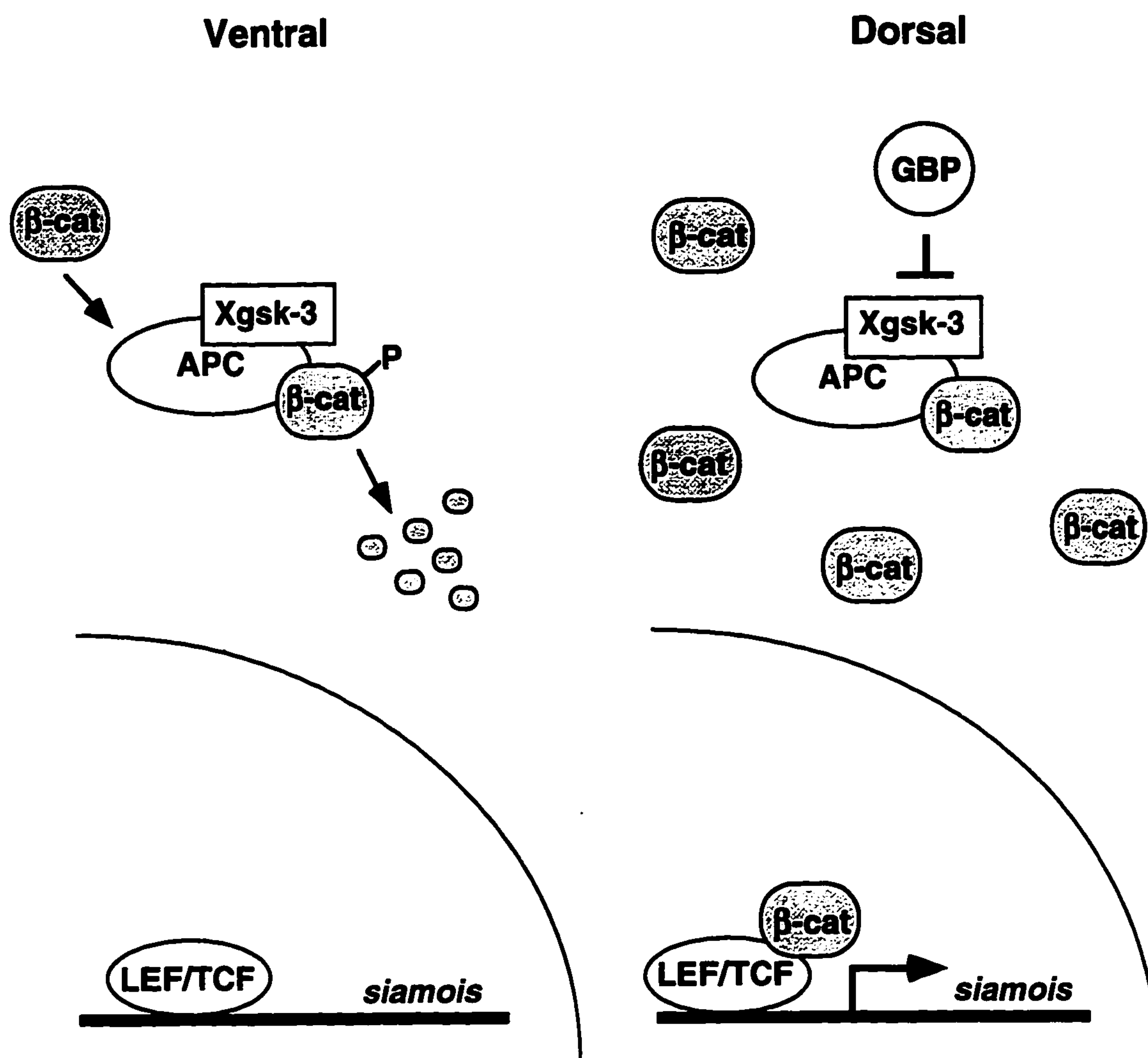


Figure 13: A model for dorsoventral axis determination in *Xenopus*. On the ventral side of the embryo β-catenin is phosphorylated by active Xgsk-3, which leads to the degradation of β-catenin. APC forms a complex with Xgsk-3 and β-catenin and is likely to cooperate with Xgsk-3 to promote β-catenin degradation. In the nucleus, transcription factors of the LEF/TCF family can bind to the promoters of specific genes, such as *siamois*, but cannot activate transcription in the absence of β-catenin. On the dorsal side, cortical rotation results in the disruption of β-catenin degradation, perhaps as a result of GBP binding to the APC complex. This leads to the accumulation of β-catenin and the formation of complexes of LEF/TCF and β-catenin. These complexes activate the transcription of dorsal genes, such as *siamois*, leading to the formation of the dorsoventral axis.

Chapter 5: Future directions

How is the Nieuwkoop organizer established?

It is now known that the activity of the Nieuwkoop organizer is due to a localized accumulation of β-catenin. This presumably results from an inhibition of or interference with the action of Xgsk-3. Data I have presented suggests that GBP could be part of this mechanism, but is it the dorsalizing activity that moves from the vegetal pole to the dorsal side during the first cell cycle? To answer this question, it would first be useful to know where GBP is expressed. GBP transcripts do not appear to be localized but the protein may be. Purified GBP would be used to produce anti-GBP antibodies that could be used to immunohistochemically localize GBP in unfertilized eggs and fertilized eggs during the first cell cycle. Localization of GBP to the vegetal pole in unfertilized eggs would suggest that it is a candidate for the translocated dorsal activity. This would be supported by visualization of an accumulation of GBP on the dorsal side during the first cell cycle. Larabell and coworkers have successfully used immunohistochemistry and confocal microscopy to visualize an accumulation of β-catenin on the dorsal side of the two-cell embryo (Larabell *et al.*, 1997; Rowning *et al.*, 1997). Because of this early dorsal accumulation of β-catenin, they have suggested that β-catenin itself could be translocated from the vegetal pole to the dorsal side.

Several experiments could be done to determine whether β-catenin or GBP could be the vegetally localized dorsalizing activity. If the accumulation of β-catenin on the dorsal side results from a localized stabilization of β-catenin, the activity at the vegetal pole would be expected to achieve this stabilization. If, on the other hand, the vegetal activity is β-catenin itself which is later moved to the

dorsal side, it would not be expected to have an effect on β-catenin stability. This could be tested by coinjecting β-catenin-*myc* RNA or ^{35}S-labeled β-catenin protein with vegetal cytoplasm into UV irradiated embryos and observing whether the ectopic β-catenin is stabilized. Animal pole cytoplasm would be used as a negative control and β-catenin-*myc* levels could be visualized by SDS-PAGE and autoradiography, immunoblotting, or immunohistochemistry of embryos. The ability of the vegetal pole cytoplasm to stabilize the ectopic β-catenin would suggest that the activity is not β-catenin itself whereas an inability to stabilize β-catenin would suggest that the activity could be β-catenin. Another approach would be to test the activity of vegetal cytoplasm from eggs in which maternal β-catenin or GBP has been depleted by antisense oligonucleotide mediated degradation. This method was used successfully to show that β-catenin is required for normal axis formation (Heasman *et al.*, 1994) and we have found that antisense oligonucleotides can be used to deplete the GBP protein in oocytes. Because it is more practical, this experiment would use vegetal cytoplasm from mature oocytes which has activity with the same characteristics as the activity in unfertilized eggs (Holowacz and Elinson, 1995). GBP or β-catenin RNA would be depleted by the injection of antisense oligonucleotides prior to maturation and the oocytes would be induced to mature by the addition of progesterone. Cytoplasm from the vegetal pole of the these oocytes would be injected into UV irradiated fertilized eggs to test whether the cytoplasm retains the inductive activity of cytoplasm from untreated oocytes. If depletion of GBP or β-catenin resulted in a loss of activity, it would suggest that this factor is a component of the vegetally localized dorsalizing activity.

Is GBP necessary for development?

Although the presence of GBP protein in the egg and the ability of ectopic GBP to induce axis formation are consistent with GBP playing an important role in early development, we have not yet demonstrated that GBP is necessary for development. This could be tested using antisense oligonucleotide mediated depletion of maternal protein. As mentioned above, this technique has been used successfully to demonstrate that β-catenin is necessary for dorsoventral axis formation (Heasman *et al.*, 1994). Because this technique targets mRNA for degradation, its success depends in part on the presence of a low level of maternal protein prior to oocyte maturation when the antisense oligonucleotides are injected. Because GBP protein does not accumulate until after maturation it is likely that maternal protein levels can be reduced dramatically. In preliminary experiments we have found this to be the case. To test the effects of protein depletion on early development, oocytes that have been injected with antisense oligonucleotides and matured with progesterone are implanted into the body cavity of a female after which they are laid and fertilized in vitro (Heasman *et al.*, 1994). Embryos depleted of β-catenin develop without any dorsal structures indicating that β-catenin is necessary for Nieuwkoop organizer function (Heasman *et al.*, 1994). Depending on the role that GBP plays in the embryo, we can predict two different outcomes of GBP depletion. If GBP normally acts to interfere with the process of β-catenin degradation, perhaps by disrupting the APC/β-catenin/GSK-3 complex, GBP depletion would be expected to have the same effect as β-catenin depletion, namely lack of dorsal structures. If, on the other hand, GBP normally acts to promote β-catenin degradation, with ectopic GBP having a dominant-negative effect, GBP depletion might have the opposite effect of causing embryos to develop with excess dorsal structures. Either of these

extreme results would be very informative for understanding the normal role of GBP. It is possible that GBP depletion could have additional or different effects that would make the results more difficult to interpret. In addition, this technique has not always been successful for establishing an in vivo role for tested proteins. In part this may be due to the difficulty of removing all of the maternal protein.

How does GBP affect the APC/β-catenin/Xgsk-3 complex?

Although we have found that GBP can associate with complexes that are immunoprecipitated with antibodies to APC, we have not determined what impact this has on the complex. For example, GBP might selectively displace or recruit a member of the complex, such as Xgsk-3. Alternatively, GBP may not change the contents of the complex but it may alter the phosphorylation state of one or more components. These questions could be examined with the following experiments.

Using size exclusion chromatography, Polakis and coworkers have demonstrated that under conditions in which cytoplasmic β-catenin levels are low, most of the cellular β-catenin is present in high molecular weight complexes (Papkoff *et al.*, 1996). Under conditions in which cytoplasmic levels of β-catenin are high, there is a dramatic increase of β-catenin in a lower molecular weight pool, presumably consisting of monomeric β-catenin. Size exclusion chromatography could be used to analyze the APC complex in more detail. Embryos would be injected with RNA encoding epitope tagged Xgsk-3, β-catenin, and GBP in various combinations. Embryo extracts would be subjected to size exclusion chromatography. The fractions would be subjected to immunoprecipitation with anti-APC antibodies and analyzed by Western blotting with antibodies to APC and the epitope tags. In this way it could be

determined whether all of these proteins are present in a single complex or whether multiple complexes exist in different configurations. In particular it could be determined whether GBP simply joins the complex with APC, β-catenin, and Xgsk-3, or whether it disrupts this complex. In addition, the Xgsk-3 activity associated with the complexes could be measured to determine the effect of GBP on Xgsk-3 activity in this context.

The phosphorylation states of β-catenin and APC in the presence and absence of GBP could be analyzed by in vivo labeling with ^{32}P. Inorganic ^{32}P phosphate would be injected into eggs soon after fertilization. RNA encoding epitope tagged β-catenin and Xgsk-3 with or without GBP would be injected at the two- to four-cell stage. A few hours later protein would be extracted, immunoprecipitated with anti-APC antibodies and analyzed by SDS-PAGE. The same experiment would be done without ^{32}P labeling and analyzed by Western blotting to determine protein levels. A change in the phosphorylation level of β-catenin or APC in the presence of GBP would support a model in which GBP interfered with (or promoted) phosphorylation of these molecules by Xgsk-3 or another unidentified associated kinase. I can see two reasons, however, why it might be difficult to see important changes in phosphorylation. First of all, the inorganic ^{32}P must be incorporated at a significant level into the ATP pool of the embryo. Because of the rapid development of the embryo it is not possible to allow very much time for this to happen so the amount of ^{32}P incorporated into the proteins of interest may be low. Second, APC is a very large protein with many potential phosphorylation sites. β-catenin is also large and is certainly phosphorylated at more sites than the identified GSK-3 sites. Thus, any changes in phosphorylation due to the presence of GBP may be obscured by background phosphorylation. One way to reduce this problem would be to work with smaller

fragments of the proteins. A central region of APC has been defined that can bind to and regulate the stability of β-catenin (Munemitsu *et al.*, 1995). This construct would be reasonable to use in this experiment. β-catenin is somewhat more difficult. It consists of a large central region containing thirteen Arm-related repeats and smaller N- and C-terminal regions (McCrea *et al.*, 1991). The central region is sufficient to induce an axis in *Xenopus* (Funayama *et al.*, 1995) but the N-terminal region contains the sites of regulation by GSK-3 (Peifer *et al.*, 1994a; Yost *et al.*, 1996). A subset of the Arm repeats have been shown to be essential for APC binding (Hulsken *et al.*, 1994). Thus it is difficult to know without further mapping experiments which regions of β-catenin could be removed without removing regions that are necessary for regulation and association with the APC complex. An alternative approach would be to make anti-phosphotyrosine antibodies directed against the N-terminal phosphorylation sites in β-catenin. These antibodies could be used in Western blotting experiments to determine whether the phosphorylation state of β-catenin is affected by the presence of GBP.

Another important question for understanding the nature and function of the APC complex is which components directly interact with each other. We know that GBP and Xgsk-3 interact and it is known that APC and β-catenin interact (Rubinfeld *et al.*, 1993; Su *et al.*, 1993). We would like to know, for example, whether GBP can directly interact with APC or β-catenin. This is difficult to assess by expressing ectopic proteins in *Xenopus* because all of these proteins are expressed endogenously, making it difficult to test an interaction between two proteins in isolation. It would be ideal to work with purified proteins but these are not all available at this time. Alternatively, these interactions could be tested using in vitro translated proteins. Two proteins could

be co-translated or mixed following individual translation and then immunoprecipitated with antibodies specific for one of the proteins. In this way, all of the possible pairwise interactions within the APC complex could be tested to determine whether they can occur in isolation. In addition, although some of the interacting domains are known for APC and β-catenin, we would like to know what regions of GBP and Xgsk-3 are important for these interactions. Because it was isolated in the two-hybrid screen, it is known that the C-terminal half of GBP interacts with Xgsk-3. Further deletion analysis with GBP could refine the interaction domain for Xgsk-3 and determine whether the N-terminus interacts with other proteins.

What is the function of GBP in other species?

The existence of GBP-related proteins in other species suggests that GBP has roles beyond amphibian dorsoventral axis specification. It would be particularly interesting to explore the function of a mammalian homolog. The sequence of the human EST could be used to isolate a full-length cDNA by library screening. Comparison of this sequence to the *Xenopus* GBP sequence for conserved regions might suggest which domains of the proteins are particularly important or carry out conserved functions. One way to test for conserved function would be to overexpress the human GBP in a Wnt-responsive cell line. Because GBP can mimic ectopic Wnt expression in *Xenopus*, I would predict that overexpression of human GBP would mimic Wnt overexpression and lead to transformation of the cells (Nusse and Varmus, 1992). Northern blot analysis could be used to determine whether it is expressed in tissues that are consistent with a role in Wnt signaling in humans.

PCR experiments in our laboratory also suggest that GBP-related proteins may be present in *Drosophila* (L. Sumoy and D. Kimelman, personal

communication). If a gene encoding a GBP-related protein is isolated from flies, this organism could be used to study the function of GBP. To test the function of GBP in Drosophila, transgenic flies could be constructed in which the *Drosophila* GBP was expressed under heat shock control. Induction of GBP expression at different times during development should indicate whether processes known to involve Wg signaling or other developmental processes are sensitive to GBP overexpression. These experiments would indicate whether GBP-related proteins are involved in development in other organisms and provide clues to the processes in which GBP is involved. The presence of GBP-related proteins in diverse organisms suggests that it is involved in a variety of processes, perhaps as a general component of Wnt signaling and the β-catenin degradation process.

How does ectopic expression of Xgsk-3 result in the enlargement of anterior ectodermal structures?

One of the unresolved questions from my experiments on the effects of Xgsk-3 on anterior ectodermal patterning is whether the effects of Xgsk-3 reflect an endogenous role for Xgsk-3 or whether these are non-specific effects of ectopic overexpression. For unknown reasons, the dominant-negative Xgsk-3 had very little effect when expressed in the future ectoderm. This could suggest that the effects of Xgsk-3 are non-specific but it may reflect some technical problem with the experiment, such as insufficient levels of dominant-negative Xgsk-3 protein by the time of anterior ectodermal patterning. For this reason, I would try alternative methods for asking the same question. If Xgsk-3 is acting in a Wnt pathway, as I would predict, β-catenin would be expected to have the same effect as dominant-negative Xgsk-3. The β-catenin mutants in which the GSK-3 phosphorylation sites have been removed or mutated are very stable in embryos (Yost *et al.*, 1996) so RNA injected into the early embryo should produce protein

which is stable through the gastrula stages when it would be expected to have an impact on ectodermal patterning. If the effects of Xgsk-3 on the cement gland and anterior gene expression reflect the action of Wnt signaling to limit the domain of these structures, I would predict that overexpression of β-catenin in this region would cause embryos to develop with reduced or absent cement glands and reduced expression of anterior genes such as *Xotx2* and *XANF-2*. An alternative method for ectopically expressing a gene at the gastrula stages is to inject DNA encoding the gene of interest under the control of a promoter that is active after MBT, such as the cytoskeletal actin (CSKA) promoter (Harland and Misher, 1988; Christian and Moon, 1993). Wild-type and dominant-negative Xgsk-3 and β-catenin could all be ectopically expressed under control of the CSKA promoter to determine whether their effects are consistent with the prediction that a *Wnt* pathway limits the expansion of the anterior region. In addition, if this model is correct, ectopic expression of dominant-negative Wnt in this region should have an effect similar to the effect of Xgsk-3 which is indeed the case. Ectopic expression of dnXwnt-8 results in the expansion of the domain of *XANF-2* expression and a loss of *Krox-20* and *En-2* expression (L. McGrew, S. Hoppler, and R. T. Moon, personal communication).

Bibliography

Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* 66, 257-270.

Ancel, P. and Vintemberger, P. (1948). Recherches sur le déterminisme de la symétrie bilatérale dans l'oeuf des amphibiens. *Bull. Biol. France Belg.* 31, 1-182.

Bartel, P., Chien, C. T., Sternglanz, R. and Fields, S. (1993). Using the two-hybrid system to detect protein-protein interactions. In Cellular interactions in development: a practical approach (ed. D. A. Hartley) Oxford: IRL Press.

Behrens, J., von Kries, J. P., Kühl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of β-catenin with the transcription factor LEF-1. *Nature* 382, 638-642.

Bianchi, M. W., Guivarc'h, D., Thomas, M. and Woodgett, J. R. (1994). *Arabidopsis* homologs of the shaggy and GSK-3 protein kinases: molecular cloning and functional expression in *Escherichia coli*. *Mol. Gen. Genet.* 242, 337-345.

Bianchi, M. W., Plyte, S. E., Kreis, M. and Woodgett, J. R. (1993). A *Saccharomyces cerevisiae* protein-serine kinase related to mammalian glycogen synthase kinase-3 and the *Drosophila melanogaster* gene *shaggy* product. *Gene* 134, 51-56.

Black, S. D. and Gerhart, J. C. (1986). High-frequency twinning of *Xenopus laevis* embryos from eggs centrifuged before first cleavage. *Dev. Biol.* 116, 228-240.

Blitz, I. L. and Cho, K. W. Y. (1995). Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*. *Development* 121, 993-1004.

Boterenbrood, E. C. and Nieuwkoop, P. D. (1973). The formation of the mesoderm in urodelean amphibians. *W. Roux' Arch. Ent. Org.* 173, 319-332.

Bourouis, M., Moore, P., Ruel, L., Grau, Y., Heitzler, P. and Simpson, P. (1990). An early embryonic product of the gene *shaggy* encodes a serine/threonine protein kinase related to the CDC28/cdc2^{+} subfamily. *EMBO J.* 9, 2877-2884.

Boyle, J. B., Smeal, T., Defize, L., Angel, P., Woodgett, J., Karin, M. and Hunter, T. (1991). Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell* 64, 573-583.

Brannon, M. and Kimelman, D. (1996). Activation of *Siamois* by the *Wnt* pathway. *Dev. Biol.* 180, 344-347.

Chakrabarti, A., Matthews, G., Colman, A. and Dale, L. (1992). Secretory and inductive properties of *Drosophila* wingless protein in *Xenopus* oocytes and embryos. *Development* 115, 355-369.

Chien, C., Bartel, P. L., Sternglanz, R. and Fields, S. (1991). The two-hybrid system: a method to identify and clone proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA* 88, 9578-9582.

Christian, J. L., Gavin, B. J., McMahon, A. P. and Moon, R. T. (1991a). Isolation of cDNAs partially encoding four *Xenopus Wnt*-1/*int*-1-related proteins and characterization of their transient expression during embryonic development . *Dev. Biol.* 143, 230-234.

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991b). *Xwnt-8*, a *Xenopus Wnt*-1/*int*-1-related gene responsive to mesoderm inducing factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* 111, 1045-56.

Christian, J. L. and Moon, R. T. (1993). Interactions between *Xwnt-8* and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of *Xenopus*. *Genes Dev.* 7, 13-28.

Christian, J. L., Olson, D. J. and Moon, R. T. (1992). *Xwnt*-8 modifies the character of mesoderm induced by bFGF in isolated *Xenopus* ectoderm. *EMBO J.* 11, 33-41.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252, 1102-1106.

Cohen, P. (1985). The role of protein phosphorylation in the hormonal control of enzyme activity. *Eur. J. Biochem.* 151, 439-448.

Cornell, R. A. and Kimelman, D. (1994). Activin-mediated mesoderm induction requires FGF. *Development* 120, 453-462.

Dale, L., Matthews, G. and Colman, A. (1993). Secretion and mesoderm-inducing activity of the TGF-β-related domain of *Xenopus* Vg1. *EMBO* 12, 4471-4480.

Dale, L., Matthews, G., Tabe, L. and Colman, N. (1989). Developmental expression of the protein product of Vg1, a localized maternal mRNA in the frog *Xenopus laevis*. *EMBO J.* 8, 1057-1065.

Dale, L. and Slack, J. M. (1987a). Fate map for the 32-cell stage of *Xenopus laevis*. *Development* 99, 527-551.

Dale, L. and Slack, J. M. W. (1987b). Regional specification within the mesoderm of early embryos of *Xenopus laevis*. *Development* 100, 279-295.

de-Groot, R. P., Auwerx, J., Bourouis, M. and Sassone-Corsi, P. (1993). Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and *Drosophila* kinase shaggy. *Oncogene* 8, 841-847.

Diaz-Benjumea, F. J. and Cohen, S. M. (1994). *wingless* acts through the *shaggy/zeste-white 3* kinase to direct dorsal-ventral axis formation in the *Drosophila* leg. *Development* 120, 1661-1670.

Dominguez, I., Itoh, K. and Sokol, S. Y. (1995). Role of glycogen synthase kinase 3β as a negative regulator of dorsoventral axis formation in *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* 92, 8498-8502.

Durfee, T., Becherer, K., Chen, P., Yeh, S., Yang, Y., Kilburn, A. E., Lee, W. and Elledge, S. J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* 7, 555-569.

Ekker, S. C., McGrew, L. L., Lai, C.-J., Lee, J. J., von Kessler, D. P., Moon, R. T. and Beachy, P. A. (1995). Distinct expression and shared activities of members of the hedgehog gene family of *Xenopus laevis*. *Development* 121, 2337-2347.

Elinson, R. P. and Pasceri, P. (1989). Two UV-sensitive targets in dorsoanterior specification of frog embryos. *Development* 106, 511-518.

Elinson, R. P. and Rowning, B. (1988). A transient array of parallel microtubules in frog eggs: potential tracks for a cytoplasmic rotation that specifies the dorso-ventral axis. *Dev. Biol.* 128, 185-197.

Fabian, J. R., Vojtek, A. B., Cooper, J. A. and Morrison, D. K. (1994). A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. *Proc. Natl. Acad. Sci. USA* 91, 5982-5986.

Fagotto, F., Guger, K. and Gumbiner, B. M. (1997). Induction of the primary dorsalizing center in *Xenopus* by the Wnt/GSK/ß-catenin signaling pathway, but not Vg1, Activin or Noggin. *Development* 124, 453-460.

Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeske, R. W. and Roach, P. J. (1987). Formation of protein kinase recognition sites by covalent modification of substrate. Molecular mechanisms for the synergistic action of casein kinase II and glycogen synthase kinase 3. *J. Biol. Chem* 262, 14042-14048.

Fiol, C. J., Williams, J., Chou, C., Wang, Q., Roach, P. and Andrisani, O. (1994). A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. *J. Biol. Chem.* 269, 32187-32193.

Fujisue, M., Kobayakawa, Y. and Yamana, K. (1993). Occurence of dorsal axis-inducing activity around the vegetal pole of an uncleaved *Xenopus* egg and displacement to the equatorial region by cortical rotation. *Development* 118, 163-170.

Funayama, N., Fagotto, F., McCrea, P. and Gumbiner, B. M. (1995). Embryonic axis induction by the Armadillo repeat domain of β-catenin: evidence for intracellular signaling. *J. Cell Biol.* 128, 959-968.

Gerhart, J. C., Danilchick, M., Doniach, T., Roberts, S., Rowning, B. and Stewart, R. (1989). Cortical rotation of the *Xenopus* egg: consequences for the anteroposterior pattern of embryonic dorsal development. *Development, Suppl.* 107, 37-51.

Gerhart, J. C., Stewart, R. and Doniach, T. (1991). Organizing the *Xenopus* organizer. In Gastrulation: movements, patterns, and molecules (ed. R. Keller, W. Clark Jr. and F. Griffin) New York: Plenum.

Gerhart, J. C., Ubbels, G., Black, S., Hara, K. and Kirschner, M. (1981). A reinvestigation of the role of the grey crescent in axis formation in *Xenopus laevis*. *Nature* 292, 511-516.

Giese, K., Kingsley, C., Kirshner, J. R. and Grosschedl, R. (1995). Assembly and function of a TCRα enhancer complex is dependent on LEF-1 induced DNA bending and multiple protein-protein interactions. *Genes Dev.* 9, 995-1008.

Gimlich, R. L. and Gerhart, J. C. (1984). Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. *Dev. Biol.* 104, 117-130.

Graff, D. M. (1997). Embryonic patterning: to BMP or not to BMP, that is the question. *Cell* 89, 171-174.

Green, J. B. A., Howes, G., Symes, K., Cooke, J. and Smith, J. C. (1990). The biological effects of XTC-MIF: quantitative comparison with *Xenopus* bFGF. *Development* 108, 173-183.

Green, J. B. A., New, H. V. and Smith, J. C. (1992). Responses of embryonic *Xenopus* cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* 71, 731-739.

Green, J. B. A. and Smith, J. C. (1990). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* 347, 391-394.

Guger, K. A. and Gumbiner, B. M. (1995). β-catenin has *Wnt*-like activity and mimics the Nieuwkoop signaling center in *Xenopus* dorsal-ventral patterning. *Dev. Biol.* 172, 115-125.

Hanger, D., Hughes, K., Woodgett, J., Brion, J. and Anderton, B. (1992). Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localization of the kinase. *Neurosc. Letters* 147, 58-62.

Harland, R. and Misher, L. (1988). Stability of RNA in developing *Xenopus* embryos and identification of a destabilizing sequence in TFIIIA messenger RNA. *Development* 102, 837-852.

He, X., Saint-Jeannet, J.-P., Woodgett, J. R., Varmus, H. E. and Dawid, I. (1995). Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. *Nature* 374, 617-622.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y. and Wylie, C. (1994). Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell* 79, 791-803.

Hemmati-Brivanlou, A. and Melton, D. A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* 359, 609-614.

Hemmings, B., Yellowlees, D., Kernohan, J. and Cohen, P. (1981). Purification of glycogen synthase kinase 3 from rabbit skeletal muscle. *Eur. J. Biochem.* 119, 443-451.

Hill, J., Donald, K. A. and Griffiths, D. E. (1991). DMSO-enhanced whole cell yeast transformations. *Nucleic Acids Res.* 19, 5791.

Hollenberg, S. M., Sternglanz, R., Cheng, P. F. and Weintraub, H. (1995). Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Mol. Cell Biol.* 15, 3813-3822.

Holley, S. A., Neul, J. L., Attisano, L., Wrana, J. L., Sasai, Y., O'Connor, M. B., De Robertis, E. M. and Ferguson, E. L. (1996). The *Xenopus* dorsalizing factor noggin ventralizes *Drosophila* embryos by preventing DPP from activating its receptor. *Cell* 86, 607-617.

Holowacz, T. and Elinson, R. P. (1993). Cortical cytoplasm, which induces dorsal axis formation in *Xenopus*, is inactivated by UV irradiation of the oocyte. *Development* 119, 277-285.

Holowacz, T. and Elinson, R. P. (1995). Properties of the dorsal activity found in the vegetal cortical cytoplasm of *Xenopus* eggs. *Development* 121, 2789-2798.

Holwill, S., Heasman, J., Crawley, C. R. and Wylie, C. C. (1987). Axis and germ line deficiencies caused by u. v. irradiation of *Xenopus* oocytes cultured in vitro. *Development* 100, 735-743.

Hoppler, S., Brown, J. D. and Moon, R. T. (1996). Expression of a dominant negative *Wnt* blocks induction of *MyoD* in *Xenopus* embryos. *Genes Dev.* 10, 2805-2817.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G. and Kemler, R. (1996). Nuclear localization of ß-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* 59, 3-10.

Hughes, K., Ramakrishna, S., Benjamin, W. and Woodgett, J. R. (1992). Identification of multifunctional ATP-citrate lyase kinase as the alpha-isoform of glycogen synthase kinase-3. *Biochem. J.* 288, 309-14.

Hulsken, J., Birchmeier, W. and Behrens, J. (1994). E-cadherin and APC compete for the interaction with ß-catenin and the cytoskeleton. *J. Cell Biol.* 127, 2061-2069.

Isaacs, H. V., Tannahill, D. and Slack, J. M. W. (1992). Expression of a novel FGF in the *Xenopus* embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. *Development* 114, 711-720.

Jones, E. A. and Woodland, H. R. (1987). The development of animal cap cells in *Xenopus*: a measure of the start of animal cap competence to form mesoderm. *Development* 101, 557-563.

Kageura, H. (1990). Spatial distribution of the capacity to initiate a secondary embryo in the 32-cell embryo of *Xenopus laevis*. *Dev. Biol.* 142, 432-438.

Kao, K. R. and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev. Biol.* 127, 64-77.

Keller, R. and Tibbetts, P. (1989). Mediolateral cell intercalation in the dorsal, axial mesoderm of *Xenopus laevis*. *Dev. Biol.* 131, 539-549.

Kelly, G. M., Erezyilmaz, D. F. and Moon, R. T. (1995). Induction of a secondary embryonic axis in zebrafish following the overexpression of β-catenin. *Mech. Dev.* 53, 261-273.

Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M. and Kirschner, M. (1988). The presence of FGF in the frog egg: its role as a natural mesoderm inducer. *Science* 242, 1053-1056.

Kimelman, D., Christian, J. L. and Moon, R. T. (1992). Synergistic principles of development: overlapping patterning systems in *Xenopus* mesoderm induction. *Development* 116, 1-9.

Klingensmith, J. and Nusse, R. (1994). Signaling by *wingless* in *Drosophila*. *Dev. Biol.* 166, 396-414.

Knecht, A. K., Good, P. J., Dawid, I. B. and Harland, R. M. (1995). Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. *Development* 121, 1927-1936.

Krieg, P. A., Varnum, S., Wormington, M. W. and Melton, D. A. (1989). The mRNA encoding elongation factor 1-α (EF-1α) is a major transcript of the midblastula transition. *Dev. Biol.* 133, 93-100.

Ku, M. and Melton, D. A. (1993). *Xwnt-11*: a maternally expressed *Xenopus Wnt* gene. *Development* 119, 1161-1173.

LaBonne, C. and Whitman, M. (1994). Mesoderm induction by activin requires FGF mediated intracellular signals. *Development* 120, 463-472.

Lai, C.-J., Ekker, S. C., Beachy, P. A. and Moon, R. T. (1995). Patterning of the neural ectoderm of *Xenopus laevis* by the amino-terminal product of hedgehog autoproteolytic cleavage. *Development* 121, 2349-2360.

Lamb, M. T., Knecht, A., Smith, W. C., Stachel, S. E., Econmides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. (1993). Neural induction by the secreted polypeptide noggin. *Science* 262, 713-718.

Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D. and Moon, R. T. (1997). Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in β-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* 136, 1123-1136.

Lemaire, P., Garrett, N. and Gurdon, J. B. (1995). Expression cloning of *Siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* 81, 85-94.

Lustig, K. D. and Kirschner, M. W. (1995). Use of an oocyte expression assay to reconstitute inductive signaling. *Proc. Natl. Acad. Sci. USA* 92, 6234-6238.

MacNicol, A. M., Muslin, A. J. and Williams, L. T. (1993). Raf-1 kinase is essential for early *Xenopus* development and mediates the induction of mesoderm by FGF. *Cell* 73, 571-584.

Malacinski, G. M., Brothers, J. and Chung, H.-M. (1977). Destruction of components of the neural induction system of the amphibian egg with ultraviolet irradiation. *Dev. Biol.* 56, 24-39.

Mandelkow, E., Drewes, G., Biernat, J., Gustke, N., Van Lint, J., Vandenheede, J. and Mandelkow, E. (1992). Glycogen synthase kinase-3 and Alzheimer-like state of microtubule-associated protein tau. *FEBS* 314, 315-321.

Massagué, J. (1987). The TGF-β family of growth and differentiation factors. *Cell* 49, 437-438.

Mathers, P. H., Miller, A., Doniach, T., Dirksen, M.-L. and Jamrich, M. (1995). Initiation of anterior head-specific gene expression in uncommitted ectoderm of *Xenopus laevis* by ammonium chloride. *Developmental Biology* 171, 641-654.

McCrea, P. D., Brieher, W. M. and Gumbiner, B. M. (1993). Induction of a secondary body axis in *Xenopus* by antibodies to beta-catenin. *J Cell Biol* 123, 477-484.

McCrea, P. D., Turck, C. W. and Gumbiner, B. (1991). A homolog of *armadillo* protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science* 254, 1359-1361.

McGrew, L. L., Lai, C.-J. and Moon, R. T. (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with *noggin* and *follistatin*. *Dev. Biol.* 172, 337-342.

McGrew, L. L., Otte, A. P. and Moon, R. T. (1992). Analysis of *Xwnt-4* in embryos of *Xenopus laevis*: A Wnt family member expressed in brain and floor plate. *Development* 115, 463-473.

McMahon, A. P. (1992). The *Wnt* family of developmental regulators. *Trends Genet.* 8, 1-5.

McMahon, A. P. and Bradley, A. (1990). The *Wnt*-1 (*int*-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 62, 1073-1085.

McMahon, A. P. and Moon, R. T. (1989). Ectopic expression of the proto-oncogene *int-1* in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* 58, 1075-1084.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* 12, 7035-7056.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates ß-catenin-induced axis formation in *Xenopus* embryos. *Cell* 86, 391-399.

Moody, S. A. (1987). Fates of the blastomeres of the 32-cell-stage *Xenopus* embryo. *Dev. Biol.* 122, 300-319.

Moon, R. T. (1993). In pursuit of the functions of the Wnt family of developmental regulators: insights from *Xenopus laevis*. *BioEssays* 15, 1-7.

Moon, R. T., Campbell, R. M., Christian, J. L., McGrew, L. L., Shih, J. and Fraser, S. (1993). *Xwnt-5A*: a maternal *Wnt* that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis*. *Development* 119, 97-111.

Moon, R. T. and Christian, J. L. (1989). Microinjection and expression of synthetic mRNAs in *Xenopus* embryos. *Technique* 1, 76-89.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* 275, 1787-1790.

Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B. and Polakis, P. (1995). Regulation of intracellular β-catenin levels by adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc. Natl. Acad. Sci. USA* 92, 3046-3050.

Newport, J. and Kirschner, M. W. (1982). A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes at the midblastula stage. *Cell* 30, 675-686.

Nieuwkoop, P. D. (1969a). The formation of the mesoderm in urodelean amphibians I. The induction by the endoderm. *W. Roux' Arch. Ent. Org.* 162, 341-373.

Nieuwkoop, P. D. (1969b). The formation of the mesoderm in urodelean amphibians II. The origin of the dorso-ventral polarity of the mesoderm. *W. Roux' Arch. Ent. Org.* 163, 298-315.

Nieuwkoop, P. D. and Faber, J. (1967). Normal table of *Xenopus laevis*. Amsterdam: North-Holland Publishing Company.

Nikolakaki, E., Coffer, P. J., Hemelsoet, R., Woodgett, J. R. and Defize, L. H. K. (1993). Glycogen synthase kinase 3 phosphorylates Jun family members *in vitro* and negatively regulates their transactivating potential in cells. *Oncogene* 8, 833-840.

Noordermeer, J., Klingensmith, J., Perrimon, N. and Nusse, R. (1994). *dishevelled* and *armadillo* act in the Wingless signalling pathway in *Drosophila. Nature* 367, 80-83.

Nusse, R. and Varmus, H. E. (1992). *Wnt* genes. *Cell* 69, 1073-1087.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* 121, 707-720.

Papkoff, J., Rubinfeld, B., Schryver, B. and Polakis, P. (1996). Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. *Mol. Cell. Biol.* 16, 2128-2134.

Pay, A., Honak, C., Bogre, L., Meskiene, I. and Mairinger, T. (1993). The MsK family of alfalfa protein kinase genes encodes homologues of shaggy/glycogen synthase kinase-3 and shows differential expression patterns in plant organs and development. *Plant J.* 3, 847-856.

Peifer, M., Pai, L.-M. and Casey, M. (1994a). Phosphorylation of *Drosophila* adherens junction protein armadillo: roles for *wingless* signal and *zeste-white 3* kinase. *Dev. Biol.* 166, 543-556.

Peifer, M., Sweeton, D., Casey, M. and Wieschaus, E. (1994b). *Wingless* signal and *zeste-white 3* kinase trigger opposing changes in the intracellular distribution of *armadillo. Development* 120, 369-380.

Pierce, S. B. and Kimelman, D. (1995). Regulation of Spemann organizer formation by the intracellular kinase *Xgsk-3. Development* 121, 755-765.

Pierce, S. B. and Kimelman, D. (1996). Overexpression of Xgsk-3 disrupts anterior ectodermal patterning in *Xenopus. Dev. Biol.* 175, 256-264.

Puziss, J. W., Hardy, T. A., Johnson, R. B., Roach, P. J. and Hieter, P. (1994). MDS1, a dosage suppressor of an mck1 mutant, encodes a putative yeast homolog of glycogen synthase kinase 3. *Mol. Cell Biol.* 14, 831-839.

Rebagliati, M. R., Weeks, D. L., Harvey, R. P. and Melton, D. A. (1985). Identification and cloning of localized maternal RNAs from *Xenopus* eggs. *Cell* 42, 769-777.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.-C., Grosschedl, R. and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signalling inputs from *wingless* and *decapentaplegic*. *Cell* In press,

Rosa, F., Roberts, A. B., Danielpour, D., Dart, L. L., Sporn, M. B. and Dawid, I. B. (1988). Mesoderm induction in Amphibians: the role of TGF-β2-like factors. *Science* 239, 783-785.

Rowning, B. A., Wells, J., Wu, M., Gerhart, J. C., Moon, R. T. and Larabell, C. A. (1997). Microtubule-mediated transport of organelles and localization of β-catenin to the future dorsal side of *Xenopus* eggs. *Proc. Natl. Acad. Sci. USA* 94, 1224-1229.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. (1996). Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. *Science* 272, 1023-1026.

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E. and Polakis, P. (1997). Stabilization of b-catenin by genetic defects in melanoma cell lines. *Science* 275, 1790-1792.

Rubinfeld, B., Souza, B., Albert, I., Müller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S. and Polakis, P. (1993). Association of the *APC* gene product with β-catenin. *Science* 262, 1731-1734.

Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S. and Polakis, P. (1995). The APC protein and E-cadherin form similar but independent complexes with α-catenin, β-catenin, and plakoglobin. *J. Biol. Chem.* 270, 5549-5555.

Saksela, K., Makela, T., Hughes, K., Woodgett, J. R. and Alitalo, K. (1992). Activation of protein kinase C increases phosphorylation of the L-myc trans-activator domain at a GSK-3 target site. *Oncogene* 7, 347-453.

Scharf, S. R. and Gerhart, J. C. (1980). Determination of the dorsal-ventral axis in eggs of *Xenopus laevis*: complete rescue of uv-impaired eggs by oblique orientation before first cleavage. *Dev. Biol.* 79, 181-198.

Schiestl, R. H. and Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* 16, 339-346.

Schmidt, J. E., Suzuki, A., Ueno, N. and Kimelman, D. (1995). Localized BMP-4 mediates dorsal-ventral patterning in the early *Xenopus* embryo. *Dev. Biol.* 169, 37-50.

Siegfried, E., Chou, T. and Perrimon, N. (1992). *wingless* signaling acts through *zeste-white 3*, the *Drosophila* homolog of *glycogen synthase kinase-3*, to regulate *engrailed* and establish cell fate. *Cell* 71, 1167-1179.

Siegfried, E., Wilder, E. L. and Perrimon, N. (1994). Components of *wingless* signaling in *Drosophila*. *Nature* 367, 76-80.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19-27.

Sive, H. L., Hattori, K. and Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. *Cell* 58, 171-180.

Slack, J. M., Isaacs, H. V. and Darlington, B. G. (1988). Inductive effects of fibroblast growth factor and lithium ion on blastula ectoderm. *Development* 103, 581-590.

Slusarski, D. C., Yang-Snyder, J., Busa, W. B. and Moon, R. T. (1997). Modulation of embryonic intracellular Ca^{2+} signaling by *Wnt-5a*. *Dev. Biol.* 182, 114-120.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Herrmann, B. G. (1991a). Expression of a *Xenopus* homolog of *brachyury* (T) is an immediate-early response to mesoderm induction. *Cell* 67, 79-87.

Smith, J. C., Price, B. M. J., Van Nimmen, K. and Huylebroeck, D. (1990). Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. *Nature* 345, 729-731.

Smith, J. C. and Slack, J. M. (1983). Dorsalization and neural induction: properties of the organizer in *Xenopus laevis*. *J. Embryol. Exp. Morphol.* 78, 299-317.

Smith, L. D., Xu, W. and Varnold, R. L. (1991b). Oogenesis and oocyte isolation. *Methods in Cell Biology* 36, 45-60.

Smith, W. C. and Harland, R. M. (1991). Injected *Xwnt-8* acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell* 67, 753-766.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of *noggin*, a new dorsalizing factor localized in the Spemann organizer in *Xenopus* embryos. *Cell* 70, 829-840.

Smith, W. C., Knecht, A. K., Wu, M. and Harland, R. M. (1993). Secreted *noggin* protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm. *Nature* 361, 547-549.

Sokol, S., Christian, J. L., Moon, R. T. and Melton, D. A. (1991). Injected *wnt* RNA induces a complete body axis in *Xenopus* embryos. *Cell* 67, 741-752.

Sokol, S. Y. (1996). Analysis of Dishevelled signalling pathways during *Xenopus* development. *Curr Biol* 6, 1456-1467.

Sokol, S. Y., Klingensmith, J., Perrimon, N. and Itoh, K. (1995). Dorsalizing and neuralizing properties of *Xdsh*, a maternally expressed *Xenopus* homolog of *dishevelled*. *Development* 121, 1637-1647.

Spemann, H. and Mangold, H. (1924). Uber induktion von embryonalanlagen durch implantation artfremder organisatoren. *W. Roux' Arch. Ent. Org.* 100, 599-638.

Su, L., K., Vogelstein, B. and Kinzler, K. W. (1993). Association of the APC tumor suppressor protein with catenins. *Science* 262, 1734-1737.

Sutherland, C., Leighton, I. A. and Cohen, P. (1993). Inactivation of glycogen synthase kinase-3β by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem. J.* 296, 15-19.

Tannahill, D. and Melton, D. A. (1989). Localized synthesis of the *Vg1* protein during early *Xenopus* development. *Development* 106, 775-785.

Thomas, K. R. and Capecchi, M. R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* 346, 847-850.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. *Cell* 63, 485-493.

Thomsen, G. H. and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in *Xenopus*. *Cell* 74, 433-441.

Turner, D. L. and Weintraub, H. (1994). Expression of *achaete-scute* homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* 8, 1434-1447.

Twamley-Stein, G. M., Pepperkok, R., Ansorge, W. and Courtneidge, S. A. (1993). The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* 90, 7696-7700.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M. and Clevers,

H. (1997). Armadillo co-activates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. *Cell* In press,

van den Eijnden Van Raaij, A. J., van Zoelent, E. J., van Nimmen, K., Koster, C. H., Snoek, G. T., Durston, A. J. and Huylebroeck, D. (1990). Activin-like factor from a *Xenopus* laevis cell line responsible for mesoderm induction. *Nature* 345, 732-734.

Vandenheede, J., Yang, S., Goris, J. and Merlevede, W. (1980). ATP-Mg-dependent protein phosphatase from rabbit skeletal muscle. *J. Biol. Chem.* 255, 11768-11774.

Vincent, J. P., Oster, G. F. and Gerhart, J. C. (1986). Kinematics of gray crescent formation in *Xenopus* eggs: the displacement of subcortical cytoplasm relative to the egg surface. *Dev. Biol.* 113, 484-500.

von Bubnoff, A., Schmidt, J. E. and Kimelman, D. (1996). The *Xenopus laevis* homeobox gene *Xgbx-2* is an early marker of anteroposterior patterning in the ectoderm. *Mech. Dev.* 54, 149-160.

Wang, Q. M., Roach, P. J. and Fiol, C. J. (1994). Use of a synthetic peptide as a selective substrate for glycogen synthase kinase 3. *Anal. Biochem.* 220, 397-402.

Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to *TGF-β*. *Cell* 51, 861-867.

Welsh, G. I., Miyamoto, S., Price, N. T., Safer, B. and Proud, C. G. (1996). T-cell activation leads to rapid stimulation of translation initiation factor eIF2B and inactivation of glycogen synthase kinase-3. *J. Biol. Chem.* 271, 11410-11413.

Whitman, M. and Melton, D. A. (1992). Involvement of $p21^{ras}$ in *Xenopus* mesoderm induction. *Nature* 357, 252-254.

Wolda, S. L. and Moon, R. T. (1992). Cloning and developmental expression in *Xenopus laevis* of seven additional members of the *Wnt* family. *Oncogene* 7, 1941-1947.

Woodgett, J. R. (1991). A common denominator linking glycogen metabolism, nuclear oncogenes and development. *Trends Biochem. Sci.* 16, 177-181.

Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D. and Moon, R. T. (1996). The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. *Genes Dev.* 10, 1443-1454.

Zimmerman, L. B., De Jesús-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86, 599-606.

Appendix A: Methods

Plasmids

Xgsk-3-LexA fusion plasmid: The plasmid pMM116 (a gift from Mike Moser) was constructed as follows. The *Sac*I site in pRS314 (Sikorski and Hieter, 1989) was converted to an *Sph*I site and the region from the *Kpn*I site to the *Bam*HI site was deleted. The *Sph*I fragment from pBTM116 (Bartel *et al.*, 1993), containing the LexA sequence, was then inserted into the *Sph*I site of pRS314 to create pMM116. The result is a LexA fusion vector containing the same promoter, polylinker, termination sequences, and yeast selectable marker (*TRP1)* as pBTM116, but with a CEN 6 origin rather than a 2μ origin. To create the Xgsk-3-LexA fusion plasmid, pMM116 (vector) and XG03 (insert) (Pierce and Kimelman, 1995) were digested with *Bam*HI and recombined to produce MM-XGSK3.

RNA expression vectors: *myc* epitope-tagged wild-type and dominant-negative Xgsk-3 were generated by PCR from pXG73 (Pierce and Kimelman, 1995) and pXG114 [generated as described for pXG73, using a fragment from pXG21 (Pierce and Kimelman, 1995)], respectively, using the GSK-F (Pierce and Kimelman, 1995) and GSK-RC (GCGATCGATTGGAGGAGTTGGAGGCAGA) primers. Amplification conditions were as described for generating full-length Xgsk-3 (Pierce and Kimelman, 1995). The amplified fragments were isolated from an agarose gel, digested with *Bam*HI and *Cla*I, and ligated into CS2^{+}MT (Turner and Weintraub, 1994) cut with *Bam*HI and *Cla*I. This generated pXG134 and pXG137 which encode wild-type and dominant-negative Xgsk-3, respectively, fused in-frame at the C-terminus to six repeats of the *myc* epitope.

To generate an RNA expression vector for GBP, the 894 bp *Sac*I-*Eco*RI fragment containing the entire GBP coding region was isolated from phage clone

1-2 and purified on an agarose gel. This fragment was inserted into pBluescriptII KS+ (Stratagene), digested with *Sac*I and *Eco*RI, to generate 1-2SacI. This plasmid was digested with *Sac*I, the ends were blunted with T4 polynucleotide kinase, and the GBP fragment was isolated by digestion with *Eco*RI and agarose gel purification. The plasmid CS2+ (Turner and Weintraub, 1994) was digested with *Bam*HI, followed by blunting of the ends with T4 polynucleotide kinase and digestion with *Eco*RI. The GBP *Eco*RI-blunt fragment and the *Eco*RI-blunt CS2+ vector were combined to create GBP-1-CS.

FLAG epitope-tagged GBP was generated by PCR from phage clone 1-2 using Vent DNA polymerase (New England Biolabs) and primers DK-54 (GCGGGATCCGCCATGCCGTGTCGCAAGGA) and DK-55 (CGCGGATCCATCGATATTGCACGGTTGTCTCA). Amplification conditions were 1 cycle at 94°C for 3 minutes; 2 cycles at 94°C for 1 minute, 49°C for 3 minutes, and 72°C for 1 minute; 25 cycles at 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute. The amplified GBP fragment was isolated from an agarose gel and used as the template in a second PCR reaction using primers DK-54 and DK-55 and *Tfl* DNA polymerase (Epicentre). The amplification conditions were 1 cycle at 94°C for 3 minutes; 2 cycles at 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute; 1 cycle at 72°C for 10 minutes. The amplified DNA was purified by phenol:chloroform extraction and ethanol precipitation and ligated into the pGEM-T vector (Promega) to generate BP8. CS2+-FLAG (a gift from Robert Cornell) was constructed by inserting a fragment encoding the FLAG epitope followed by a stop codon between the *Xba*I and *Sna*BI sites of CS2+, destroying the *Sna*BI site and the T7 promoter. GBP was isolated from BP8 by digestion with *Bam*HI and *Cla*I and ligated into CS2+-FLAG digested with *Bam*HI and *Cla*I to generate BP20. This construct contains the complete coding

sequence of GBP fused in-frame at the C-terminus to a single copy of the FLAG epitope. *Myc*-epitope-tagged GBP was constructed by ligating the *Bam*HI/*Cla*I GBP fragment from BP20 into CS2$^+$-MT, digested with *Bam*HI and *Cla*I, to generate BP25.

Construction of the plasmid encoding *myc*-tagged β-catenin has been described (Yost *et al.*, 1996).

Yeast two-hybrid library construction

Total RNA was isolated from late stage oocytes and unfertilized eggs by homogenization in an SDS-proteinase K buffer (Cornell and Kimelman, 1994). To select poly(A)$^+$ RNA, the RNA was subjected to two rounds of oligo(dT) purification using the PolyATtract kit (Promega). The two-hybrid library was constructed essentially as described (Hollenberg *et al.*, 1995). Random-primed cDNA was synthesized from 0.6 mg each of oocyte and egg mRNA using the RiboClone kit with M-MLV (H^-) reverse transcriptase (Promega). The cDNA was ligated overnight with a 100-fold molar excess of *Not*I adaptors (Hollenberg *et al.*, 1995) and size-fractionated (500-1300 bp) by agarose gel electrophoresis. One tenth of the purified cDNA was amplified by the polymerase chain reaction (PCR) with the NL-s primer (Hollenberg *et al.*, 1995). The PCR reaction contained 1.5 mM Mg^{2+} and amplification conditions were 1 cycle at 94°C for 3 minutes and 17 cycles at 94°C for 30 seconds; 58°C for 30 seconds; 72°C for 1 minute. To assure that all of the cDNA was in duplex form, the reaction was diluted 5-fold with fresh PCR reagents and subjected to one additional amplification cycle. The cDNA was digested overnight with *Not*I, purified by agarose gel electrophoresis, and 30% was ligated into pVP16 (*Not*I digested; dephosphorylated) (Hollenberg *et al.*, 1995). 80% of the ligation was transformed by electroporation into DH10B cells (BioRad) to yield 1.4 x 10^7 transformants.

Yeast two-hybrid library screen

Yeast strain L40 containing the bait plasmid MM-XGSK3 was transformed with library DNA essentially as described (Hollenberg *et al.*, 1995). Two hundred fifty ml of L40 containing MM-XGSK3 was transformed with 100 μg library DNA mixed with 5 mg sheared salmon sperm carrier DNA by the lithium acetate method (Schiestl and Gietz, 1989), with dimethyl sulfoxide added to a final concentration of 10% (Hill *et al.*, 1991). Transformants were allowed to recover for 4 hours in 500 ml of yeast complete media (Trp^- Leu^- Ura^-) before being plated on media to select for histidine prototrophy (Trp^- Leu^- Ura^- Lys^- His^-). Histidine positive colonies were assayed for β-galactosidase activity using a filter assay (Durfee *et al.*, 1993). After loss of MM-XGSK3, β-galactosidase positive clones were mated to AMR 70 strains carrying either MM-XGSK3 or pLex-Lamin (Hollenberg *et al.*, 1995). The resulting diploids were again assayed for β-galactosidase activity. Library plasmids which showed no activity with the Lex-Lamin construct but which were positive with MM-XGSK3 were isolated and transformed into XL-1 Blue (Stratagene). Clones were manually sequenced using Sequenase (Amersham) in the anti-sense direction with the M-13 $^-$20 primer and in the sense direction with a pVP16 sense primer (GCCGACTTCGAGTTTGAGCAGATG). Sequences were analyzed by translating in 6 reading frames and searching the non-redundant peptide sequence database using the BLASTX program.

cDNA library screen

To isolate a full-length GBP cDNA, the 247 bp fragment of GBP isolated in the two-hybrid screen was used as a probe to screen $1x10^6$ pfu of a *Xenopus* oocyte cDNA library (Rebagliati *et al.*, 1985) at high stringency. Six different positive cDNA fragments were isolated and cloned into pBluescriptII SK^+.

Restriction mapping and sequencing of the ends of the clones identified the 5' and 3' most clones. The 5' most clone, 1-2, was easily sequenced due to its short length (~1.2 Kb) and was found to contain all of the GBP sequence present in the two-hybrid clone, upstream of which was an in-frame methionine just downstream of stop codons in all three frames, and downstream of which was an in-frame stop codon. A poly-A sequence was not present in this clone, but was identified at the 3' end of the 3' most phage clone recovered. The poly-A sequence is approximately 2.6 Kb downstream of the stop codon. Thus the entire coding region of the gene along with 360 bp of 3' UTR was contained in phage clone 1-2 and the entire 3' UTR along with 110 bp of 3' coding region was contained in another clone, 2-2.

RNA isolation and RNase protection

RNA was prepared by homogenization in an SDS-Proteinase K buffer (Cornell and Kimelman, 1994) and analyzed by the RNase protection assay (Melton *et al.*, 1984). For GBP, 10 μg of total RNA from whole oocytes or embryos or from dissected embryos was used. To make the GBP probe, a 427 bp *Eco*RI-*Sma*I fragment from GBP phage clone 1-2 was cloned into pBluescriptII SK^+ (Stratagene) to generate p1-2(400). p1-2(400) was linearized with *Eco*RI and transcribed with T7 polymerase to generate an antisense riboprobe of approximately 490 bp which protected 192 bases of the 5' end of the GBP transcript. A probe for the ubiquitously expressed *EF-1α* gene (Krieg *et al.*, 1989) was synthesized to a lower specific activity and included in every hybridization reaction (Cornell and Kimelman, 1994). Probes were hybridized with RNA samples overnight at 45°C and then treated with 10 μg/ml of RNase A and approximately 500 units/ml RNase T_1 (Sigma) for 1 hour at room temperature. Protected fragments were separated on 8% acrylamide-urea gels.

Embryos and oocytes

Fertilized embryos were obtained as previously described (Newport and Kirschner, 1982). Eggs were fertilized in 0.5x MMR (1x MMR is 0.1 M NaCl, 2.0 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.8). The jelly coat was removed with 2% cysteine in water, pH 7.8, and eggs were rinsed in 0.1x MMR. Embryos were kept at 14°C to 23°C. Staging was as previously described (Nieuwkoop and Faber, 1967).

Ovaries were surgically removed from female frogs and the oocytes defolliculated with 0.5 mg/ml collagenase (Sigma) in 0.1 M phosphate buffer, pH 7.5. Oocytes were washed extensively with OR2 (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, and 5.0 mM HEPES, pH 7.8) and cultured at 14°C to 23°C in OCM [50% Liebowitz medium (Gibco), 0.4 mg/ml bovine serum albumin (BSA), 1.0 mM glutamine]. Staging was as described (Smith *et al.*, 1991b). Oocytes were matured by culturing overnight in OCM with 5 µg/ml progesterone (Sigma).

RNA synthesis and microinjection

All RNA was synthesized from the $CS2^+$ derived vectors, linearized with *Not*I, using the SP6 mMESSAGE mMACHINE kit (Ambion) following the manufacturer's instructions. Phenol:chloroform (1:1) extracted RNA was separated from unincorporated nucleotides with a Microcon 100 microconcentrator (Amicon) and injected without further purification. RNA was microinjected as published (Moon and Christian, 1989), in volumes of 10 nl or less per blastomere.

Immunoprecipitation and Western blotting

For Xgsk-3 kinase assays embryos were lysed in 10 μl per embryo of GSK lysis buffer containing 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2 mM DTT, 0.2 mM sodium orthovanadate, 20 μg/ml leupeptin, 1 mM NaF, 2 μg/ml pepstatin A and 0.5 mM PMSF. For other immunoprecipitations, embryos were lysed in 10 μl per embryo of Triton X-100 lysis buffer (Rubinfeld *et al.*, 1993) without Pefabloc. Lysates were centrifuged at full speed in a microcentrifuge for 15 minutes at 4°C and half of the clear supernatant was retained. Immunoprecipitation was performed by adding 1 μl of mouse anti-*myc* monoclonal (Oncogene Science) or rabbit anti-FLAG polyclonal antibody (Santa Cruz Biotechnology) per 10 embryos or 1 μg of rabbit polycloncal anti-APC2 antibody (Rubinfeld *et al.*, 1995) per sample and incubating 1 hour to overnight at 4°C. Immune complexes were isolated by adding 10 μl of Protein G-sepharose beads (Sigma) and incubating 1 hour at 4°C with agitation. For detection by Western blotting or kinase labeling, the beads were washed three times with 750 μl of buffer B (Rubinfeld *et al.*, 1993) and boiled in SDS-PAGE sample buffer or labeled as described below, respectively. Unless otherwise noted, all SDS-PAGE was performed in 12% polyacrylamide gels and electroblotting used standard Tris-glycine buffers containing 25% methanol. Proteins were detected using mouse anti-*myc* monoclonal (Oncogene Science) or rabbit anti-FLAG polyclonal (Santa Cruz Biotechnology) primary antibody, followed by the appropriate horseradish peroxidase-conjugated goat anti-immunoglobulin secondary antibody, and blots were developed using Enhanced Chemiluminescence (Amersham).

Kinase labeling

Immunoprecipitates were washed with 0.5 ml protein kinase A (PKA) buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM β-glycerophosphate, 100 μM Na_3VO_4, 1 mM DTT, 0.2 mM PMSF). The beads were incubated in 20 μl of PKA buffer with 1 mU PKA catalytic subunit (Boehringer Mannheim) and 0.5 μl γ-^{32}P-ATP (3000 Ci/mmol, 10 mCi/ml) for 30 min at 30°C, transferred to ice, and EDTA was added to 10 mM. The beads were washed twice with 1 ml of buffer B, boiled in SDS-PAGE sample buffer, and samples were analyzed on polyacrylamide gels.

Xgsk-3 kinase assay

Immunoprecipitates were washed twice with 50 mM Tris, pH 7.5, 1 M NaCl and 1 mM DTT, and twice with 50 mM Tris, pH 7.5. For quantitation of Xgsk-3-*myc* protein levels, 10% of each sample was analyzed by SDS-PAGE and immunoblotting with anti-*myc* antibody. To measure GSK-3 kinase activity, the remainder of the immunoprecipitates were incubated in a 30 μl reaction containing 100 μM p-CREB or CREB peptide (Genosys), 30 mM Tris,pH 7.4, 100μM [γ-^{32}P] ATP, 10 mM $MgCl_2$, 1 mM DTT, 5% glycerol and 0.1 mg/ml BSA for 30 minutes at 30°C (Wang *et al.*, 1994). ^{32}P-labeled peptide was analyzed by recovery on p81 phosphocellulose paper (Whatman), followed by four washes with 75 mM phosphoric acid over 20 minutes, and liquid scintillation spectrometry. The activity of purified Xgsk-3 was assayed as described (Yost *et al.*, 1996).

In-gel protease mapping

In-gel protease mapping was performed by a modification of the published procedure (Cleveland *et al.*, 1977). Gel slices containing proteins to be

analyzed were excised from wet unfixed gels, using an autoradiograph of the wet gel for localization. The buffers for soaking and proteolysis were as described with the addition of 2.5 mM DTT, and 0.1% bromophenol blue was added to the protease dilution buffer. Proteins were digested with 1 μg *Staphylococcus aureus* V8 protease (Sigma, P-2922) during electrophoresis on 16% SDS-polyacrylamide gels.

Appendix B: Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3

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Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3

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SUMMARY

Dorsal axis formation in the *Xenopus* embryo can be induced by the ectopic expression of several *Wnt* family members. In *Drosophila*, the protein encoded by the *Wnt* family gene, *wingless*, signals through a pathway that antagonizes the effects of the serine/threonine kinase zeste-white 3/shaggy. We describe the isolation and characterization of a *Xenopus* homolog of *zeste-white 3/shaggy*, *Xgsk-3*. A kinase-dead mutant of Xgsk-3, Xgsk-3K→R, has a dominant negative effect and mimics the ability of Wnt to induce a secondary axis by induction of an ectopic Spemann organizer. Xgsk-3K→R, like Wnt, induces dorsal axis formation when expressed in the deep vegetal cells, which do not contribute to the axis. These results indicate that the dorsal fate is actively repressed by Xgsk-3, which must be inactivated for dorsal axis formation to occur. Furthermore, our work suggests that the effects of Xgsk-3K→R are mediated by an additional intercellular signal.

Key words: *Xenopus*, Spemann organizer, kinase XGSK-3, dorsal axis formation, *Wnt*

INTRODUCTION

Pattern formation in the early *Xenopus* embryo involves a series of intercellular signaling events which occur during the early cleavage stages. With the identification of many of the key signaling factors, it has become increasingly clear that they are likely to act in combination to regulate early inductive events (Kimelman et al., 1992; Sive, 1993; Cornell and Kimelman, 1994b). A member of the fibroblast growth factor (FGF) family and an activin-like molecule, perhaps Vg1, appear to act together to induce mesoderm to form at the equator of the embryo (R. Cornell, T. Musci, and D. Kimelman, unpublished data). During this same time period, a dorsalizing signal, present in the dorsal vegetal and perhaps dorsal marginal zone blastomeres, acts to induce the dorsal organizing center, known as the Spemann organizer, in the dorsal mesoderm (Gimlich and Gerhart, 1984). The Spemann organizer then generates signals that dorsalize the adjacent mesoderm, creating the dorsoventral axis (Spemann and Mangold, 1924). Several factors have been proposed to be the dorsalizing signal that induces the Spemann organizer, including members of the *Wnt* family, noggin, and high levels of Vg1, all of which have the ability to induce ectopic axis formation when expressed in the ventral region of the early embryo (Smith and Harland, 1991, 1992; Sokol et al., 1991; Thomsen and Melton, 1993).

Although many of the signaling factors involved in dorsoventral patterning have been identified, little is known about the intracellular signaling mechanisms by which they act. FGFs act via a MAP kinase-dependent pathway, whereas activin-mediated mesoderm induction occurs through a different and, as of yet, unknown pathway (Graves et al., 1994; Hartley et al., 1994; LaBonne and Whitman, 1994). Receptors for Wnt and noggin have not been identified, and hence these pathways are poorly understood. Lithium chloride, which causes embryos to develop an ectopic axis (Kao et al., 1986), appears to act by disrupting the phosphoinositide (PI) cycle (Busa and Gimlich, 1989; Maslanski et al., 1992), suggesting that PI turnover may be an important aspect of the intracellular signaling pathways stimulated by dorsalizing factors. In addition, there is enhanced gap junction activity on the dorsal side of the 32-cell embryo (Guthrie, 1984), an effect that can be produced on the ventral side of embryos by the expression of Xwnt-8 (Olson et al., 1991) or the addition of lithium chloride (Nagajski et al., 1989), indicating that intercellular communication through gap junctions may be involved in early patterning of the dorsoventral axis (Warner et al., 1984; Guthrie et al., 1988).

In a few cases, large parts of intracellular signaling pathways are conserved between species, even though the signals are used to accomplish different objectives (Nishida and Gotoh, 1993). The *Drosophila* gene *wingless* (*wg*) encodes a member of the *Wnt* family, which has axis-inducing properties when expressed in the *Xenopus* embryo (Chakrabarti et al., 1992). Several genes have been identified that potentially act downstream of *wg*, including *zeste-white 3/shaggy* (*zw3/shaggy*), *armadillo* and *dishevelled* (Siegfried et al., 1992, 1994; Noordermeer et al., 1994). *Zw3/shaggy* encodes a cytoplasmic Ser/Thr kinase whose effects are antagonized by *wg* signaling (Bourouis et al., 1990; Siegfried et al., 1992). The mammalian homologs of *zw3/shaggy*, glycogen synthase kinase-3α and β (GSK-3α and β), have recently been shown to be regulated by targets of growth factor signaling (Sutherland et al., 1993), and *zw3/shaggy*/GSK-3 homologs have also been identified in yeast and plants (Bianchi et al., 1993, 1994; Pay et al., 1993;

Puziss et al., 1994). The conservation of *zw3/shaggy*/GSK-3 among species and its regulation by Wg and other growth factors suggested to us that it may be involved in intracellular signaling in *Xenopus*, particularly during dorsoventral axis determination.

We have isolated and characterized a *Xenopus* homolog of *zw3/shaggy*/GSK-3 (*Xgsk-3*) which is expressed as a maternal transcript. A kinase-dead mutant of Xgsk-3 was constructed, which, when overexpressed in embryos, causes ectopic axis formation, similar to the effects of Xwnt-8, noggin and Vg1. The kinase-dead Xgsk-3 appears to act as a dominant negative mutant by blocking the function of endogenous Xgsk-3. Our results indicate that the dorsal fate is actively repressed by a Ser/Thr kinase which must be inactivated for dorsal determination to take place. In addition, our results suggest that *Wnt* signaling in vertebrates may be mediated by an additional intercellular signal.

MATERIALS AND METHODS

Isolation of an *Xgsk-3* cDNA

The polymerase chain reaction (PCR) was used to isolate a fragment of *Xgsk-3*, using primers based on the *Drosophila zw3/shaggy* gene (Bourouis et al., 1990) and the rat GSK-3α and β genes (Woodgett, 1990). Oligo(dT)-primed cDNA was first synthesized using mouse Moloney leukemia virus polymerase (BRL) with RNA isolated from stage 7 and stage 17 *Xenopus* embryos. The degenerate oligonucleotides used in the PCR, designed to amplify a 703 bp region of the kinase domain, were: 5′ primer: CGCGGATCCAA-(A/G)GTXAT(A/C/T)GGXAA(C/T)GG and 3′ primer: GCG-GAATTCGG(G/A)AA(T/C)TT(G/A)AA(T/C)TCXGT. PCR conditions were 1 cycle at 94°C for 3 minutes; 2 cycles at 94°C for 1 minute, 42°C for 3 minute, and 72°C for 1 minute; 40 cycles at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute. Vent polymerase (New England Biolabs) was used in all PCR reactions. After the PCR, amplified fragments were isolated from polyacrylamide gels, digested with *Pst*I and *Kpn*I to produce a 528 bp fragment, and cloned into a BluescriptII KS+ plasmid (Stratagene) for DNA sequence analysis (Sanger et al., 1977). All isolates had the same nucleotide sequence. One of the PCR fragments was used as a probe to isolate cDNAs from a stage 17 phage library (Kintner and Melton, 1987), which were then inserted into the *Eco*RI site of a BluescriptII SK+ vector. Nucleotide sequence analysis of several cDNAs and comparison with the rat and *Drosophila* sequences revealed that at least 200 bp of coding region were missing from the 3′ end of the transcripts due to an internal *Eco*RI site, and that the cDNAs contained a large 5′ untranslated region (UTR). To obtain the nucleotide sequence of the missing 3′ end, PCR was employed using DNA from one of the phage isolates (ϕ7) as the template, a phage primer, the 5′ degenerate primer and the PCR conditions used above. The amplified fragment was gel-isolated, digested with *Eco*RI, and inserted into a BluescriptII SK+ vector. To obtain a DNA fragment containing the entire coding region without the 5′ UTR, primers with the sequences CGCCGGATCCATATGAGCG-GAAGGCCGAGAACC (GSK-F) and CGCGGATCCTCGATACAT-GTGGCC (GSK-R) were used to amplify the *Xgsk-3* coding region from the ϕ7 DNA. The PCR conditions were 1 cycle at 94°C for 3 minutes; 2 cycles at 94°C for 1 minute, 42°C for 3 minutes, and 72°C for 1.5 minutes; 15 cycles at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1.5 minutes; 1 cycle at 72°C for 10 minutes. The amplified fragment was isolated from an agarose gel, digested with *Bam*HI, which cleaves at the ends of the PCR primers, and inserted into the *Bam*HI site of a BluescriptII KS+ vector, creating pXG03 and into the *Bgl*II site of a pSP64T vector, creating pXG30. The complete nucleotide sequence of the PCR-generated cDNA (*Xgsk-3*) was determined and deposited in GenBank under accession number L38492.

A frame-shift mutant of *Xgsk-3* (*Xgsk-3FS*) was made by digesting pXG30 with *Eco*RV, which cleaves in the middle of the *Xgsk-3* gene, ligating in the presence of *Bgl*II linkers (8-mer; New England Biolabs), digesting with *Bgl*II and then ligating to recircularize the plasmid. This created an insertion resulting in a frame-shift mutation.

Construction of the *Xgsk-3* kinase-dead mutant

The kinase-dead mutant of *Xgsk-3* (*Xgsk-3K→R*) was constructed using a PCR-based overlap extension method (Ho et al., 1989). Complimentary primers were designed to cover the region to be mutated, incorporating a single nucleotide change to convert a lysine to an arginine residue. These primers, GSK-K85R-R and GSK-K85R-F, had the sequences CAGCAGCTTCCTGATAGCCAC and GTGGC-TATCAGGAAGGTGCGG, respectively. ϕ7 DNA was used as the template in two separate PCR reactions with the primer pairs GSK-F/GSK-K85R-R and GSK-K85R-F/GSK-R. The resulting overlapping fragments were purified on agarose gels and used together as the template in a PCR reaction with GSK-F and GSK-R as primers. PCR conditions for these reactions were 2 cycles at 94°C for 1 minute, 42°C for 3 minutes, and 72°C for 1.5 minutes; 15 cycles at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1.5 minutes. The full-length amplified fragment with the incorporated mutation was purified on an agarose gel, digested with *Bam*HI and inserted into the *Bam*HI site of a BluescriptII KS+ vector, creating pXG21 and into the *Bgl*II site of a pSP64T vector, creating pXG40. The presence of the correct mutation was confirmed by nucleotide sequence analysis.

Embryos

Fertilized embryos were obtained as previously described (Newport and Kirschner, 1982). Eggs were fertilized in 0.5× MMR (1× MMR is 0.1 M NaCl, 2.0 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 0.1 mM EDTA and 5.0 mM Hepes, pH 7.8). The jelly coat was removed with 2% cysteine in water, pH 7.8, and eggs were rinsed in 0.1× MMR. Embryos were kept at 14°C to 23°C. Staging was as previously described (Nieuwkoop and Faber, 1967). For dissection experiments, the dorsal side of embryos was marked with Nile blue at the 4-cell stage.

Animal caps

The upper portion of the animal hemisphere was dissected from stage 9 embryos with a fine wire knife. Care was taken to remove any adherent vegetal cells. Caps were cultured in 1× MMR with 1 mg/ml BSA and 50 μg/ml gentamicin sulfate (Sigma) alone or in the presence of 15 ng/ml *Xenopus* basic FGF, prepared as previously described (Kimelman et al., 1988).

RNA synthesis and microinjection

Xgsk-3, *Xgsk-3K→R*, and *Xgsk-3FS* RNA was synthesized from the pSP64T derived vectors, linearized with *Bam*HI. *ΔXgsk-3* RNA was synthesized from pXG30 linearized with *Sma*I, which produces a form of the Xgsk-3 protein that is truncated within the kinase domain. The template for β-galactosidase RNA was CS-β-galactosidase (Turner and Weintraub, 1994), linearized with *Not*I. RNA was synthesized using the SP6 mMESSAGE mMACHINE kit (Ambion) following the manufacturer's instructions. Phenol:chloroform (1:1) extracted RNA was separated from unincorporated nucleotides with a Microcon 100 microconcentrator (Amicon) and injected without further purification. RNA was microinjected as published (Moon and Christian, 1989), in volumes of 10 nl or less per blastomere.

RNA isolation and RNase protection

RNA was prepared by homogenization in an SDS-proteinase K buffer (Cornell and Kimelman, 1994a) and analyzed by the RNase protection assay (Melton et al., 1984). For *Xgsk-3*, 20 μg of whole embryo RNA or RNA isolated from 10 dissected embryos was used. For muscle actin, 2.5 animal cap equivalents of RNA were used. The muscle actin and *EF-1α* genes were used as previously described (Cornell and Kimelman, 1994a). To make the *Xgsk-3* probe, a *Bam*HI-

PstI fragment was cloned into BluescriptII SK+. This plasmid (pXG-C) was linearized with *Bam*HI and transcribed with T7 polymerase, producing a probe which protects 291 bases of the 5′ end of the *Xgsk-3* transcript. A probe for the ubiquitously expressed *EF-1α* gene (Krieg et al., 1989) was synthesized to a lower specific activity and included in every hybridization reaction (Cornell and Kimelman, 1994a). When RNA from dissected 32-cell stage embryos was analyzed, the *EF-1α* probe was synthesized at the same specific activity as the *Xgsk-3* probe. Probes were hybridized with RNA samples overnight at 45°C and then treated with 10 μg/ml of RNase A (Sigma) for 1 hour at room temperature. Protected fragments were separated on 8% acrylamide-urea gels and exposed to film for 2-4 days.

In situ hybridization

Whole-mount in situ hybridization followed the procedure of Harland (Harland, 1991) with a variety of modifications (R. Harland, personal communication). The *goosecoid* and *Xnot* probes were used as previously described (Cho et al., 1991; von Dassow et al., 1993).

RESULTS

Isolation and characterization of the *Xenopus* glycogen synthase kinase-3 gene

DNA encoding a fragment of *Xgsk-3* was isolated using degenerate primers derived from published sequences of the *Drosophila zw3/shaggy* and rat GSK-3α and β kinase domains (Bourouis et al., 1990; Woodgett, 1990). These primers were used in a reverse transcription polymerase chain reaction (RT-PCR) to amplify a 703 bp fragment from blastula and neurula stage RNA. This fragment was used as a probe to screen a *Xenopus* stage 17 cDNA library, from which several clones were isolated. The predicted amino acid sequence encoded by one of these clones is shown in Fig. 1, aligned with the amino acid sequences of *zw3/shaggy* and rat GSK-3β. The amino acid sequence of *Xgsk-3* shows 75% identity to *zw3/shaggy*, 92% to GSK-3β, and 75% to GSK-3α, with the highest regions of identity located within the putative kinase domain (Fig. 1). The *Drosophila* gene *zw3/shaggy* is also more closely related to GSK-3β than GSK-3α, which may indicate an evolutionary relationship among these three genes.

In order to determine whether the *Xgsk-3* transcript is present during the blastula stages, when mesoderm is induced and patterned (Jones and Woodland, 1987), RNA was isolated from early *Xenopus* embryos and analyzed by the RNase protection assay. Using a probe derived from the 5′ end of the *Xgsk-3* cDNA, *Xgsk-3* was found to be expressed at a relatively constant level in the unfertilized egg and throughout the early embryonic stages (Fig. 2A), demonstrating that *Xgsk-3* is present in the embryo as a maternal transcript. To determine whether the maternal *Xgsk-3* transcripts are localized in the *Xenopus* embryo, RNA was isolated from dissected embryos and analyzed by the RNase protection assay. When 32-cell-stage embryos were dissected into dorsal and ventral halves, *Xgsk-3* was found to be expressed at the same level in both halves (Fig. 2B, lanes 1 and 2). Stage 9 embryos were dissected into dorsal and ventral halves or the animal hemisphere was dissected away from the rest of the embryo. At stage 9, *Xgsk-3* was also found to be expressed at equal levels in all parts of the embryo (Fig. 2B, lanes 3-6).

A kinase-dead mutant of Xgsk-3 causes axis duplication

In order to explore the function of Xgsk-3, we generated a kinase-dead mutant of Xgsk-3. All kinases have a conserved lysine residue in the ATP-binding region, which is necessary for kinase activity (Hanks et al., 1988). Conversion of this lysine residue to another amino acid abolishes kinase activity and, in some cases, results in the creation of a dominant negative mutant (MacNicol et al., 1993). With the hope of creating a dominant negative mutant of *Xgsk-3*, we used a PCR-based strategy to change a single nucleotide in the *Xgsk-3* cDNA, converting the conserved lysine to an arginine residue (Fig. 1). This mutant is referred to as *Xgsk-3K→R*.

The function of Xgsk-3K→R was investigated by ectopic overexpression in embryos. Injection of 0.5 ng per blastomere of RNA encoding Xgsk-3K→R into the lateral sides of 2-cell embryos resulted in tadpoles with two heads, indicating duplication of the anterior dorsal axis (Fig. 3). Although some variability was observed when embryos from different mothers were used, the duplicated axis phenotype was typically observed in 10% of the laterally injected embryos. 20% of the embryos were normal while the remaining 70% exhibited various axial and dorsoanterior defects. When the site of

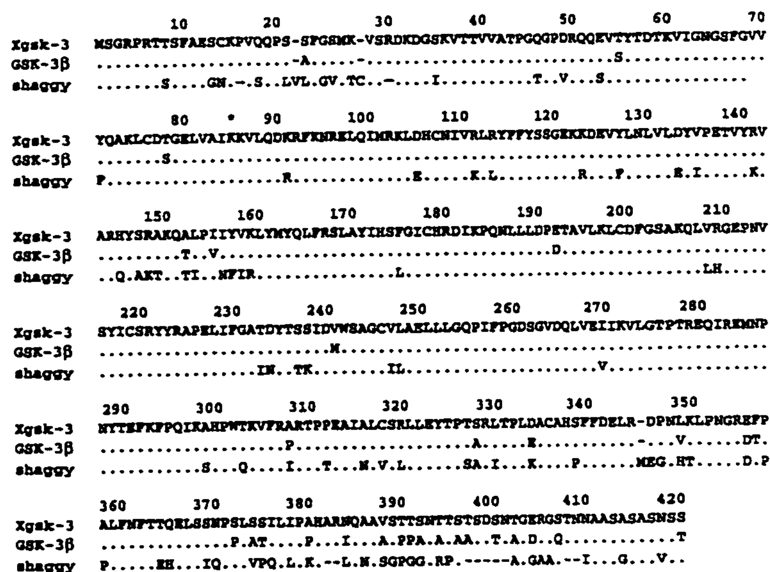

Fig. 1. Amino acid sequence of *Xgsk-3*. The predicted amino acid sequences of *Xgsk 3*, GSK-3β, and *zw3/shaggy* are compared, with non-identical residues in GSK-3β and *zw3/shaggy* indicated. The putative kinase region encompasses residues 54 to 325. The lysine residue which was altered in the *Xgsk-3K→R* mutant is indicated by an asterisk at position 85.

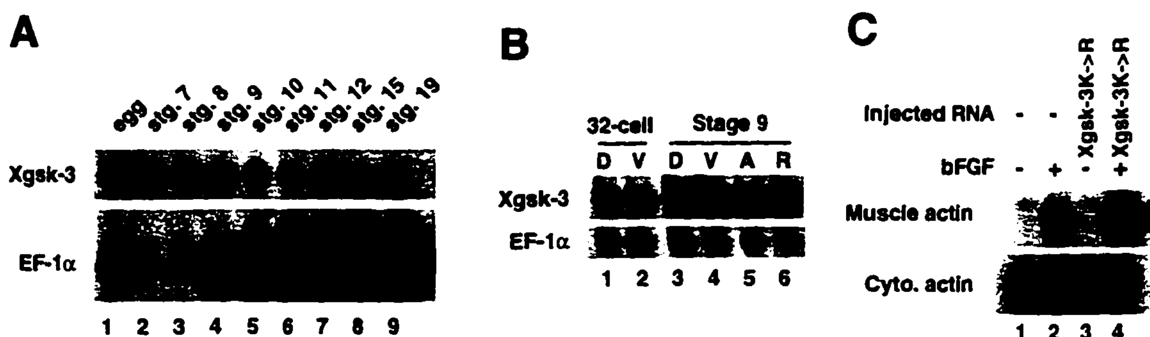

Fig. 2. Temporal and spatial expression of *Xgsk-3*. (A,B) RNA levels were determined with the RNase protection assay using a mixture of *Xgsk-3* and *EF-1α* probes. *EF-1α* is a ubiquitously expressed gene in the *Xenopus* embryo; *EF-1α* levels increase from the mid-blastula transition at stage 8 (Krieg et al., 1989). (A) Analysis of 20 μg of total RNA from unfertilized eggs and embryos at the following stages: stage 7 (blastula); stage 9 (late blastula); stage 11 (mid-gastrula); stage 15 (mid-neurula); stage 19 (late neurula). (B) Analysis of RNA isolated from dissected embryos (10 embryos per sample). 32-cell-stage embryos were dissected into dorsal and ventral halves (lanes 1 and 2). Stage-9 embryos were dissected into dorsal and vental halves (lanes 3 and 4), or the animal hemisphere was dissected away from the rest of the embryo (lanes 5 and 6). Note that the total amount of RNA in the samples from 32-cell-stage and stage-9 embryos cannot be compared because the *EF-1α* probes are not at the same specific activity. (C) Animal caps were explanted at the late blastula stage from uninjected embryos or from embryos injected in the animal pole of each cell at the 2-cell stage with 1 ng of *Xgsk-3K→R* RNA. Animal caps were cultured alone or with XbFGF for approximately 20 hours. Total RNA was extracted from the caps and analyzed by RNase protection using the muscle actin probe. The muscle actin probe also protects a portion of the cytoplasmic actin (cyto. actin) gene which is ubiquitously expressed at this stage and thus serves as an internal control.

injection was confined to the future ventral side, the percentage of embryos with axis duplication went up to 86%, while little effect was seen when *Xgsk-3K→R* RNA was injected on the future dorsal side (>90% of the embryos were normal). The duplicated axis phenotype was somewhat variable, most commonly including two well-formed heads and a shortened body (Fig. 3C). Less commonly, the embryos had two perfectly formed heads and a normal trunk and tail (Fig. 3B) or one head with widened or fused features (data not shown). At higher doses of injected RNA, the resulting embryos were severely dorsoanteriorized (data not shown). Since injection of RNA encoding a kinase-dead version of Xgsk-3 resulted in a specific phenotype, we hypothesized that Xgsk-3K→R acts as a dominant negative mutant, and thus was interfering with a normal developmental pathway involved in dorsal axis formation.

Xgsk-3K→R, like Xwnt-8, rescues dorsal axis formation in UV-irradiated embryos

Dorsal axis duplication can also be caused by ectopic overexpression of members of the *Wnt* family, including Xwnt-8 (Christian et al., 1991; Sokol et al., 1991). Since the Xgsk-3 homolog, zw3/shaggy, is involved in signaling by the *Wnt* family member, wg, in *Drosophila* (Siegfried et al., 1992, 1994; Diaz-Benjumea and Cohen, 1994), we hypothesized that Xgsk-3K→R might mimic another known effect of Xwnt-8. If fertilized eggs are treated with ultraviolet (UV) radiation early in the first cell cycle, dorsal axis development is inhibited and the resulting embryos develop a ventralized phenotype (Malacinski et al., 1977; Scharf and Gerhart, 1980). If these embryos are injected with RNA encoding Xwnt-8, dorsal axis formation is restored and nearly normal tadpoles develop (Smith and Harland, 1991; Sokol et al., 1991). We tested whether the Xgsk-3K→R mutant could mimic the ability of Xwnt-8 to rescue dorsal axis formation in UV-irradiated embryos.

Fertilized eggs were irradiated for 60 seconds with UV light within 40 minutes after fertilization. At the 2-cell stage, the embryos were injected in one cell with 1 ng of *Xgsk-3K→R* RNA or 2 ng of an RNA encoding a truncated form of Xgsk-3 (ΔXgsk-3). While injection of *ΔXgsk-3* RNA had no effect on the UV-irradiated embryos (Fig. 4A,B), injection of *Xgsk-3K→R* RNA rescued dorsal axis development, resulting in virtually normal embryos (Fig. 4C). The results were quantitated by scoring the dorsoanterior index (DAI) of the embryos, in which 5 represents normal embryos and zero represents the most severely ventralized embryos (Kao and Elinson, 1988). Uninjected UV-irradiated embryos had an average DAI of 1.4, while embryos injected with *Xgsk-3K→R* RNA had an average DAI of 4.0 (Fig. 4D). Therefore, Xgsk-3K→R, like Xwnt-8, can induce the formation of a normal dorsal axis in a ventralized embryo.

Wild-type Xgsk-3 opposes the Xgsk-3K→R phenotype

If Xgsk-3K→R is having a dominant negative effect and interfering with the action of endogenous Xgsk-3, we expected that the addition of excess Xgsk-3 would be able to overcome the effect of Xgsk-3K→R. This was tested by asking whether injection of *Xgsk-3* RNA could prevent dorsal axis rescue by Xgsk-3K→R in UV-irradiated embryos. Embryos were UV-irradiated as described above and injected with 0.5 ng of *Xgsk-3K→R* RNA, or with 0.5 ng of *Xgsk-3K→R* RNA in combination with 1 ng of *Xgsk-3* RNA. After 3 days, the DAI of the embryos was scored. Injection of RNA encoding a frameshift mutant of Xgsk-3, that produces a phenotype indistinguishable from that of uninjected embryos or embryos injected with *ΔXgsk-3* RNA, did not rescue the dorsal axis and

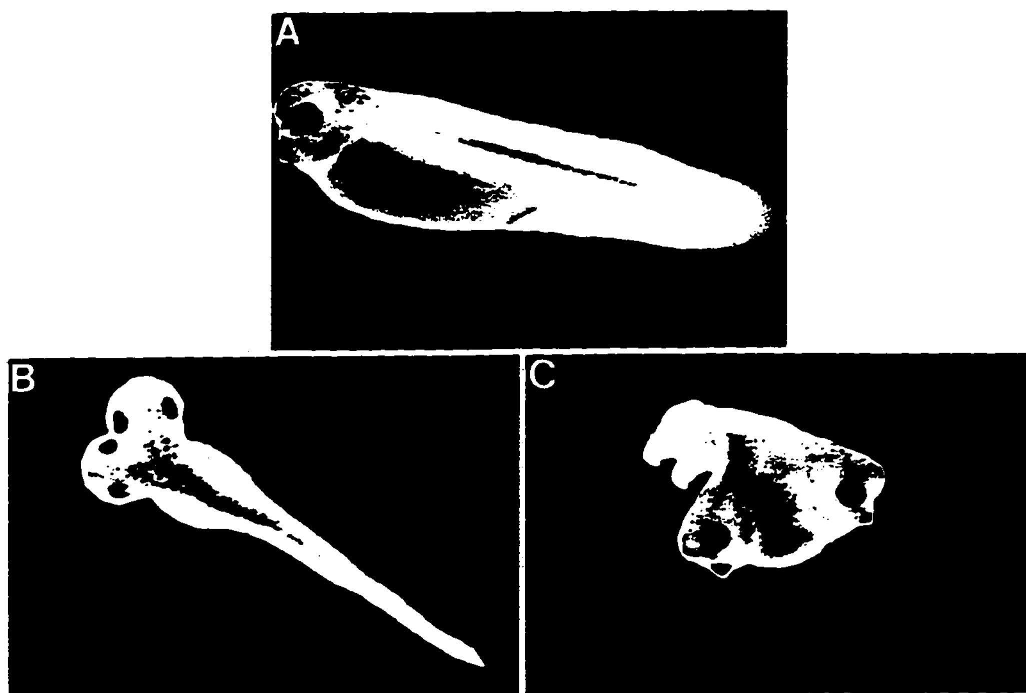

Fig. 3. Xgsk-3K→R causes dorsal axis duplication. Both cells of two-cell embryos were injected laterally with 2 ng per blastomere of *ΔXgsk-3* RNA (A) or 0.5 ng per blastomere of *Xgsk-3K→R* RNA (B,C) and allowed to develop for 3 days. (B) A dorsal view and (C) a dorsoanterior view of embryos with different degrees of axis duplication.

the resulting embryos had an average DAI of 0.7 (n=32). In contrast, UV-irradiated embryos injected with *Xgsk-3K→R* RNA had an average DAI of 3.3 (n=30), indicating restoration of dorsal axis formation. Co-injection of a two-fold excess of *Xgsk-3* RNA completely blocked the rescuing activity of Xgsk-3K→R, producing embryos with an average DAI of 1.0 (n=30). These results indicate that Xgsk-3K→R functions by interfering with endogenous Xgsk-3.

Effect of Xgsk-3 and Xgsk-3K→R on *goosecoid* and *Xnot*, markers of prospective dorsal mesoderm

We reasoned that the effects on dorsal axis development caused by overexpression of Xgsk-3K→R should be reflected in the early expression of dorsal specific genes if Xgsk-3 is involved in the early patterning events. Embryos were injected ventrally with 2 ng of *Xgsk-3K→R* RNA and cultured until the early gastrula (stage 10) or late gastrula (stage 12) stages. In addition, to test the effects of excess Xgsk-3 on the dorsal side, embryos were injected dorsally with 4 ng of *Xgsk-3* RNA and cultured until stage 10 or stage 12. The embryos were fixed and the expression of the head-specific and notochord-specific homeobox genes *goosecoid* (*gsc*) and *Xnot* was determined by in situ hybridization. *Gsc* is expressed at stage 10 in a patch above the dorsal lip, marking the future head mesoderm (Cho et al., 1991). Uninjected embryos (Fig. 5A) and embryos injected either dorsally or ventrally with *ΔXgsk-3* RNA (data not shown) showed the normal pattern of expression. Embryos injected ventrally with *Xgsk-3K→R* RNA typically had two dorsal lips at this stage with *gsc* expressed in a patch above each of them (Fig. 5B). In contrast, over 50% of the embryos injected dorsally with *Xgsk-3* RNA have not begun to gastrulate at this stage and did not have visible *gsc* staining (Fig. 5C). In the remaining embryos, *gsc* staining was present but was fainter than in control embryos. At stage 12, *Xnot* is expressed in the presumptive notochord (von Dassow et al., 1993), as seen in uninjected embryos (Fig. 5D) and in embryos injected either dorsally or ventrally with *ΔXgsk-3* RNA (data not shown). In addition to causing duplication of *gsc* expression, ventral injection of *Xgsk-3K→R* RNA resulted in duplication of *Xnot* expression (Fig. 5E). Embryos injected dorsally with *Xgsk-3* RNA generally showed *Xnot* staining, but the region of staining was not as tightly defined and was generally fainter than in uninjected embryos (Fig. 5F). These results demonstrate that the effects of Xgsk-3K→R are due to early perturbations of mesodermal patterning.

Although Xwnt-8 can induce the formation of a secondary axis in the *Xenopus* embryo and can induce the expression of mesodermal genes such as *Xnot* (von Dassow et al., 1993), it cannot directly induce mesoderm (Christian et al., 1992). However, Xwnt-8 has been shown to synergize with bFGF in the induction of dorsal mesoderm (Christian et al., 1992), suggesting that it acts primarily to regulate the type of mesoderm that forms. To determine whether Xgsk-3K→R shares these properties with Xwnt-8, the mesoderm-inducing properties of Xgsk-3K→R were examined in an animal cap assay. The upper region of the animal hemisphere (the animal cap) was

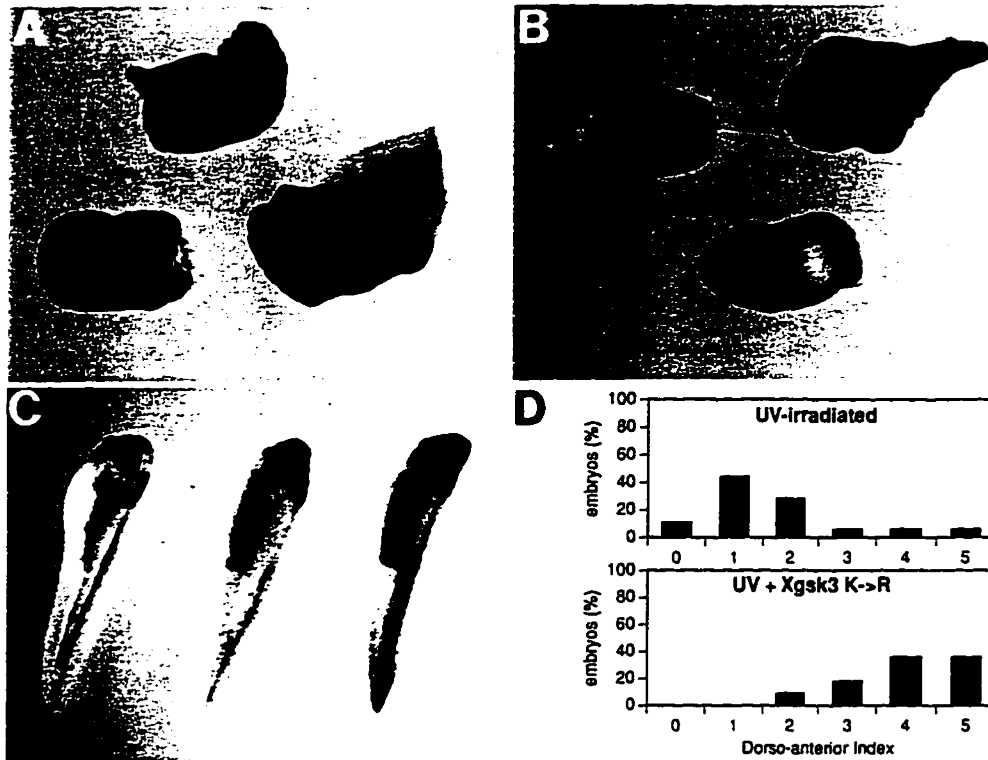

Fig. 4. Xgsk-3K→R rescues dorsal axis formation in UV-irradiated embryos. Fertilized eggs were UV-irradiated for 60 seconds within 40 minutes after fertilization. At the 4-cell stage, one cell was injected with the indicated RNA and the embryos were allowed to develop for three days. (A) Uninjected embryo; (B) Embryo injected with 2 ng *ΔXgsk-3* RNA; (C) Embryo injected with 1 ng *Xgsk-3K→R* RNA; (D) The dorsoanterior index (DAI) of the uninjected (*n*=54) (upper panel) and *Xgsk-3K→R* RNA injected (*n*=22) (lower panel) embryos was scored. The percentage of embryos with each score is shown.

explanted at stage 9 from uninjected embryos or embryos injected in the animal pole with 1 ng of *Xgsk-3K→R* RNA at the 2-cell stage. The animal caps were then cultured with or without bFGF for approximately 20 hours. RNA extracted from the caps was analyzed by the RNase protection assay using a probe for the mesoderm marker, muscle actin. Animal caps from uninjected or *Xgsk-3K→R* RNA-injected embryos express no muscle actin when cultured without bFGF (Fig. 2C, lanes 1 and 3), but muscle actin is strongly induced in the presence of bFGF (Fig. 2C, lanes 2 and 4). The level of muscle actin transcript induced in Xgsk-3K→R-expressing animal caps is approximately 3-fold greater than in animal caps from uninjected embryos. These results demonstrate that Xgsk-3K→R is not able to directly induce mesoderm, and, like Xwnt-8, it can synergize with bFGF in the induction of mesoderm.

Xgsk-3K→R rescues dorsal axis formation from the deep vegetal cells

The deep vegetal cells of the *Xenopus* embryo are able to induce the formation of the Spemann organizer in the marginal zone, even though they do not become incorporated into the mesoderm (Gimlich and Gerhart, 1984). Similarly, Xwnt-8 can induce the formation of a complete dorsal axis when expressed in deep vegetal cells of embryos ventralized by exposure to UV light, even though none of the cells expressing Xwnt-8 become part of the induced axis (Smith and Harland, 1991). It is believed that these results are due to the ability of Xwnt-8 protein to be secreted from the deep vegetal cells and therefore to alter the fate of the overlying marginal zone cells. Since we expected that Xgsk-3 was an intracellular target of a *Wnt*-like signaling pathway, based on the studies of *wingless* and *zw3/shaggy* in *Drosophila* (Siegfried et al., 1992; Diaz-Benjumea and Cohen, 1994), we predicted that Xgsk-3K→R would not be able to induce axis formation when expressed in the deep vegetal cells.

UV-irradiated 32-cell embryos were selectively injected in the marginal zone (tier C) or deep vegetal cells (tier D) with *Xgsk-3K→R* RNA in combination with RNA encoding β-galactosidase, to identify the cells expressing the injected RNA (Smith and Harland, 1991). As shown in Fig. 6, Xgsk-3K→R was able to rescue dorsal axis formation when expressed in either tier C or tier D cells (Fig. 6B,C). These results were quantitated by scoring the DAI of the embryos

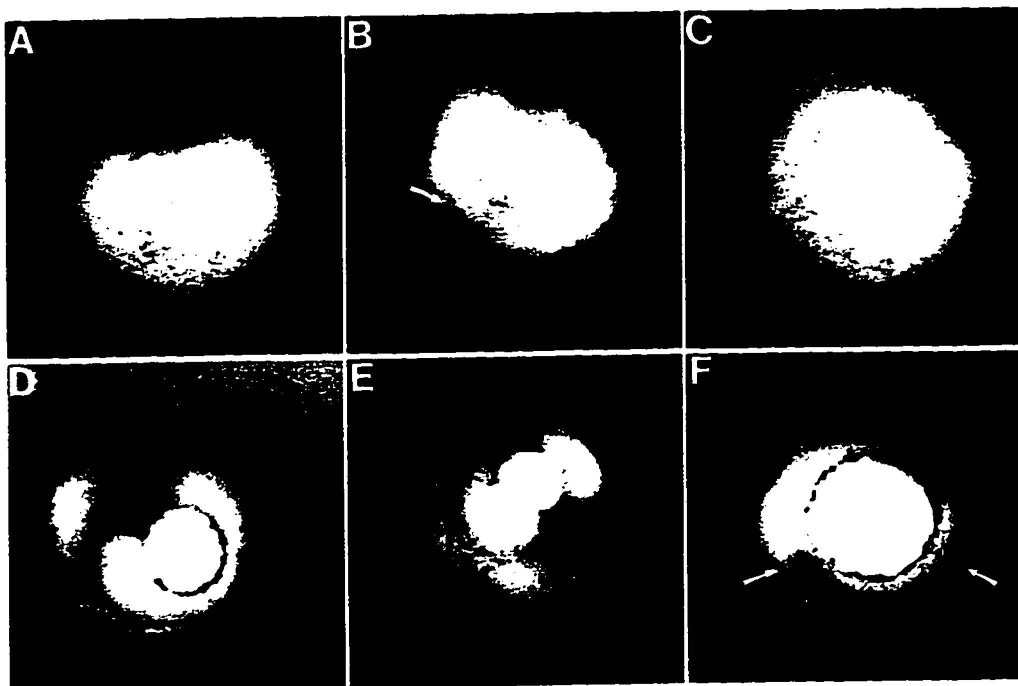

Fig. 5. Regulation of dorsal genes by Xgsk-3K→R and Xgsk-3. 4-cell embryos were injected with RNA into either the two dorsal or two ventral blastomeres, allowed to develop to stage 10 and stained by in situ hybridization for *goosecoid* (A-C), or to stage 12 and stained for *Xnot* (D-F). (A,D) Uninjected embryos; (B,E) Embryos injected ventrally with 1 ng per blastomere *Xgsk-3K→R* RNA. In B, the arrows indicate the two dorsal lips. (C,F) Embryos injected dorsally with 2 ng per blastomere *Xgsk-3* RNA. Note the faint, dispersed *Xnot* staining between the arrows in F.

(Fig. 6D). β-galactosidase staining confirmed that tier C cells contribute to the induced axis while tier D cells contribute only to endoderm (Fig. 6B,C). *Xgsk-3K→R* RNA was somewhat less effective at rescuing dorsal axis formation when injected at the 32-cell stage than when injected at the 4-cell stage (Fig. 4). These results indicate that elimination of Xgsk-3 signaling either from the cells that contribute to Spemann's organizer, or from the deep vegetal cells that do not contribute to the organizer, is sufficient to induce the formation of a dorsal axis.

DISCUSSION

We have described the isolation of a *Xenopus* homolog of the mammalian GSK-3 and *Drosophila zw3/shaggy* genes, *Xgsk-3*, which is involved in dorsoventral patterning in the early embryo. The amino acid sequence of *Xgsk-3*, like *zw3/shaggy*, has a greater degree of identity to GSK-3β (92%) than to GSK-3α (75%). The high degree of sequence conservation between *Xgsk-3*, *zw3/shaggy* and GSK-3β suggests a possible conserved role for these proteins among flies, frogs and mammals. *Xgsk-3* is expressed as a maternal transcript, which is present throughout the early embryonic stages, consistent with a role for *Xgsk-3* in early patterning events, which are thought to take place during the blastula stages. Analysis of dissected embryos shows that the *Xgsk-3* transcript is present at equal levels throughout the embryo. In situ hybridization experiments also indicate that *Xgsk-3* transcripts are not localized during the blastula stages, but instead are uniformly distributed throughout the embryo (unpublished results), although we cannot entirely exclude the possibility that *Xgsk-3* transcripts are absent from a small area of the embryo.

Induction of the Spemann organizer by a kinase-dead mutant of Xgsk-3

Genetic studies in *Drosophila* have defined a role for *zw3/shaggy* in the *wg* signaling pathway. During embryonic segmentation, *wg* signaling is required for the maintenance of *engrailed* (*en*) expression in a group of adjacent cells (Bejsovec and Martinez-Arias, 1991). In loss-of-function *zw3/shaggy* mutant embryos, the region of *en* expression is expanded, and its maintenance is independent of *wg* signaling (Siegfried et al., 1992). These results have led to a model in which the regulation of *en* expression by Wg is mediated by the inactivation of zw3/shaggy (Siegfried et al., 1992). Similarly, the inactivation of zw3/shaggy by *wg* signaling has been proposed to be necessary for the specification of the ventral cells in the *Drosophila* leg (Diaz-Benjumea and Cohen, 1994). If this mechanism of action has been conserved by the *Wnt* signaling pathway in *Xenopus*, we would predict that *Xwnt-8* signaling would result in the inactivation of the zw3/shaggy homolog, Xgsk-3. To test this prediction, we constructed a kinase-dead version of Xgsk-3, Xgsk-3K→R, with the hope that it would act as a dominant negative mutant.

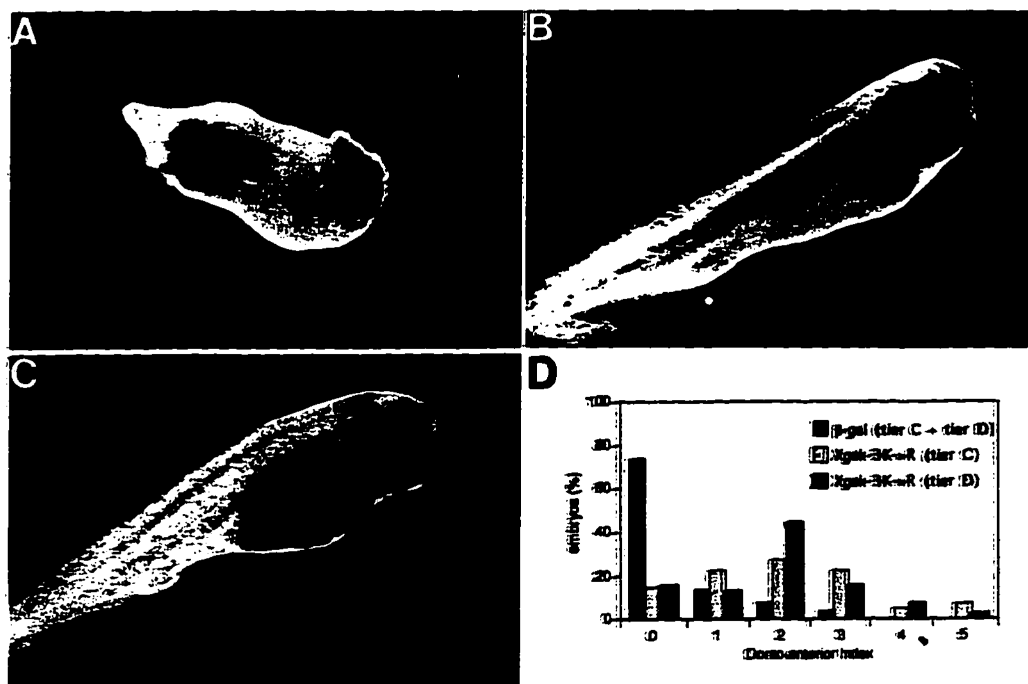

Fig. 6. Xgsk-3K→R rescues dorsal axis formation from deep vegetal cells. Fertilized eggs were UV irradiated for 60 seconds within 40 minutes after fertilization. At the 32-cell stage, the embryos were injected in two adjacent cells of the same tier with 200 pg of β-galactosidase (β-gal) RNA per cell in tier C (n=24) (A) or 200 pg β-gal and 1 ng of *Xgsk-3K→R* RNA per cell in tier C (n=39) (B) or tier D (n=38) (C). After 3 days, the DAI of the embryos was scored. The percentage of embryos with each score is shown in D. The scores of embryos injected with β-gal RNA in tier C (n=24) and tier D (n=25) are combined.

When RNA encoding Xwnt-8 is injected into the ventral side of a *Xenopus* embryo, a second dorsal axis is formed due to the induction of an ectopic Spemann organizer (Smith and Harland, 1991; Sokol et al., 1991). Ventral injection of RNA encoding Xgsk-3K→R also resulted in dorsal axis duplication, suggesting that Xgsk-3 acts on the same pathway as Xwnt-8. This conclusion is supported by the ability of Xgsk-3K→R to rescue dorsal axis formation in embryos ventralized by UV-irradiation. In addition, like Xwnt-8, Xgsk-3K→R has no effect on normal dorsal axis formation when it is ectopically expressed on the dorsal side of the embryo. Since expression of a kinase-dead form of Xgsk-3 caused a specific phenotype, and since addition of excess wild-type Xgsk-3 eliminated the effects of Xgsk-3K→R, we conclude that Xgsk-3K→R acts as a dominant negative mutant by interfering with the function of endogenous Xgsk-3. Xgsk-3K→R may act as a dominant inhibitory mutant by competing with endogenous Xgsk-3 for substrates or upstream regulatory molecules. Since there is no evidence that zw3/shaggy/GSK-3 acts as a dimer, it is not likely to function by directly interfering with the endogenous Xgsk-3.

Xgsk-3 functions as an endogenous repressor of dorsal fate

Our results suggest that dorsal fate is normally repressed throughout the embryo by Xgsk-3, and that dorsal determination requires the inhibition or opposition of Xgsk-3 activity on the dorsal side of the embryo. This hypothesis is consistent with results obtained by overexpressing Xgsk-3. When Xgsk-3 is ectopically expressed on the dorsal side of the embryo, dorsal development is compromised (unpublished results). At lower doses of *Xgsk-3* RNA, the resulting tadpoles have a somewhat ventralized phenotype lacking eyes, with small heads and a shortened dorsal axis. Higher doses of *Xgsk-3* RNA result in complete loss of heads. However, even at high doses of *Xgsk-3* RNA, the embryos are not completely ventralized, retaining some axial development, while comparatively low doses of *Xgsk-3K→R* RNA can completely rescue dorsal axis formation in a ventralized embryo. These results suggest that the endogenous dorsalizing signal may have the capacity to regulate much higher levels of Xgsk-3 than are normally present in the embryo.

The ability of Xgsk-3K→R to mimic Xwnt-8 suggests that the function of Xgsk-3 is necessary for one of the earliest steps of dorsoventral axis determination, the induction of the Spemann organizer. This is confirmed by the expression patterns of *gsc* and *Xnot*, two genes that are expressed in the organizer region and which mark prospective dorsal tissue. When Xgsk-3K→R is expressed on the ventral side of the embryo, the expression patterns of *gsc* and *Xnot* are duplicated, indicating duplication of the organizer itself. In contrast,

expression of Xgsk-3 on the dorsal side of the embryo alters the expression patterns of *gsc* and *Xnot*, indicating that organizer formation has been disrupted. These observations are supported by changes in the pattern of gastrulation in response to ectopic expression of Xgsk-3K→R and Xgsk-3. Expression of Xgsk-3K→R on the ventral side of the embryo causes gastrulation to initiate simultaneously on the dorsal and ventral sides of the embryo, indicating that the ventral side of the embryo has acquired the properties of the Spemann organizer. However, when Xgsk-3 is expressed on the dorsal side, gastrulation initiates later than in control *ΔXgsk-3* RNA-injected embryos, suggesting that cells that would normally form the dorsal lip have acquired a more lateral quality and therefore do not begin the movements of gastrulation at the normal time.

Xgsk-3, like zw3/shaggy, acts non-cell autonomously

The prevailing view of *Wnts* when we began this project was that they acted directly as secreted morphogens to change the fate of the receiving cell and that their range of action is determined by the distance they diffuse from the signaling cell (Struhl and Basler, 1993; Thuringer and Bienz, 1993). Since *zw3/shaggy* is epistatic to *wg* (Siegfried et al., 1992), we predicted that Xgsk-3K→R would mimic the axis inducing ability of Xwnt-8 when expressed in cells that become part of the induced axis. However, since the Xgsk-3 protein is not secreted, we expected that Xgsk-3K→R would not mimic the ability of Xwnt-8 to induce axis formation when expressed in the deep vegetal cells, which do not contribute to the axis. We were therefore surprised to find that Xgsk-3K→R is as effective at rescuing axis formation in UV-irradiated embryos when expressed in the deep vegetal cells as in the marginal zone cells. There are two possible explanations for this result. If Xgsk-3 functions downstream of Xwnt-8, as predicted from the results with *Drosophila*, our results suggest that Xwnt-8 triggers the release of an intercellular morphogen. An alternative possibility is that *Wnt* signaling in *Xenopus* is significantly different from *wg* signaling in *Drosophila* and that Xgsk-3 is necessary for the release of an Xwnt-8-like signal, placing it upstream of Wnt in the signaling cascade. Although the second scenario is formally possible, we favor the first possibility in light of recent work in *Drosophila*.

Diaz-Benjumea and Cohen (1994) have found that, in the *Drosophila* leg imaginal disc, clones of cells that have lost the *zw3/shaggy* function have all the properties of clones of *wg*-expressing cells, including the ability to determine the fate of cells around them. This indicates that *wg*-expressing cells normally inhibit *zw3/shaggy* function in neighboring cells and that these cells in turn affect the fate of cells outside the clone. They suggest that this is due either to the release of a second secreted factor or to effects mediated by cell-cell contact. This view is supported by the results of experiments which show that Wg protein probably does not diffuse more than one cell diameter from its source (Vincent and Lawrence, 1994). Since *wg* signaling can be effective over distances of several cell diameters (Struhl and Basler, 1993; Thuringer and Bienz, 1993), its action may be mediated in some cases by a long-range gradient of a morphogen that is activated by Wg (Vincent and Lawrence, 1994).

Our results suggest that the emerging picture of the mechanism of *wg* signaling may reflect the mechanism employed by the vertebrate *Wnts* as well. The ablility of Xgsk-3K→R to rescue dorsal axis formation from the deep vegetal cells suggests that *Xwnt-8* signaling is mediated by a subsequent intercellular interaction. Although another secreted factor may be involved, an alternate possibility is that the repression of Xgsk-3 on the dorsal side of the embryo activates gap junctional communication in that region. Gap junctional activity is normally higher on the dorsal side of the embryo than on the ventral side (Guthrie, 1984). Expression of Xwnt-8 on the ventral side of the embryo enhances gap junctional communication there during the early cleavage stages (Olson et al., 1991), raising the possibility that the hypothetical long-range morphogen could pass through gap junctions.

The function of Xgsk-3 in mesoderm induction

Our results are consistent with the view that Xgsk-3 acts to repress an activity necessary for dorsal axis formation. Wnt (and perhaps noggin) is expected to inactivate Xgsk-3 via an intracellular signaling pathway triggered by its binding to an extracellular receptor. Although both Xwnt-8 and noggin can induce dorsal axis formation, neither is able to directly induce mesoderm (Christian et al., 1992; Smith and Harland, 1992), indicating that another factor, most likely Vg1, needs to cooperate with these factors in the induction of dorsal mesoderm (Thomsen and Melton, 1993). Complicating this

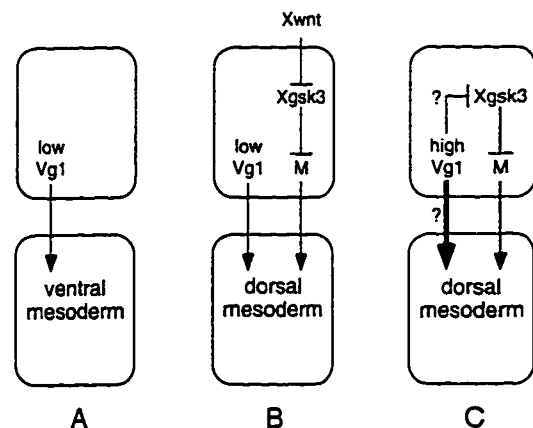

Fig. 7. Model for the function of Xgsk-3 in dorsal mesoderm patterning. (A) A low level of Vg1, acting in combination with FGF (not shown in the figure) induces ventral mesoderm (R. Cornell, T. Musci, and D. K., unpublished data; and see Kimelman et al., 1992). Dorsal mesoderm may arise by one of two pathways: (B) *Wnt*-like signals are suggested to inactivate Xgsk-3, leading to the activation of dorsal-specific genes, possibly through signals from an unidentified morphogen (M). Low level Vg1 signaling is still required for mesoderm induction since neither addition of Xwnt-8 (Smith and Harland, 1991; Sokol et al., 1991) or inhibition of Xgsk-3 is able to induce mesoderm directly. (C) High levels of Vg1 can also induce dorsal mesoderm (Thomsen and Melton, 1993) either by overriding the effects of Xgsk-3, or by inhibiting Xgsk-3. The intracellular signaling pathway used by noggin is still unclear, although it may be similar to the *Wnt* pathway since noggin, like Xwnt-8, is unable to directly induce mesoderm (Smith and Harland, 1992).

view is the observation that high levels of Vg1 can also induce a complete dorsal axis, bypassing the need for Wnt or noggin (Thomsen and Melton, 1993). These observations can be reconciled in a model in which Xgsk-3 is either directly inhibited or its effects are overridden by the various dorsalizing factors, resulting in the formation of dorsal mesoderm. In this model, ventral mesoderm is induced by a low level of Vg1 signaling (Fig. 7A). Dorsal mesoderm can be induced by a combination of low Vg1 and Wnt signals (Smith and Harland, 1991; Sokol et al., 1991) which leads to the repression of Xgsk-3, resulting in the activation of dorsal-specific genes (Fig. 7B). *Vg1* signaling is also required, since neither Wnt (Smith and Harland, 1991; Sokol et al., 1991) nor the dominant-negative Xgsk-3 mutant are able to induce mesoderm. High levels of Vg1 could either override the repressive effects of Xgsk-3, and thus directly induce dorsal mesoderm, or Vg1 might inactivate Xgsk-3, leading indirectly to the induction of dorsal mesoderm (Fig. 7C). It is not yet clear which of the candidate dorsal-inducing factors are used in vivo, nor which pathway is used to antagonize the effects of Xgsk-3. Since Xwnt-8 is expressed only on the ventral side of the embryo after the early dosoventral patterning events, Xwnt-8 cannot be regulating Xgsk-3 in vivo. This may be accomplished by a maternal Wnt or, as discussed above, by noggin or Vg1. With the isolation and characterization of *Xgsk-3*, and the ability to perform biochemical studies on intracellular signaling pathways in *Xenopus* embryos and explants (Graves et al., 1994; LaBonne and Whitman, 1994), we hope to distinguish between these various possibilities.

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REFERENCES

Bejsovec, A. and Martinez-Arias, A. (1991). Roles of *wingless* in patterning the larval epidermis of *Drosophila*. *Development* **113**, 471-485.

Bianchi, M. W., Guivarc'h, D., Thomas, M. and Woodgett, J. R. (1994). Arabidopsis homologs of the shaggy and GSK-3 protein kinases: molecular cloning and functional expression in Escherichia coli. *Mol. Gen. Genet.* **242**, 337-45.

Bianchi, M. W., Plyte, S. E., Kreis, M. and Woodgett, J. R. (1993). A saccharomyces cerevisiae protein-serine kinase related to mammalian glycogen synthase kinase-3 and the *Drosophila* melanogaster gene *shaggy* product. *Gene* **134**, 51-56.

Bourouis, M., Moore, P., Ruel, L., Y., G., Heitzler, P. and Simpson, P. (1990). An early embryonic product of the gene *shaggy* encodes a serine/threonine protein kinase related to the CDC28/cdc2+ subfamily. *EMBO J.* **9**, 2877-2884.

Busa, W. B. and Gimlich, R. L. (1989). Lithium induced teratogenesis in frog embryos prevented by a polyphosphoinositide cycle intermediate or a diacylglycerol analog. *Dev. Biol.* **132**, 315-24.

Chakrabarti, A., Matthews, G., Colman, A. and Dale, L. (1992). Secretory and inductive properties of *Drosophila* wingless protein in *Xenopus* oocytes and embryos. *Development* **115**, 355-369.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene *goosecoid*. *Cell* **67**, 1111-1120.

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). *Xwnt-8*, a *Xenopus Wnt-1/int-1*-related gene responsive to mesoderm inducing factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-56.

Christian, J. L., Olson, D. J. and Moon, R. T. (1992). *Xwnt-8* modifies the character of mesoderm induced by bFGF in isolated *Xenopus* ectoderm. *EMBO J.* **11**, 33-41.

Cornell, R. A. and Kimelman, D. (1994a). Activin-mediated mesoderm induction requires FGF. *Development* **120**, 453-462.

Cornell, R. A. and Kimelman, D. (1994b). Combinatorial signaling in development. *Bioessays* **16**, 577-581.

Diaz-Benjumea, F. J. and Cohen, S. M. (1994). *wingless* acts through the *shaggy/zeste-white 3* kinase to direct dorsal-ventral axis formation in the *Drosophila* leg. *Development* **120**, 1661-1670.

Gimlich, R. L. and Gerhart, J. C. (1984). Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. *Dev. Biol.* **104**, 117-130.

Graves, L. M., Northrop, J. L., Potts, B. C., Krebs, E. G. and Kimelman, D. (1994). FGF, but not activin, is a potent inducer of MAP kinase in *Xenopus* explants. *Proc. Natl. Acad. Sci. USA* **91**, 1662-1666.

Guthrie, S., Turin, L. and Warner, A. (1988). Patterns of junctional communication during development of the early amphibian embryo. *Development* **103**, 769-783.

Guthrie, S. C. (1984). Patterns of junctional communication in the early amphibian embryo. *Nature* **311**, 149-151.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Harland, R. M. (1991). *In situ* hybridization: an improved whole mount method for *Xenopus* embryos. In *Xenopus laevis: Practical Uses in Cell and Molecular Biology* (ed. B. K. Kay and H. J. Peng). San Diego, Calif.: Academic Press Inc.

Hartley, R. S., Lewellyn, A. L. and Maller, J. L. (1994). MAP kinase is activated during mesoderm induction in *Xenopus laevis*. *Dev. Biol.* **163**, 521-524.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51-59.

Jones, E. A. and Woodland, H. R. (1987). The development of animal cap cells in *Xenopus*: a measure of the start of animal cap competence to form mesoderm. *Development* **101**, 557-563.

Kao, K. R. and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev. Biol.* **127**, 64-77.

Kao, K. R., Masui, Y. and Elinson, R. P. (1986). Lithium induced respecification of pattern in *Xenopus laevis* embryos. *Nature* **322**, 371-373.

Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M. and Kirschner, M. (1988). The presence of FGF in the frog egg: its role as a natural mesoderm inducer. *Science* **242**, 1053-1056.

Kimelman, D., Christian, J. L. and Moon, R. T. (1992). Synergistic principles of development: overlapping patterning systems in *Xenopus* mesoderm induction. *Development* **116**, 1-9.

Kintner, C. R. and Melton, D. A. (1987). Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311.

Krieg, P. A., Varnum, S., Wormington, M. W. and Melton, D. A. (1989). The mRNA encoding elongation factor 1-α (EF-1α) is a major transcript of the midblastula transition. *Dev. Biol.* **133**, 93-100.

LaBonne, C. and Whitman, M. (1994). Mesoderm induction by activin requires FGF mediated intracellular signals. *Development* **120**, 463-472.

MacNicol, A. M., Muslin, A. J. and Williams, L. T. (1993). Raf-1 kinase is essential for early *Xenopus* development and mediates the induction of mesoderm by FGF. *Cell* **73**, 571-584.

Malacinski, G. M., Brothers, J. and Chung, H.-M. (1977). Destruction of components of the neural induction system of the amphibian egg with ultraviolet irradiation. *Dev. Biol.* **56**, 24-39.

Maslanski, J. A., Leshko, L. and Busa, W. B. (1992). Lithium-sensitive production of inositol phosphates during amphibian embryonic mesoderm induction. *Science* **256**, 243-245.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12**, 7035-7056.

Moon, R. T. and Christian, J. L. (1989). Microinjection and expression of synthetic mRNAs in *Xenopus* embryos. *Technique* **1**, 76-89.

Nagajski, D. J., Guthrie, S. C., Ford, C. C. and Warner, A. E. (1989). The

correlation between patterns of dye transfer through gap junctions and future developmental fate in *Xenopus*: the consequences of u.v. irradiation and lithium treatment. *Development* **105**, 747-752.
Newport, J. and Kirschner, M. W. (1982). A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675-686.
Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis.* Amsterdam: North-Holland Publishing Company.
Nishida, E. and Gotoh, Y. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18**, 128-131.
Noordermeer, J., Klingensmith, J., Perrimon, N. and Nusse, R. (1994). *dishevelled* and *armadillo* act in the Wingless signalling pathway in *Drosophila*. *Nature* **367**, 80-83.
Olson, D. J., Christian, J. L. and Moon, R. T. (1991). Effect of *wnt-1* and related proteins on gap junctional communication in *Xenopus* embryos. *Science* **252**, 1173-1176.
Pay, A., Honak, C., Bogre, L., Meskiene, I. and Mairinger, T. (1993). The MsK family of alfalfa protein kinase genes encodes homologues of *shaggy*/glycogen synthase kinase-3 and shows differential expression patterns in plant organs and development. *Plant J.* **3**, 847-856.
Puziss, J. W., Hardy, T. A., Johnson, R. B., Roach, P. J. and Hieter, P. (1994). MDS1, a dosage suppressor of an mck1 mutant, encodes a putative yeast homolog of gycogen synthase kinase 3. *Mol. Cell Biol.* **14**, 831-839.
Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5436-5467.
Scharf, S. R. and Gerhart, J. C. (1980). Determination of the dorsal-ventral axis in eggs of *Xenopus laevis*: complete rescue of uv-impaired eggs by oblique orientation before first cleavage. *Dev. Biol.* **79**, 181-198.
Siegfried, E., Chou, T. and Perrimon, N. (1992). *wingless* signaling acts through *zeste-white 3*, the *Drosophila* homolog of *glycogen synthase kinase-3*, to regulate *engrailed* and establish cell fate. *Cell* **71**, 1167-1179.
Siegfried, E., Wilder, E. L. and Perrimon, N. (1994). Components of *wingless* signaling in *Drosophila*. *Nature* **367**, 76-80.
Sive, H. L. (1993). The frog prince-cess: A molecular formula for dorsoventral patterning in *Xenopus*. *Genes Dev.* **7**, 1-12.
Smith, W. C. and Harland, R. M. (1991). Injected *Xwnt-8* acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-766.
Smith, W. C. and Harland, R. M. (1992). Expression cloning of *noggin*, a new dorsalizing factor localized in the Spemann organizer in *Xenopus* embryos. *Cell* **70**, 829-840.
Sokol, S., Christian, J. L., Moon, R. T. and Melton, D. A. (1991). Injected *wnt* RNA induces a complete body axis in *Xenopus* embryos. *Cell* **67**, 741-752.
Spemann, H. and Mangold, H. (1924). Uber induction von embryonalagen durch implantation artfremder organis atoren. *W. Roux' Arch. Ent. Org.* **100**, 599-638.
Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in *Drosophila*. *Cell* **72**, 527-540.
Sutherland, C., Leighton, I. A. and Cohen, P. (1993). Inactivation of glycogen synthase kinase-3β by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem. J.* **296**, 15-19.
Thomsen, G. H. and Melton, D. A. (1993). Processed Vg-1 protein is an axial mesoderm inducer in *Xenopus*. *Cell* **74**, 433-441.
Thuringer, F. and Bienz, M. (1993). Indirect regulation of a homeotic *Drosophila* gene mediated by extracellular signaling. *Proc. Natl. Acad. Sci. USA* **90**, 3899-3903.
Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.
Vincent, J. and Lawrence, P. A. (1994). *Drosophila wingless* sustains *engrailed* expression only in adjoining cells: evidence from mosaic embryos. *Cell* **77**, 909-915.
von Dassow, G., Schmidt, J. E. and Kimelman, D. (1993). Induction of the *Xenopus* organizer: expression and regulation of *Xnot*, a novel FGF and activin-regulated homeobox gene. *Genes Dev* **7**, 355-366.
Warner, A. E., Guthrie, C. and Gilula, N. B. (1984). Antibodies to gap-junctional protein selectively disrupt gap junctional communication in the early amphibian embryo. *Nature* **311**, 127-131.
Woodgett, J. R. (1990). Molecular cloning and expression of glycogen synthase kinase-3/Factor A. *EMBO J.* **9**, 2431-2438.

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Appendix C: Regulation of dorsal-ventral axis formation in Xenopus by intercellular and intracellular signaling

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Overexpression of Xgsk-3 Disrupts Anterior Ectodermal Patterning in *Xenopus*

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The *Xenopus* homolog of glycogen synthase kinase-3, Xgsk-3, plays a major role in regulating the formation of the dorsal–ventral axis, most likely through effects on the mesoderm. To determine whether Xgsk-3 is involved in ectodermal patterning, Xgsk-3 was ectopically overexpressed in the presumptive ectoderm. This approach resulted in a dramatically expanded cement gland, which is due to early changes in cement gland specification at the anterior end of the embryo. Explant experiments were used to show that Xgsk-3 overexpression enhances the response of ectoderm to cement-gland-inducing signals from the mesoderm and to the intercellular signaling factor noggin. Expression of two other noggin-inducible genes, *Xotx2* and *XANF-2*, was also expanded in whole embryos, while the expression of the epidermal marker, *Xgbx-2*, was eliminated. These results suggest that Xgsk-3 may play a role in anterior ectodermal patterning as a component of an intracellular pathway that regulates the ectodermal responsiveness to endogenous inducing signals. © 1996 Academic Press, Inc.

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) is a mammalian intracellular serine/threonine kinase represented by two isoforms, GSK-3α and GSK-3β, which has highly conserved homologs in many organisms, including invertebrates, other vertebrates, and plants. GSK-3 and its homologs have been shown to play roles in several intracellular signaling pathways which are known to be important for both cell growth and cell fate determination (reviewed in Woodgett, 1991; Plyte *et al.*, 1992). One of these is the pathway utilized by the Wnt family of intercellular signaling factors, originally characterized genetically as the pathway activated by the *Drosophila* Wnt homolog, *wingless* (wg). *wg* acts at several times in development by inhibiting the function of the *Drosophila* homolog of GSK-3β, *zeste-white 3/shaggy* (*zw3/shaggy*), leading to specific cell-fate decisions (Bourouis *et al.*, 1990; Siegfried *et al.*, 1992). Two other components of the *wg* pathway have been identified: *armadillo*, which is epistatic to *zw3/shaggy*, and *dishevelled*, which acts upstream of *zw3/shaggy* (Noordermeer *et al.*, 1994; Siegfried *et al.*, 1994).

The *Xenopus* GSK-3 homolog, Xgsk-3, like *zw3/shaggy*, is more closely related to GSK-3β than to GSK-3α (Dominguez *et al.*, 1995; Pierce and Kimelman, 1995). During the early development of the *Xenopus* embryo, Xgsk-3 appears to regulate the determination of the dorsal–ventral axis by functioning as an inhibitor of dorsal structures (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995). Inhibition of Xgsk-3 with a dominant inhibitory mutant causes ectopic head formation, whereas expression of wild-type Xgsk-3 on the dorsal side of the embryo leads to a partially ventralized embryo. Since members of the Wnt family can also cause ectopic head formation (Christian *et al.*, 1991; Sokol *et al.*, 1991; Chakrabarti *et al.*, 1992), these results suggest that the wg pathway is conserved between *Drosophila* and *Xenopus*. This is supported by the discovery that homologs of two other components of the wg pathway are likely to be involved in this process. Elimination of the maternal mRNA encoding the *Xenopus* homolog of *armadillo*, β-catenin, eliminates all dorsal structures (Heasman *et al.*, 1994), whereas injection of RNA encoding β-catenin, or the related protein plakoglobin, induces ectopic heads (Funayama *et al.*, 1995; Karnovsky and Klymkowsky, 1995). Similarly, injection of RNA encoding the *Xenopus* homolog of *dishevelled* induces ectopic heads (Sokol *et al.*, 1995).

In regulating the *Xenopus* dorsal–ventral axis, Xgsk-3 appears to primarily affect the developing mesoderm (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995). In *Drosophila*, *zw3/shaggy* also functions to regulate the decision between neural and epidermal fates (Heitzler and Simpson, 1991; Ruel *et al.*, 1993). Since *zw3/shaggy* is involved in the specification of ectodermal cell fates, we asked whether Xgsk-3 might also regulate patterning of

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Xenopus ectoderm. To investigate this possibility, Xgsk-3 was ectopically overexpressed in the presumptive ectodermal region of early *Xenopus* embryos. The most striking effect of Xgsk-3 overexpression was a broad expansion of the cement gland, an induced ectodermal tissue at the anterior end of the embryo which is neither a neural nor an epidermal structure. We show that Xgsk-3 overexpression enhances the responsiveness of naive ectoderm to cement-gland-inducing signals originating in the mesoderm and show that Xgsk-3 promotes the response of ectoderm to the intercellular signaling factor, noggin. In addition, ectopic overexpression of Xgsk-3 expands the expression of *Xotx2* and *XANF-2*, two noggin-inducible anterior genes. Concomitantly, the expression of *Xgbx-2*, an epidermal marker, is lost, suggesting that expansion of anterior ectodermal structures occurs at the expense of anterior epidermis. These results suggest that Xgsk-3 may play a role in ectodermal patterning and that, as in *Drosophila*, GSK-3 homologs function in multiple developmental processes.

MATERIALS AND METHODS

Embryos. Fertilized embryos were obtained as previously described (Newport and Kirschner, 1982). Eggs were fertilized in 0.5× MMR (1× MMR is 0.1 *M* NaCl, 2.0 m*M* KCl, 1.0 m*M* $MgSO_4$, 2.0 m*M* $CaCl_2$, 0.1 m*M* EDTA, and 5.0 m*M* Hepes, pH 7.8). The jelly coat was removed with 2% cysteine in water, pH 7.8, and eggs were rinsed in 0.1× MMR. Embryos were kept at 14 to 23°C. Staging was as previously described (Nieuwkoop and Faber, 1967).

Tissue explants and conjugates. For ectoderm explants, the upper portion of the animal hemisphere was dissected from stage 9 embryos with a fine wire knife. Care was taken to remove any adherent vegetal cells. Explants were cultured in 1× MBS (88 m*M* NaCl, 1 m*M* KCl, 0.41 m*M* $CaCl_2$, 0.33 m*M* $Ca(NO_3)_4$, 0.82 m*M* $MgSO_4$, 2.4 m*M* $NaHCO_3$, 10 m*M* Hepes, pH 7.4) with 1 mg/ml BSA and 50 μg/ml gentamicin sulfate. For ectoderm/mesoderm conjugates, ectoderm was explanted as above. All involuted dorsal mesoderm with the attached superficial layer was dissected from stage 11 embryos and the overlying ectoderm was removed and discarded. Ectoderm and mesoderm were cultured in contact or alone in 1× MBS, 1 mg/ml BSA, 50 μg/ml gentamicin sulfate, 5 units/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin. All antibiotics were from Sigma.

RNA expression vectors. An RNA expression vector encoding Xgsk-3 was constructed by removing the *Bam*HI fragment from pXG03 (Pierce and Kimelman, 1995), and inserting it into the *Bam*HI site of a CS2⁺ vector (Turner and Weintraub, 1994), creating pXG73. A CS2⁺ construct of the frame-shift mutant of *Xgsk-3* (*Xgsk-3FS*) was made by removing a *Bst*EII–*Sph*I fragment from pXG30FS (Pierce and Kimelman, 1995) and inserting it into pXG73, which had been digested with *Bst*EII and *Sph*I, creating pXG92.

RNA synthesis and microinjection. *Xgsk-3* and *Xgsk-3FS* RNA was synthesized from the CS2⁺-derived vectors, linearized with *Not*I. The template for β-galactosidase RNA was CS-β-galactosidase (Turner and Weintraub, 1994), linearized with *Not*I. *Noggin* RNA was synthesized from p*noggin*Δ5' (Smith and Harland, 1992), linearized with *Eco*RV. RNA was synthesized using the SP6 mMESSAGE mMACHINE kit (Ambion) following the manufacturer's instructions. Phenol:chloroform (1:1)-extracted RNA was separated from unincorporated nucleotides with a Microcon 100 microconcentrator (Amicon) and injected without further purification. RNA was microinjected as published (Moon and Christian, 1989), in volumes of 10 nl or less per blastomere. Embryos were injected with 4–8 ng *Xgsk-3* or *Xgsk-3FS* RNA, 400 pg β-galactosidase RNA, or 37.5 or 150 pg *noggin* RNA.

β-galactosidase activity staining. β-galactosidase activity staining followed the procedure of Turner and Weintraub (1994), using 6-chloro-3-indolyl β-D-galactopyranoside (Salmon-gal; Biosynth AG) as a substrate to produce a red stain.

In situ hybridization. Whole-mount *in situ* hybridization followed the procedure of Harland (1991), with a variety of modifications (Knecht *et al.*, 1995). For antisense probe synthesis, *XAG-1* plasmid (Sive *et al.*, 1989) was digested with *Not*I, *XANF-2* (Mathers *et al.*, 1995) with *Hin*dIII, *Xotx2* (Blitz and Cho, 1995) with *Kpn*I, and *Xgbx-2* (von Bubnoff *et al.*, 1996) with *Eco*RV. *XAG-1* was transcribed with T7 polymerase, and *XANF-2*, *Xotx2*, and *Xgbx-2* were transcribed with T3 polymerase.

RESULTS

Ectopic Expression of Xgsk-3 Causes Expansion of the Cement Gland

In order to investigate a possible role for Xgsk-3 in ectodermal patterning, Xgsk-3 was overexpressed in the prospective ectodermal region of early *Xenopus* embryos. Embryos were injected at the two-cell stage in the animal pole region of one blastomere with RNA encoding Xgsk-3 or a nonfunctional frameshift version of Xgsk-3 (Xgsk-3FS). Xgsk-3FS contains a frameshift approximately 40% into the protein and has no effect on early development (Pierce and Kimelman, 1995). By the end of gastrulation, the left–right axis in many embryos corresponds approximately to the first cleavage plane that divided the embryo into two cells (Danilchik and Black, 1988). Thus, injection in one of two cells with RNA encoding a nonsecreted protein results in one-half of the embryo being affected while the other half acts as an internal control. This was the case for the majority of the embryos in our experiments as determined by the use of β-galactosidase as a lineage tracer (see below). All embryos injected in one cell at the two-cell stage with *Xgsk-3* RNA developed with expanded or ectopic cement glands on the injected sides (Fig. 1B), while the cement glands were normal on the uninjected sides of these embryos (Fig. 1A) and in embryos injected with *Xgsk-3FS* RNA (not shown). When the embryos injected with *Xgsk-3* RNA developed to late tailbud stages, their bodies were shorter and curved toward the injected side, and the eye typically failed to develop on that side (not shown).

Although Xgsk-3 overexpression caused dramatic expansion of the cement gland, the ectopic cement gland was limited to approximately the anterior half of the embryo and was never seen in the posterior region. To determine whether this was due to a restricted distribution of the injected RNA, embryos were co-injected in one cell at the two-cell stage with *Xgsk-3* RNA and RNA encoding β-galactosidase, so that cells expressing the injected RNAs could

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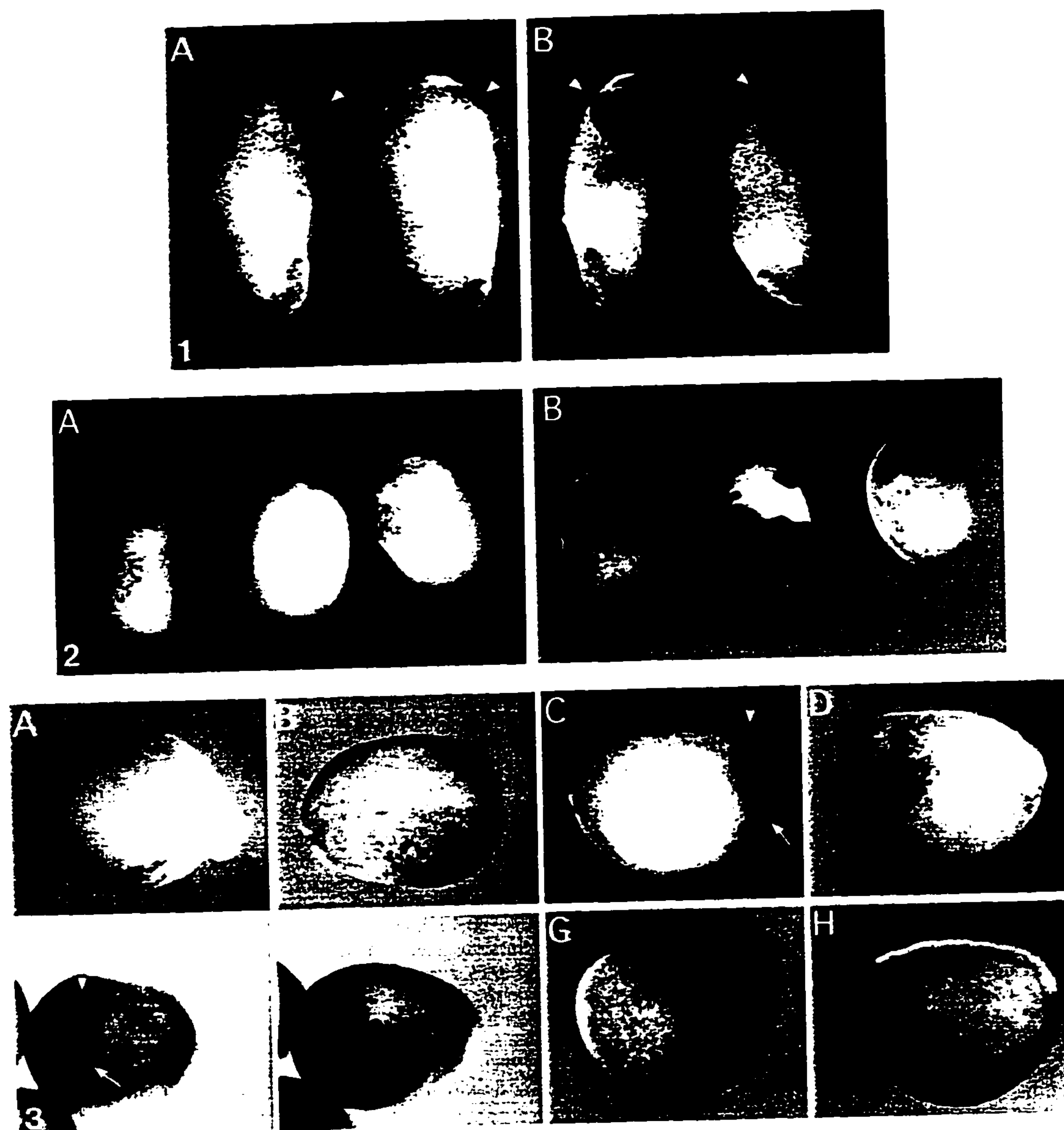
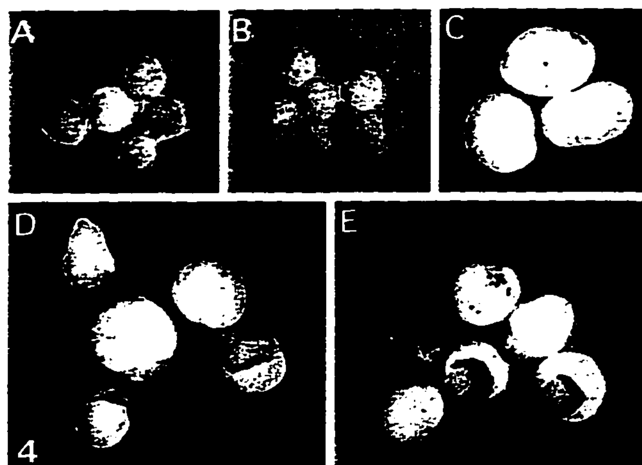


FIG. 1. Ectopic Xgsk-3 expression causes cement gland expansion. Embryos were injected in one cell at the two-cell stage with *Xgsk-3* RNA and cultured until stage 24. (A) Uninjected sides; dorsal is to the left and anterior is at the top. (B) Injected sides of embryos in (A); dorsal is to the right. The sites of the endogenous cement glands are indicated (arrowheads).

FIG. 2. Cement gland expansion is limited to the anterior region of ectopic Xgsk-3 expression. Embryos were injected with *Xgsk-3* and β-galactosidase RNA in one cell at the two-cell stage, cultured until stage 20, and stained for β-galactosidase activity (red) and *XAG-1* expression (purple). (A) Uninjected sides; dorsal is to the right and anterior is at the top. (B) Injected sides of the embryos in (A); dorsal is to the left. Note that *XAG-1* expression is limited to the anterior region of β-galactosidase activity. Occasional β-galactosidase expressing cells on the uninjected side of the embryo indicate that the first cleavage does not always perfectly divide the embryo into left and right halves.



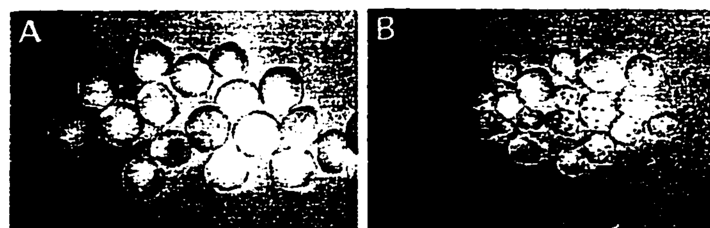


FIG. 4. Xgsk-3 enhances ectodermal response to mesoderm. Ectoderm was explanted from embryos previously injected with *Xgsk-3FS* or *Xgsk-3* RNA, cultured alone or in contact with dorsal mesoderm, and stained for *XAG-1* expression. (A) Ectoderm from embryos injected with *Xgsk-3FS* RNA, cultured alone. (B) Ectoderm from embryos injected with *Xgsk-3* RNA, cultured alone. (C) Mesoderm from uninjected embryos. (D) Ectoderm as in (A) cultured with mesoderm. (E) Ectoderm as in (B) cultured with mesoderm. The spot of stain in (C) is debris stuck to the explant.

FIG. 5. Xgsk-3 enhances ectodermal responsiveness to noggin. Embryos were injected with *Xgsk-3FS* RNA (A), *Xgsk-3* RNA (B), 37.5 pg of *noggin* RNA in combination with *Xgsk-3FS* (C) or *Xgsk-3* (D) RNA, or 150 pg of *noggin* RNA alone (E). Ectoderm was explanted, cultured, and stained for *XAG-1* expression. The diffuse pale purple color in some explants is background staining. One of three experiments is shown.

FIG. 3. Ectopically expressed Xgsk-3 disrupts expression of anterior and lateral genes. Embryos were injected in one cell at the two-cell stage with *Xgsk-3FS* (E) or *Xgsk-3* (A–D, F–H) and β-galactosidase RNA, cultured until stage 13.5 (A) or stage 14 (B–H), and stained for β-galactosidase activity and the indicated gene expression. (A and B) *XAG-1* expression in embryos viewed from the dorsal side. Anterior is facing to the left in (A) and to the right in (B), with the injected sides facing down. Note the presence of β-galactosidase activity and the absence of *XAG-1* expression within the neural plate region (arrowhead) in (B). Although not apparent in the photograph, the neural folds, which mark the borders of the neural plate, are readily seen with a microscope. (C and D) *Xotx2* expression on the uninjected (C) and injected (D) sides of one embryo. Note anterior (arrow) and posterior (arrowhead) regions of expression in (C). In the embryo in (D), only the anterior region of expression has expanded. (E and F) Anterior-lateral view of *XANF-2* expression in embryos injected with *Xgsk-3FS* (E) and *Xgsk-3* (F) RNA. Note the appearance of anterior (arrow) and posterior (arrowhead) regions of expression in (E). In the embryo in (F), both the anterior and posterior regions of expression have expanded on the injected side (facing down). (G and H) *Xgbx-2* expression on the uninjected (G) and injected (H) sides of one embryo. The epidermal expression is visible in (G). The neural domain of expression is mostly hidden in this view by the neural folds. The pairs of embryos in (C and D) and (G and H) are oriented with dorsal at the top and anterior facing the center. Staining for β-galactosidase activity is visible in the microscope although it is not visible in all photographs.

be identified. In this experiment, cement gland was detected by *in situ* hybridization using the cement gland marker gene *XAG-1* (Sive *et al.*, 1989). As shown in Fig. 2, expanded and ectopic cement gland formation was limited to the region in which β-galactosidase activity was detected (Fig. 2B). However, while the β-galactosidase staining was often spread along the length of the body, the cement gland marker was found only in the anterior region of the embryo. These results indicate that while inappropriate cement gland formation occurred only where Xgsk-3 was overexpressed, this was not sufficient for cement gland formation in the posterior region of the embryo.

Although cement gland differentiation is not observed in the embryo until the late neurula stages, cement gland specification begins during the gastrula stages (Sive *et al.*, 1989). We asked whether Xgsk-3 altered early specification of the cement gland. Embryos were co-injected in one cell at the two-cell stage with *Xgsk-3* and β-galactosidase RNAs, and presumptive cement gland was detected by *in situ* hybridization with *XAG-1*, which is first expressed late in gastrulation (Sive *et al.*, 1989). We found that as early as the beginning of neurulation, the region of *XAG-1* expression was expanded both laterally (Figs. 3A and 3B) and ventrally (not shown) on the side of the embryo where Xgsk-3 was ectopically expressed. At this early stage, *XAG-1* is expressed in a somewhat broader area than will be occupied by the differentiated cement gland. Therefore, the effects of Xgsk-3 on cement gland localization can be seen at a time soon after cement gland specification begins.

Ectopic Expression of Xgsk-3 Does Not Decrease the Size of the Neural Plate

Although cement gland and neural tissue are both of ectodermal origin, gastrulation results in progressively more anterior dorsal tissue becoming specified as cement gland until the cement gland is finally positioned in the most dorsal-anterior region of the embryo, anterior to the neural plate (Sive *et al.*, 1989). This region is neither neural ectoderm nor epidermis (Sive *et al.*, 1989). We hypothesized that the expansion of cement gland caused by Xgsk-3 might reflect the specification of a larger region of the embryo as nonneural ectoderm at the expense of neural ectoderm. However, observation of embryos at the early neurula stage that had been injected with *Xgsk-3* RNA in one cell at the two-cell stage and stained for *XAG-1* expression indicated that the presumptive cement gland was expanded ventrally and laterally around the neural plate (Figs. 3A and 3B). *XAG-1* expression was never seen to expand posteriorly onto the neural plate, as defined by the emerging neural folds, even when β-galactosidase activity indicated the presence of *Xgsk-3* RNA in the neural region (Fig. 3B). From these results we conclude that the cement gland expansion caused by overexpression of Xgsk-3 did not occur at the expense of neural tissue, indicating that Xgsk-3 causes ventral and lateral ectodermal cells to convert to a cement gland fate.

Xgsk-3 Disrupts Patterning within Anterior and Lateral Ectoderm

The above results demonstrate that Xgsk-3 overexpression causes the expansion of cement gland into more lateral regions of the embyro, rather than posteriorly into the neural region. To investigate whether general anterior patterning and epidermal patterning are also altered, we determined the effects of Xgsk-3 overexpression on the expression of genes in the anterior and epidermal regions.

We first determined the effects of Xgsk-3 overexpression on the expression of two anterior markers which are expressed in and adjacent to the cement gland region. *Xotx2* is expressed in the early neurula in two regions, one more anterior, corresponding to the prospective cement gland, and the other more posterior, overlapping the anterior neural plate (Blitz and Cho, 1995; Pannese *et al.*, 1995) (Fig. 3C). When *Xgsk-3* RNA was injected in one cell at the two-cell stage, the anterior domain of *Xotx2* expression expanded laterally on the injected side in 95% of the embryos (Fig. 3D), similar to the effect on *XAG-1* expression. In addition, in approximately 25% of the embryos, the posterior region of expression was also distorted or expanded laterally. *XANF-2* is expressed in a region extending from the anterior neural fold to just posterior to the prospective cement gland. Its expression also appears as two bands, with the more anterior expression corresponding to the prospective anterior pituitary (Mathers *et al.*, 1995) (Fig. 3E). The region corresponding to the prospective pituitary is not coincident with the prospective cement gland but is just posterior to it (Mathers *et al.*, 1995). Xgsk-3 overexpression on one side of the embryo caused lateral expansion of the anterior *XANF-2* expression in 85% of the embryos (Fig. 3F), as was seen with *Xotx2* and *XAG-1*. The posterior region of expression was also expanded in some embryos, but this effect was more variable than the effect on the *Xotx2* posterior expression. These results demonstrate that there is a general expansion of markers for anterior ectoderm in response to ectopic Xgsk-3 expression.

Since the expansion of *Xotx2* and *XANF-2* mimics that of *XAG-1*, in that the expression of these genes expands laterally and not dorsally in the neural plate, it may occur at the expense of genes which are normally expressed in the epidermal region. To investigate this possibility, we determined the effect of Xgsk-3 overexpression on *Xgbx-2*, which is expressed at the early neurula stage in two anterior epidermal patches (Fig. 3G) in addition to two anterior dorsal stripes in the neural plate (von Bubnoff *et al.*, 1996). The epidermal patches have sharp anterior boundaries, at the anterior–posterior level of the midbrain–hindbrain boundary, with expression tapering off posteriorly (von Bubnoff *et al.*, 1996). Injection of *Xgsk-3* RNA resulted in significant weakening or elimination of the epidermal component of *Xgbx-2* expression on the injected side (Fig. 3H). In addition, 50% of the embryos showed weakening or elimination of the neural *Xgbx-2* expression as well (not shown). The loss of *Xgbx-2* epidermal expression appeared to extend further

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TABLE 1
Xgsk-3 Enhances Ectodermal Response to Mesoderm

Sample	n	Relative *XAG-1* expression			
		None	Weak	Moderate	Strong
Experiment 1					
Xgsk-3FS ectoderm	6	6 (100%)	0	0	0
Xgsk-3 ectoderm	6	1 (17%)	5 (83%)	0	0
Mesoderm	3	3 (100%)	0	0	0
Xgsk-3FS conjugates	5	2 (40%)	0	3 (60%)	0
Xgsk-3 conjugates	6	2 (33%)	0	2 (33%)	2 (33%)
Experiment 2					
Xgsk-3FS ectoderm	19	19 (100%)	0	0	0
Xgsk-3 ectoderm	21	19 (90%)	2 (10%)	0	0
Mesoderm	20	20 (100%)	0	0	0
Xgsk-3FS conjugates	20	16 (80%)	0	3 (15%)	1 (5%)
Xgsk-3 conjugates	19	3 (16%)	0	10 (53%)	6 (31%)

Note. Ectoderm was explanted from embryos previously injected with *Xgsk-3FS* or *Xgsk-3* RNA and cultured alone (indicated as ectoderm) or in contact with mesoderm (indicated as conjugates). Experiment 1 is shown in Fig. 4.

posteriorly than the extent of anterior gene expression caused by *Xgsk-3* RNA injection. Although this suggests that Xgsk-3 may be affecting ectoderm at the posterior end of the embryo, the posterior expression of *Xgbx-2* is very weak (Fig. 3G) and difficult to measure reliably. We conclude that the loss of *Xgbx-2* epidermal staining suggests that the expansion of the prospective cement gland and pituitary regions caused by Xgsk-3 occurred at the expense of epidermis.

Xgsk-3 Increases Ectodermal Responsiveness to Mesoderm

Since cement gland expansion is a specific effect of animal pole injection of *Xgsk-3* RNA and is different from the effect of injection in the presumptive mesoderm (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995), we asked if the effects of Xgsk-3 were due to a direct effect on the ectoderm. To examine this, and to determine whether Xgsk-3 could directly induce cement gland, we cultured isolated blastula stage ectoderm from embryos injected with *Xgsk-3* RNA and evaluated *XAG-1* expression by whole-mount *in situ* hybridization. Ectoderm expressing Xgsk-3 expressed very low levels of *XAG-1*, visible as small specks of stain, while control explants, isolated from embryos that were injected with *Xgsk-3FS* RNA, showed no *XAG-1* induction (Figs. 4A and 4B and Table 1). This indicated that Xgsk-3 was capable of inducing low levels of cement gland, but this seemed unlikely to account for the dramatic expansion of cement gland seen in the whole embryo. We hypothesized that Xgsk-3 might be enhancing the response of ectoderm to endogenous cement-gland-inducing signals originating in the mesoderm. To test this hypothesis, we analyzed cement gland induction in conjugates of ectodermal tissue from embryos injected with *Xgsk-3* RNA and mesodermal tissue isolated from the dorsal side of uninjected embryos. When dorsal mesodermal explants from midgastrula-stage embryos were cultured alone, they showed no *XAG-1* staining (Fig. 4C), but when control ectoderm was combined with dorsal mesoderm, a low level of *XAG-1* expression was observed (Fig. 4D and Table 1). Although we would have observed a higher level of *XAG-1* induction by selectively using anterior mesoderm as the inducing tissue (Sive *et al.*, 1989), the lower level of *XAG-1* expression was advantageous for our experiments. When ectoderm expressing Xgsk-3 was combined with mesoderm, *XAG-1* expression was seen in a larger number of explants and in larger regions within the explants (Fig. 4E and Table 1). In two experiments, greater than 30% of conjugates with ectoderm expressing Xgsk-3 showed strong *XAG-1* expression, whereas 0–5% of the conjugates expressing Xgsk-3FS showed strong staining (Table 1). The *XAG-1* expression in both types of conjugates was significantly greater than that in Xgsk-3 expressing ectoderm alone (compare Figs. 4D and 4E to 4B). The reciprocal experiment, with Xgsk-3 expressed in the mesoderm, was not done, since expression of Xgsk-3 in the mesoderm eliminates anterior structures (He *et al.*, 1995; Pierce and Kimelman, 1995), indicating a loss of dorsal character which is necessary for CG induction (Sive *et al.*, 1989). Our results demonstrate that overexpression of Xgsk-3 in ectodermal explants increases cement gland induction in the presence of dorsal mesoderm.

Xgsk-3 Synergizes with Noggin

To explore the mechanism by which ectopic Xgsk-3 expression enhances cement gland induction in whole embryos and ectoderm/mesoderm conjugates, we tested the ability of Xgsk-3 to enhance the effects of factors known to induce cement gland. Noggin is a secreted factor that is expressed in the dorsal mesoderm (Smith and Harland, 1992) and that can induce cement gland and the expression of neural genes in isolated ectoderm (Lamb *et al.*, 1993). In addition, in isolated ectoderm, noggin induces *Xotx2* (Lamb *et al.*, 1993; Blitz and Cho, 1995) and *XANF-2* (Mathers *et al.*, 1995), the expression of which are expanded in whole embryos by Xgsk-3 overexpression. Embryos were injected in both cells at the two-cell stage with 37.5 pg of RNA encoding noggin in combination with RNA encoding Xgsk-3 or Xgsk-3FS. Ectoderm was explanted at the blastula stage, cultured until the late neurula stage, and analyzed by whole-mount *in situ* hybridization for *XAG-1* expression. Noggin induced a low level of *XAG-1* (Fig. 5C), as did Xgsk-3 alone (Fig. 5B). In three independent experiments, a much higher level of *XAG-1* was induced by noggin in the presence of Xgsk-3 (Fig. 5D), similar to the level induced by 150 pg of *noggin* RNA alone (Fig. 5E). In three independent experiments, Xgsk-3 had no effect on cement gland induction by *Xenopus* banded-hedgehog (not shown), which is also an effective inducer of cement gland in isolated ecto-

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derm (Ekker *et al.*, 1995; Lai *et al.*, 1995). Thus, Xgsk-3 appears to function by enhancing ectodermal responsiveness to specific cement-gland-inducing signals, such as noggin.

DISCUSSION

We show here that injection of RNA encoding Xgsk-3 in the presumptive ectoderm results in a dramatic expansion of the cement gland, the most anterior structure in the *Xenopus* embryo. Using β-galactosidase as a lineage tracer, we found that while the injected RNA is often spread along the length of the body, the cement gland expansion is limited to approximately the anterior half of the embryo. This suggests that ectopic Xgsk-3 may be enhancing the responsiveness of the ectoderm to endogenous cement-gland-inducing signals originating in the anterior dorsal mesoderm. This enhanced responsiveness would be expected to result in cement gland induction in a broader region than normal, but the extent of this region would still be limited by the spread of the inducing signal. We tested this hypothesis by making conjugates of ectoderm and dorsal mesoderm and found that the cement gland marker *XAG-1* is induced by mesoderm more effectively in ectoderm that overexpresses Xgsk-3 than in control ectoderm. This result demonstrates that overexpression of Xgsk-3 in the ectoderm is sufficient for the induction of excess cement gland and suggests that in the whole embryo, excess Xgsk-3 enhances ectodermal responsiveness to endogenous cement-gland-inducing signals derived from the mesoderm.

To explore the mechanism by which ectodermal responsiveness to cement-gland-inducing signals is increased by Xgsk-3 overexpression, we tested whether Xgsk-3 could enhance the response of ectoderm to specific signals known to induce cement gland. We found that in ectodermal explants, Xgsk-3 significantly increased the response to noggin, a secreted factor that is expressed in the dorsal mesoderm (Smith and Harland, 1992), but that it had no effect on ectodermal responsiveness to *Xenopus* banded-hedgehog, which also induces cement gland in ectodermal explants (Ekker *et al.*, 1995; Lai *et al.*, 1995). These results indicate that Xgsk-3 is affecting a specific signaling pathway or pathways, rather than ubiquitously enhancing the response to all cement-gland-inducing signals. We also observed that overexpression of Xgsk-3 alone was able to weakly induce the expression of *XAG-1* in ectodermal explants, although at a much lower level than is observed in combination with noggin or in whole embryos. It is possible that noggin and Xgsk-3 function in two parallel pathways that interact or, alternatively, Xgsk-3 might directly participate in transduction of the noggin signal.

Ectopic expression of Xgsk-3 also caused expansion of the domains of expression of two anteriorly expressed noggin-inducible genes, *Xotx2* and *XANF-2*. The more anterior of the two regions of *Xotx2* expression is coincident with the presumptive cement gland (Blitz and Cho, 1995; Pannese *et al.*, 1995), while the more anterior region of the *XANF-2* expression domain marks the presumptive anterior pituitary (Mathers *et al.*, 1995). Both of these genes can be induced in naive ectoderm by noggin (Lamb *et al.*, 1993; Blitz and Cho, 1995; Lai *et al.*, 1995), and *Xotx2* can directly induce cement gland (Blitz and Cho, 1995; Pannese *et al.*, 1995). Although noggin has been shown to induce the anterior genes tested here, it has not been found to induce neural genes which are expressed posterior to *Xotx2*, such as *en-2* (Lamb *et al.*, 1993). In keeping with this, we have found that more posterior neural genes, such as *Krox-20* and the neural component of *Xgbx-2*, are not expanded in whole embryos by the ectopic expression of Xgsk-3 (unpublished results). In addition, although Xgsk-3 enhances the ability of noggin to induce *XAG-1* (Fig. 5) and *XANF-2* (unpublished results) in ectodermal explants, we have not found that it allows noggin to induce the neural gene *en-2* (unpublished results). These results are consistent with the hypothesis that Xgsk-3 is interacting with the noggin signaling pathway in its effects on anterior patterning. The reduction of the neural expression of *Xgbx-2* in some embryos, as well as effects on other genes within the neural plate and neural crest (unpublished results), suggests that although the size of the neural plate is not appreciably altered, Xgsk-3 overexpression may disrupt signals involved in neural patterning.

Sive and co-workers have shown that at least two mesoderm-derived signals are required for correct cement gland localization: an inducing signal that specifies cement gland formation from anterior ectoderm and a dominant inhibitory signal that could produce a stable border between neural and nonneural ectoderm (Sive *et al.*, 1989) Noggin is well positioned to act as the endogenous inducer of cement gland, since *noggin* RNA is expressed in the gastrula stages in the dorsal midline (Smith and Harland, 1992). We suggest that ectopic overexpression of Xgsk-3 is enhancing the responsiveness of ectoderm to endogenous cement-gland-inducing signals without affecting the inducing signal itself or the proposed inhibitory signal. Thus, cement gland induction would be limited posteriorly by the inhibitory signal forming the border of the neural plate and laterally and ventrally by the spread of noggin and the responsiveness of the ectoderm.

Microdissection and RNase protection analysis of late blastula and midneurula-stage embryos, as well as whole-mount *in situ* hybridization, have shown that *Xgsk-3* RNA is expressed at similar levels throughout the embryo during these stages (unpublished results), indicating that localization of anterior structures is not dependent on localization of *Xgsk-3* RNA. Instead, our results suggest that Xgsk-3 could be a component of the ectodermal intracellular pathway, which responds to endogenous anterior-inducing signals by activating the transduction of genes such as *XAG-1*, *Xotx2*, and *XANF-1*. As we develop methods to measure the endogenous activity of Xgsk-3, it will be interesting to determine whether it is constitutively active, thus providing a baseline responsiveness to inducing signals, or

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whether its activity is directly regulated by factors such as noggin or members of the Wnt family.

In demonstrating and elucidating the *in vivo* role of Xgsk-3 in *Xenopus* dorsal-ventral axis determination, the use of a dominant inhibitory mutant of Xgsk-3 (Xgsk-3K → R) was very successful (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995). In our study of anterior development, we found that Xgsk-3K → R caused only minor defects in cement gland and head development (unpublished results). However, for a dominant inhibitory mutant to be effective, it must form a stable interaction with a target such that it interferes with normal signaling. It is possible that the appropriate targets during these two developmental processes are different and that Xgsk-3K → R is unable to interact with a suitable target strongly enough to inhibit signaling during anterior development. Alternatively, Xgsk-3 may artificially amplify the effects of anterior inducing signals. This will become clear when the noggin pathway is known. In addition, at least four signals have been suggested to be involved in regulating this part of the embryo, including noggin (Lamb *et al.*, 1993), follistatin (McGrew *et al.*, 1995), X-bhh (McGrew *et al.*, 1995), and chordin (Sasai *et al.*, 1995), and elimination of the intracellular pathway used by one of them may cause only minor perturbations. Until it is possible to selectively inactivate noggin, for example, it will not be possible to test this hypothesis.

Although the dominant inhibitory mutant of Xgsk-3 was not useful for investigating the role of Xgsk-3 during anterior development, recent results indicate an interaction between noggin and Wnt signals, suggesting that the effects of Xgsk-3 on anterior development may be mediated by the *Wnt* pathway. Using a system in which ectodermal explants were cultured in contact with oocytes expressing Wnt-1 and/or noggin, it was shown that Wnt-1 significantly inhibits the ability of noggin to induce cement gland (Lustig and Kirschner, 1995). In addition, when noggin was coexpressed in ectodermal explants with Xwnt-3a or β-catenin, an intracellular component of the *Wnt* signal transduction pathway, the ability of noggin to induce *XAG-1* expression was inhibited (McGrew *et al.*, 1995). Since *Wnt* signaling appears to antagonize the function of Xgsk-3 (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995), parallel to the results observed in *Drosophila* (Siegfried *et al.*, 1992), these results indicate that inhibition of Xgsk-3 can lead to a diminished response to noggin. This observation fits well with our results in which excess Xgsk-3, which is expected to antagonize *Wnt* signaling, leads to an enhancement of noggin signaling in ectodermal explants. This suggests that in the embryo, Xgsk-3 could function in a *Wnt* signaling pathway to spatially regulate the responsiveness to noggin or noggin-like signals. McGrew and co-workers also found that *Xwnt-3a* was able to inhibit induction of *XAG-1* expression by *X-bhh* (McGrew *et al.*, 1995) a result which is in apparent contradiction with the lack of synergism seen between Xgsk-3 and *X-bhh* (this study). At present we do not know the reason for this discrepancy. However, while *Xwnt-3a* caused an elevation of posterior neural gene expression when combined with noggin, only anterior genes were affected when *Xwnt-3a* was combined with *X-bhh* (McGrew *et al.*, 1995), indicating that different signaling pathways may be involved.

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REFERENCES

Blitz, I. L., and Cho, K. W. Y. (1995). Anterior neurectoderm is progressively induced during gastrulation: The role of the *Xenopus* homeobox gene *orthodenticle*. *Development* 121, 993–1004.

Bourouis, M., Moore, P., Ruel, L. Y. G., Heitzler, P., and Simpson, P. (1990). An early embryonic product of the gene *shaggy* encodes a serine/threonine protein kinase related to the CDC28/cdc2⁺ subfamily. *EMBO J.* 9, 2877–2884.

Chakrabarti, A., Matthews, G., Colman, A., and Dale, L. (1992). Secretory and inductive properties of *Drosophila* wingless protein in *Xenopus* oocytes and embryos. *Development* 115, 355–369.

Christian, J. L., McMahon, J. A., McMahon, A. P., and Moon, R. T. (1991). *Xwnt-8*, a *Xenopus Wnt-1/int-1*-related gene responsive to mesoderm inducing factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* 111, 1045–1056.

Danilchik, M. V., and Black, S. D. (1988). The first cleavage plane and the embryonic axis are determined by separate mechanisms in *Xenopus laevis*. *Dev. Biol.* 128, 58–64.

Dominguez, I., Itoh, K., and Sokol, S. Y. (1995). Role of glycogen synthase kinase 3β as a negative regulator of dorsoventral axis formation in *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* 92, 8498–8502.

Ekker, S. C., McGrew, L. L., Lai, C.-J., Lee, J. J., von Kessler, D. P., Moon, R. T., and Beachy, P. A. (1995). Distinct expression and shared activities of members of the hedgehog gene family of *Xenopus laevis*. *Development* 121, 2337–2347.

Funayama, N., Fagotto, F., McCrea, P., and Gumbiner, B. M. (1995). Embryonic axis induction by the Armadillo repeat domain of β-catenin: Evidence for intracellular signaling. *J. Cell Biol.* 128, 959–968.

Harland, R. M. (1991). *In situ* hybridization: An improved whole mount method for *Xenopus* embryos. *In* "Methods in Cell Biology" (B. K. Kay and H. J. Peng, Eds.). Academic Press, San Diego, CA.

He, X., Saint-Jeannet, J.-P., Woodgett, J. R., Varmus, H. E., and Dawid, I. (1995). Glycogen synthase kinsase-3 and dorsoventral patterning in Xenopus embryos. *Nature* 374, 617–622.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P.,

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Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y., and Wylie, C. (1994). Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell* 79, 791-83.

Heitzler, P., and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* 64, 1083-1092.

Karnovsky, A., and Klymkowsky, M. W. (1995). Anterior axis duplication in *Xenopus* induced by the overexpression of the cadherin-binding protein plakoglobin. *Proc. Natl. Acad. Sci. USA* 92, 4522-4526.

Knecht, A. K., Good, P. J., Dawid, I. B., and Harland, R. M. (1995). Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. *Development* 121, 1927-1936.

Lai, C.-J., Ekker, S. C., Beachy, P. A., and Moon, R. T. (1995). Patterning of the neural ectoderm of *Xenopus laevis* by the amino-terminal product of hedgehog autoproteolytic cleavage. *Development* 121, 2349-2360.

Lamb, M. T., Knecht, A., Smith, W. C., Stachel, S. E., Econmides, A. N., Stahl, N., Yancopolous, G. D., and Harland, R. (1993). Neural induction by the secreted polypeptide noggin. *Science* 262, 713-718.

Lustig, K. D., and Kirschner, M. W. (1995). Use of an oocyte expression assay to reconstitute inductive signaling. *Proc. Natl. Acad. Sci. USA* 92, 6234-6238.

Mathers, P. H., Miller, A., Doniach, T., Dirksen, M.-L., and Jamrich, M. (1995). Initiation of anterior head-specific gene expression in uncommitted ectoderm of *Xenopus laevis* by ammonium chloride. *Dev. Biol.* 171, 641-654.

McGrew, L. L., Lai, C.-J., and Moon, R. T. (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with *noggin* and *follistatin*. *Dev. Biol.* 172, 337-342.

Moon, R. T., and Christian, J. L. (1989). Microinjection and expression of synthetic mRNAs in *Xenopus* embryos. *Technique* 1, 76-89.

Newport, J., and Kirschner, M. W. (1982). A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes at the midblastula stage. *Cell* 30, 675-686.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis*." North-Holland, Amsterdam.

Noordermeer, J., Klingensmith, J., Perrimon, N., and Nusse, R. (1994). *dishevelled* and *armadillo* act in the Wingless signalling pathway in *Drosophila*. *Nature* 367, 80-83.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., and Boncinelli, E. (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* 121, 707-720.

Pierce, S. B., and Kimelman, D. (1995). Regulation of Spemann organizer formation by the intracellular kinase *Xgsk-3*. *Development* 121, 755-765.

Plyte, S. E., Hughes, K., Nikolakaki, E., Pulverer, B. J., and Woodgett, J. R. (1992). Glycogen synthase kinase-3: Functions in oncogenesis and development. *Biochem. Biophys. Acta* 1114, 147-162.

Ruel, L., Bourouis, M., Heitzler, P., Pantesco, V., and Simpson, P. (1993). Drosophila *shaggy* kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of *Notch*. *Nature* 362, 557-560.

Sasai, Y., Lu, B., Steinbeisser, H., and DeRobertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. *Nature* 376, 333-336.

Siegfried, E., Chou, T., and Perrimon, N. (1992). *wingless* signaling acts through *zeste-white* 3, the Drosophila homolog of *glycogen synthase kinase-3*, to regulate *engrailed* and establish cell fate. *Cell* 71, 1167-1179.

Siegfried, E., Wilder, E. L., and Perrimon, N. (1994). Components of *wingless* signaling in *Drosophila*. *Nature* 367, 76-80.

Sive, H. L., Hattori, K., and Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. *Cell* 58, 171-180.

Smith, W. C., and Harland, R. M. (1992). Expression cloning of *noggin*, a new dorsalizing factor localized in the Spemann organizer in *Xenopus* embryos. *Cell* 70, 829-840.

Sokol, S., Christian, J. L., Moon, R. T., and Melton, D. A. (1991). Injected *wnt* RNA induces a complete body axis in *Xenopus* embryos. *Cell* 67, 741-752.

Sokol, S. Y., Klingensmith, J., Perrimon, N., and Itoh, K. (1995). Dorsalizing and neuralizing properties of Xdsh, a maternally expressed *Xenopus* homolog of *dishevelled*. *Development* 121, 1637-1647.

Turner, D. L., and Weintraub, H. (1994). Expression of *achaete-scute* homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* 8, 1434-1447.

von Bubnoff, A., Schmidt, J. E., and Kimelman, D. (1996). The *Xenopus laevis* homeobox gene *Xgbx-2* is an early marker of anteroposterior patterning in the ectoderm. *Mech. Dev.* in press.

Woodgett, J. R. (1991). A common denominator linking glycogen metabolism, nuclear oncogenes and development. *Trends Biochem. Sci.* 16, 177-181.

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