

GBP, an Inhibitor of GSK-3, Is Implicated in *Xenopus* Development and Oncogenesis

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Summary

Dorsal accumulation of β -catenin in early *Xenopus* embryos is required for body axis formation. Recent evidence indicates that β -catenin is dorsally stabilized by the localized inhibition of the kinase Xgsk-3, utilizing a novel Wnt ligand-independent mechanism. Using a two-hybrid screen, we identified GBP, a maternal Xgsk-3-binding protein that is homologous to a T cell protooncogene in three well-conserved domains. GBP inhibits *in vivo* phosphorylation by Xgsk-3, and ectopic GBP expression induces an axis by stabilizing β -catenin within *Xenopus* embryos. Importantly, antisense oligonucleotide depletion of the maternal GBP mRNA demonstrates that GBP is required for the establishment of the dorsal-ventral axis in *Xenopus* embryos. Our results define a family of GSK-3-binding proteins with roles in development and cell proliferation.

Introduction

A major issue faced by all developing organisms is how to break the symmetry of the oocyte or egg in order to establish the embryonic axes. In *Xenopus laevis*, sperm entry triggers a rotation of the cortex relative to the underlying cytoplasm that moves an unknown determinant from the vegetal pole to the future dorsal side of the embryo, creating an asymmetry near the equator (Gerhart et al., 1991). Emerging evidence from a number of avenues strongly supports the view that the rotation activates the Wnt pathway as a critical first step in determining the future dorsal-ventral axis and, subsequently, the anterior-posterior axis (Moon and Kimelman, 1998).

By the first cell division following fertilization, β -catenin, a key cytoplasmic effector of the Wnt pathway (reviewed in Miller and Moon, 1996) accumulates on the future dorsal side of the *Xenopus* embryo (Larabell et al., 1997). β -catenin binds the XTcf-3 transcription factor (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996) and activates the transcription of *siamois*

(Brannon and Kimelman, 1996; Brannon et al., 1997; Fagotto et al., 1997), a dorsal-specific gene required for the activation of the Spemann organizer (Lemaire et al., 1995; Carnac et al., 1996; Fan and Sokol, 1997) and the subsequent development of the embryonic axes.

A key member of the Wnt intracellular pathway is GSK-3, a negative regulator of β -catenin (Peifer et al., 1994b; Siegfried et al., 1994) that can phosphorylate it at an amino-terminal site (Yost et al., 1996). Mutations in the serine and threonine residues at this site prevent the ubiquitination and degradation of β -catenin in *Xenopus* (Yost et al., 1996; Aberle et al., 1997), indicating that phosphorylation of β -catenin by GSK-3 targets it for degradation. The phosphorylation of β -catenin by GSK-3 is strongly promoted by axin (Zeng et al., 1997; Ikeda et al., 1998), which binds both GSK-3 and β -catenin (Ikeda et al., 1998; Sakana et al., 1998). GSK-3 also phosphorylates APC, the tumor suppressor protein required for negative regulation of β -catenin in colon cancer cells (Munemitsu et al., 1995; Rubinfeld et al., 1996). However, the role of APC in *Xenopus* axis formation is not clear (Vleminckx et al., 1997).

The importance of GSK-3 in *Xenopus* development has been shown with a dominant-negative mutant, which stabilizes β -catenin and induces an ectopic axis (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995; Yost et al., 1996). The same effects are seen with lithium (Kao et al., 1986; Hedgepeth et al., 1997; Larabell et al., 1997), a noncompetitive inhibitor of GSK-3 (Klein and Melton, 1996; Stambolic et al., 1996). These results have led to the proposal that localized inhibition of GSK-3 on the future dorsal side of the embryo soon after fertilization leads to the localized stabilization of β -catenin within the first few hours of embryonic life (Larabell et al., 1997). How GSK-3 could be locally inhibited remains a major question. Because ectopic expression of Wnt induces an ectopic axis (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991), it is possible that the cortical rotation moves a Wnt ligand to the future dorsal side of the embryo. However, no Wnt with the correct spatial distribution and axis-inducing properties has been found in *Xenopus* embryos (Cui et al., 1995). Moreover, ectopic expression of dominant-negative mutants of Wnt or Dishevelled, a cytoplasmic protein required for Wnt signaling (Noordermeer et al., 1994; Siegfried et al., 1994), does not block axis formation in *Xenopus* even though they inhibit gastrula-stage Wnt signaling (Hoppler et al., 1996; Sokol, 1996). These results raise the possibility that the Wnt pathway could be activated independently of Wnt in the early embryo, utilizing maternal factors.

We report here the identification of a novel maternal *Xenopus* GSK-3-binding protein, GBP, which we show is required for the establishment of the dorsal-ventral axis in *Xenopus*. We demonstrate that GBP binds GSK-3 in *Xenopus* embryos, prevents GSK-3-dependent phosphorylation of an *in vivo* substrate, induces an ectopic axis, and increases β -catenin levels. Using the similarity of GBP to a recently discovered T cell protooncogene of unknown function, *Frat1* (Jonkers et al., 1997), and a

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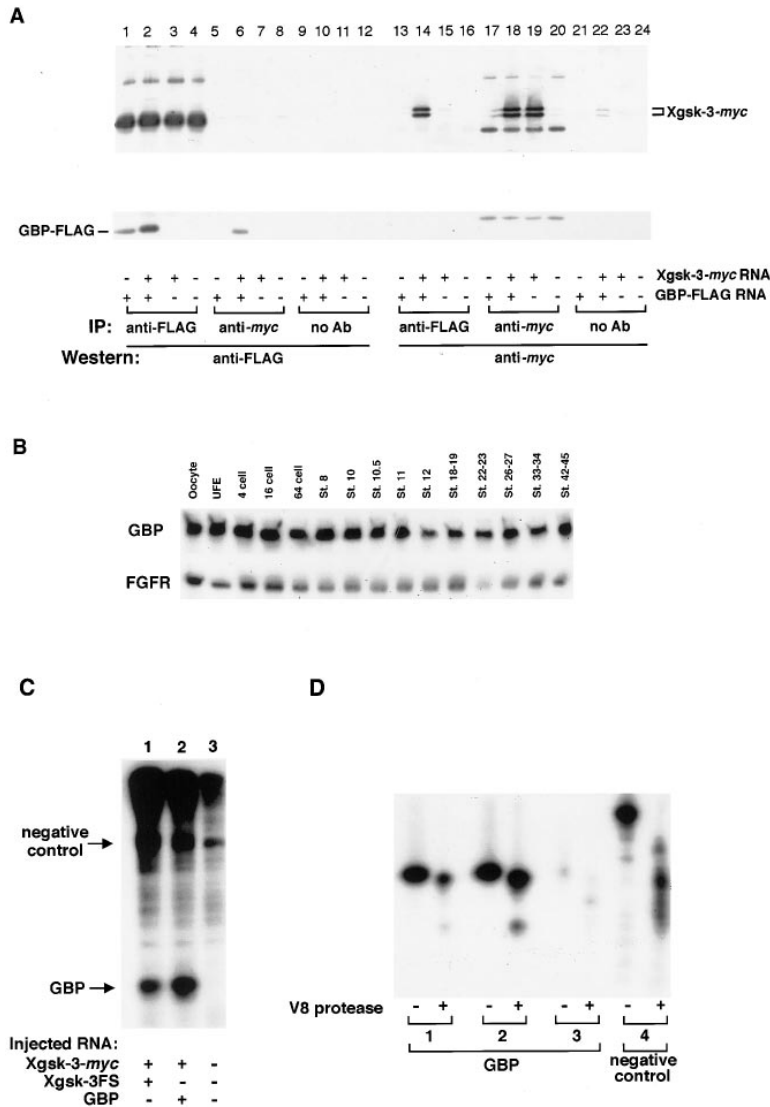


Figure 1. Expression and Binding of GBP in *Xenopus* Embryos

(A) GBP and Xgsk-3 associate in vivo. Embryos were injected in the animal pole at the two-cell stage with 1.0 ng of RNA encoding Xgsk-3-myc or GBP-FLAG, as indicated. After 4 hr, proteins were isolated by immunoprecipitation with or without antibodies to the myc or FLAG epitopes, as indicated. Xgsk-3-myc and GBP-FLAG were detected by immunoblotting with anti-FLAG (left side) and anti-myc (right side) antibodies. The positions of GBP-FLAG and Xgsk-3-myc are marked. Each lane represents ten embryos.

(B) GBP is expressed in oocytes and embryos. RNA levels were determined with the RNase protection assay using GBP or FGFR probes. FGFR is a ubiquitously expressed gene in the *Xenopus* embryo. UFE, unfertilized eggs; oocyte, mixture of stages I-VI.

(C) GBP protein is present in *Xenopus* embryos. 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 with anti-myc antibodies 2.5 hr after injection. Samples were labeled with ³²P as described in Experimental Procedures, and 20% of the samples were analyzed by SDS-PAGE. The locations of the GBP and negative control bands analyzed in (B) are indicated. Lane 1 represents 40 embryos, and lanes 2 and 3 represent 15 embryos.

(D) Eighty percent of the samples in (C) 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 were run on a 16% gel in the absence or presence of V8 protease.

human EST, we demonstrate that GBP contains a highly conserved domain required for GSK-3 binding and inhibition. These studies define a family of GSK-3-binding proteins with multiple roles in vertebrates.

Results

Cloning and Expression of a Novel GSK-3-Binding Protein

To search for a cytoplasmic regulator of Xgsk-3, we used a modification of the yeast two-hybrid system (Hollenberg et al., 1995). We produced a *Xenopus* maternal cDNA library by PCR and isolated 13 clones containing the same insert that interacted strongly with Xgsk-3. A full-length clone encoding a predicted protein of 169 amino acids that we named GBP (GSK-3-binding protein) was isolated from a *Xenopus* oocyte library.

To assess whether the interaction between Xgsk-3 and GBP observed in the two-hybrid screen also occurs in *Xenopus* embryos, RNA expression constructs were utilized that encode Xgsk-3 fused to a myc epitope tag

(GSK-3-myc) and GBP fused to a FLAG epitope tag (GBP-FLAG). RNA synthesized from these constructs was injected, separately or in combination, into the animal pole of both cells at the two-cell stage. After approximately 4 hr, the embryos were lysed and proteins were incubated with anti-myc or anti-FLAG antibodies, and the resulting immunoprecipitates were analyzed on a Western blot. As shown in Figure 1A, GBP-FLAG was immunoprecipitated indirectly by Xgsk-3 almost as efficiently as it was precipitated directly with the anti-FLAG antibody (Figure 1A, compare lane 6 to 1 or 2). Conversely, Xgsk-3-myc was precipitated indirectly by GBP as efficiently as it was precipitated by the anti-myc antibody (Figure 1A, compare lane 14 to 18 or 19). Moreover, GBP and Xgsk-3 remain associated in washes of 1 M NaCl (data not shown), reflecting the strength of this interaction. These results demonstrate that ectopically expressed Xgsk-3 and GBP bind each other efficiently in *Xenopus* embryos.

The dorsal-ventral axis in *Xenopus* is initiated prior to zygotic transcription by maternal components stored in

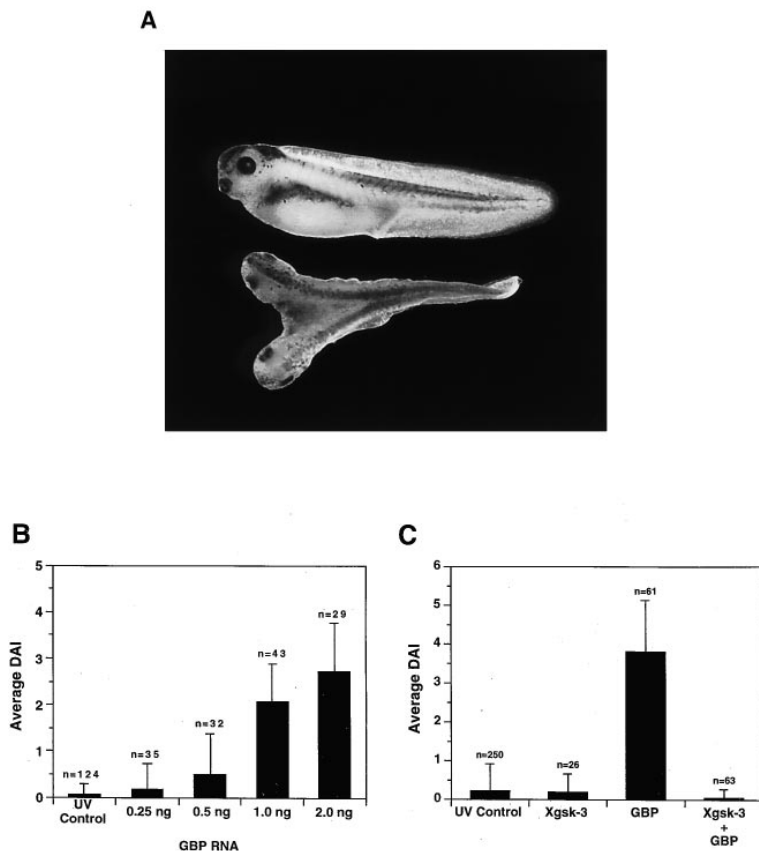


Figure 2. GBP Contains Axis-Inducing Activity and Is Blocked by Xgsk-3

(A) GBP induces ectopic axis formation in *Xenopus* embryos. Two ventral or dorsal cells were injected at the equator of 2- to 16-cell stage embryos with 0.75 ng per blastomere of GBP RNA, and embryos were allowed to develop for 3 days. Representative dorsally injected (top embryo) and ventrally injected (bottom embryo) embryos are shown.

(B) GBP rescues axis formation in UV-irradiated embryos. Fertilized eggs were UV-irradiated within 40 min after fertilization. At the 2- to 4-cell stage, one cell was injected with GBP RNA at the doses 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.

(C) Xgsk-3 blocks the axis rescuing activity of GBP. Fertilized eggs were UV-irradiated within 40 min after fertilization. At the 2- to 4-cell stage, one cell was injected at the marginal zone with 2 ng of Xgsk-3-myc RNA or 1 ng of GBP RNA, alone or in combination, as indicated, and the DAI was scored at 3 days. UV control embryos were uninjected.

the oocyte. To determine whether the GBP transcript is present maternally, as would be predicted for a gene that is involved in dorsal-ventral axis determination, RNA was isolated from *Xenopus* oocytes and early embryos and analyzed by the RNase protection assay. Using a probe derived from the 3' end of the GBP cDNA coding region, we found that GBP transcripts are expressed at fairly constant levels in the oocyte and early embryo (Figure 1B). Analysis of RNA distribution by RNase protection and in situ hybridization did not show any specific localization of GBP transcripts in the early embryo (data not shown).

In the course of our experiments, we found that when $\gamma^{32}\text{P}$ -ATP was added to anti-myc immunoprecipitates from embryos expressing ectopic Xgsk-3-myc and untagged GBP or GBP-FLAG, a protein became phosphorylated that was the expected size for GBP (Figure 1C, lane 2). When GBP was not ectopically expressed in embryos, a phosphorylated protein of the same size was coimmunoprecipitated by Xgsk-3-myc, but it was less abundant than the ectopic GBP (Figure 1C, lane 1). We hypothesized that this protein could be the endogenous GBP. To test this, we compared the partial proteolysis patterns of ectopically expressed GBP and the putative endogenous GBP using an in-gel proteolysis method (Cleveland et al., 1977). Ectopically expressed GBP and the putative endogenous GBP have identical V8 protease digestion patterns that are clearly different from the digestion pattern for a control protein (Figure 1D). This result was confirmed by digestion with chymotrypsin

(data not shown). We conclude that endogenous GBP protein is present in early embryos and that it binds to ectopically expressed Xgsk-3.

GBP Causes Axis Duplication and Rescues Dorsal Axis Formation in UV-Irradiated *Xenopus* Embryos

To explore the function of GBP, RNA encoding GBP was ectopically expressed in *Xenopus* embryos. Injection of 0.25 or 0.75 ng per blastomere of GBP RNA into two cells of the ventral marginal region (0.5 or 1.5 ng total) at the 2- to 16-cell stage resulted in the development of tadpoles with split body axes, indicating duplication of anterior dorsal structures (Figure 2A, bottom embryo). Axis duplication was typically observed in greater than 90% of the embryos injected with 1.5 ng of GBP RNA (Table 1, experiment 1). The higher dose of RNA also included more complete axis formation, as evidenced by the presence of eyes in the secondary axis. When GBP RNA was injected on the dorsal side, normal embryos developed, and axis duplication was never observed (Figure 2A, top embryo; Table 1, experiment 1).

Exposure of fertilized eggs to UV radiation prevents endogenous axis formation that can be rescued by ectopic expression of various dorsalizing factors. We tested whether ectopic expression of GBP could also rescue axis formation in UV-irradiated embryos. Fertilized eggs were irradiated with UV light and embryos were injected with 0.25 to 2 ng of GBP RNA at the 2- to 4-cell stage, and the results were quantitated by

Table 1. Axis Duplication by GBP and FRAT2

Experiment	Injections	Complete 2° Axis n (%)	Partial 2° Axis n (%)	No 2° Axis n (%)
1	Dorsal 0.5 ng GBP	0	0	27 (100)
	Dorsal 1.5 ng GBP	0	0	26 (100)
	Ventral 0.5 ng GBP	1 (3)	12 (33)	23 (64)
	Ventral 1.5 ng GBP	15 (44)	17 (50)	2 (6)
2	Uninjected	0	0	55 (100)
	Ventral Injections			
	0.25 ng C-GBP	14 (67)	6 (29)	1 (4)
	0.5 ng C-GBP	23 (85)	4 (15)	0
	1.0 ng C-GBP	31 (97)	1 (3)	0
	0.25 ng GBP	0	6 (27)	16 (73)
	0.5 ng GBP	0	22 (73)	8 (27)
	1.0 ng GBP	3 (10)	21 (70)	6 (20)
3	Uninjected	0	0	45 (100)
	Ventral Injections			
	0.5 ng FRAT2	0	8 (29)	20 (71)
	1.0 ng FRAT2	0	23 (79)	6 (21)
4	Uninjected	0	0	22 (100)
	Ventral Injections			
	2.0 ng FRAT2 & 4.0 ng Xgsk-3FS	1 (3)	23 (74)	7 (23)
	2.0 ng FRAT2 & 4.0 ng Xgsk-3	0	3 (12)	22 (88)

scoring the dorsoanterior index (DAI) of the embryos (Kao and Elinson, 1988), where 5 represents a normal embryo and 0 indicates an embryo lacking axial structures. While UV-irradiated control embryos are severely ventralized, injection of GBP RNA causes significant axis rescue (Figure 2B).

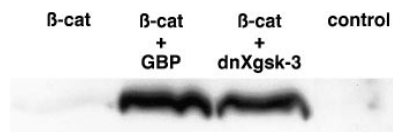
If the binding of GBP to endogenous Xgsk-3 inhibits the function of Xgsk-3, leading to the formation of an ectopic axis, it should be possible to overcome the effects of GBP by ectopically expressing additional Xgsk-3. Using the UV-rescue assay, we measured the effects of GBP on axis formation in the presence or absence of ectopic Xgsk-3 and found that coinjection of RNA encoding Xgsk-3 blocked induction of a secondary axis (Figure 2C). These results support the contention that GBP functions by binding and inhibiting endogenous Xgsk-3.

GBP Regulates the Stability of β -Catenin in *Xenopus* Embryos

Inhibition of endogenous Xgsk-3, either with a dominant-negative mutant or by lithium, results in elevated levels of β -catenin that promote the formation of a dorsal axis. To determine whether GBP functions by the same mechanism, RNA encoding myc epitope-tagged β -catenin (β -catenin-myc; Yost et al., 1996) was injected into embryos alone or in combination with GBP or dominant-negative Xgsk-3 RNA, and β -catenin-myc protein levels were measured by Western blotting with an anti-myc antibody. When β -catenin-myc was expressed alone, very low levels of the protein accumulated (Figure 3). In contrast, when β -catenin-myc was coexpressed with either GBP or dominant-negative Xgsk-3, high levels of β -catenin-myc protein were detected. This result indicates that, like dominant-negative Xgsk-3, ectopic expression of GBP stabilizes β -catenin in the embryo.

The Predicted Amino Acid Sequence of *Xenopus* GBP and Potential Family Members

We initially could not find any relatives of GBP in the nucleotide or protein databases and did not find any informative motifs within the GBP protein sequence. However, two recently entered sequences have provided important clues to the function of GBP, both in early development and normal cell growth. Molecular cloning of a proviral insertion site identified in T cell lymphomas experimentally induced by murine retroviruses (Sorensen et al., 1996; Jonkers et al., 1997) identified a protooncogene of unknown function named *Frat1* (for frequently rearranged in advanced T cell lymphomas). An ortholog of *Frat1* was found in humans (*FRAT1*; Jonkers et al., 1997). Both the mouse and human proteins contain three regions that are well-conserved within GBP (Figure 4A). We found a portion of an additional human gene, which we designate *FRAT2*, in the expressed sequence tag (EST) database. This *FRAT2* fragment contains two of the three conserved domains shared between *Frat1*/*FRAT1* and GBP (Figure 4A) and is 59% identical to *FRAT1* over the available amino acid sequence, indicating that it is a considerably diverged

Figure 3. GBP Stabilizes β -Catenin Levels

Embryos were injected in two 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 hr, β -catenin-myc levels were determined by immunoblotting of protein extracts with anti-myc antibodies.

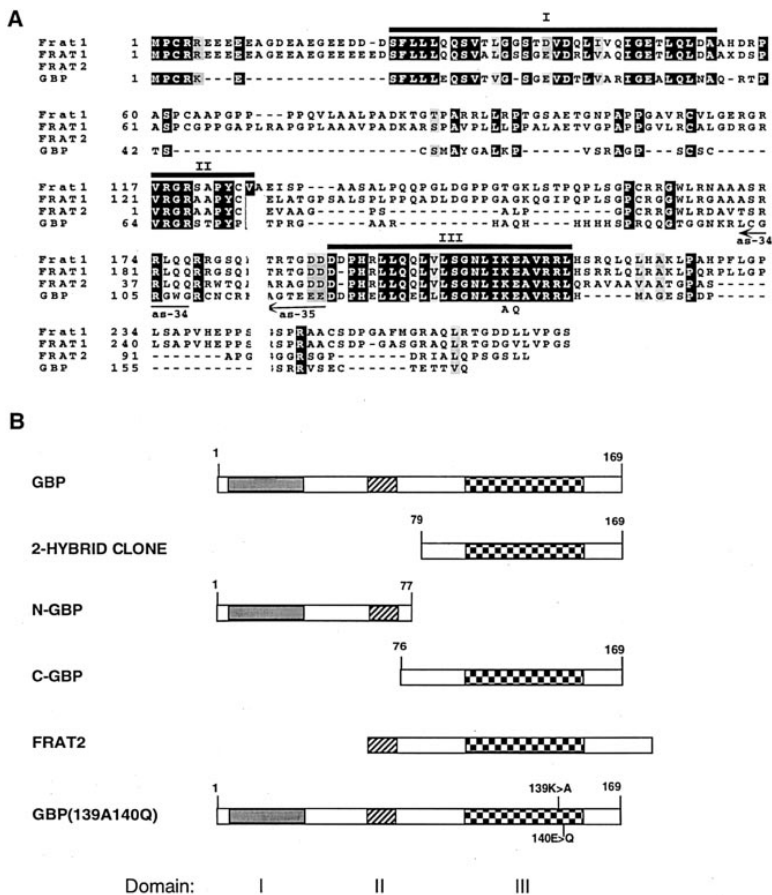


Figure 4. Amino Acid Alignment of GBP and the Frat Proteins and Injection Constructs

(A) The amino acid alignment of GBP, mouse Frat1, human FRAT1, and human FRAT2. Black boxes depict conserved amino acids, and gray boxes show similar amino acids. The three conserved domains are indicated by roman numerals. A comparison of GBP and Frat1/FRAT1 shows that domain I is 63% identical, II is 70% identical, and III is 83% identical. The amino acids changed in GBP(139A140Q) are shown below domain III. Arrows below the GBP sequence indicate the positions of the two antisense oligonucleotides used in the antisense depletion experiments.

(B) RNA injection constructs used in this study with the conserved domains indicated. The two-hybrid clone is shown for comparison.

gene. These results suggest that GBP belongs to a family of vertebrate genes.

The C-Terminal Domain of GBP Binds GSK-3 and Is Sufficient for Axis Duplication

As the clone identified from the yeast two-hybrid screen corresponds to the C-terminal half of GBP (Figure 4B), this portion of GBP is predicted to contain the GSK-3-binding domain. To determine whether the conserved C-terminal domain of GBP was sufficient for binding to GSK-3 in vivo, FLAG epitope-tagged constructs were made that encode either the carboxyl or amino half of GBP, C-GBP and N-GBP, respectively (Figure 4B). RNA synthesized from these constructs was expressed in *Xenopus* embryos along with Xgsk-3-myc. The N-terminal fragment was not stable in embryos, so we were unable to analyze it further (Figure 5, lane 2). However, Western analysis revealed that C-GBP coimmunoprecipitated GSK-3 as efficiently as the full-length GBP (Figure 5, compare lanes 1 and 3).

To test whether C-GBP also was able to inhibit Xgsk-3 function, embryos were injected ventrally with C-GBP RNA. C-GBP was a potent inducer of a secondary axis, causing a complete secondary axis in 97% of the embryos (Table 1, experiment 2). The increased activity of the C-terminal fragment relative to full-length GBP is probably due to the greater level of expression of C-GBP in *Xenopus* embryos (Figure 5, lanes 1 and 3). These

results demonstrate that the axis-inducing and Xgsk-3-binding activities of the full-length protein are contained within the C-terminal region.

Ectopic Expression of FRAT2 Causes Axis Duplication

Because the C terminus of GBP contains the GSK-3 binding and axis-duplicating activity and this region shares a conserved domain with FRAT2 (domain III, Figures 4A and 4B), we asked whether FRAT2 could also bind Xgsk-3 and promote axis duplication. Coinjection of FRAT2 fused to a FLAG epitope tag with Xgsk-3-myc demonstrated that it, like GBP, can be immunoprecipitated with antibodies to Xgsk-3-myc (data not shown). Ectopic expression of FRAT2 induced an ectopic axis (Table 1, experiment 3), and coexpression of Xgsk-3 with FRAT2 blocked its axis-inducing activity (Table 1, experiment 4). These results demonstrate GBP and the *Frat* genes constitute a family of GSK-3 inhibitory proteins.

The Axis-Inducing Activity of GBP Requires GSK-3 Binding

The experiments with C-GBP and FRAT2 demonstrate that the Xgsk-3 binding and axis-duplicating activities are contained within the C terminus of these proteins, which share domain III. To determine whether these

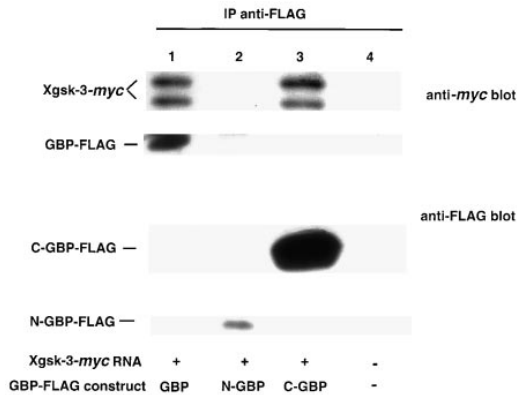


Figure 5. C-GBP Coimmunoprecipitates Xgsk-3
Embryos were injected with 2 ng of RNA encoding either GBP-FLAG (lane 1), C-GBP-FLAG (lane 2), or N-GBP-FLAG (lane 3) together with 2 ng of Xgsk-3-myc RNA. Lane 4 is from uninjected embryos. After 4 hr, proteins were isolated by immunoprecipitation with antibodies to the FLAG epitope. FLAG-containing proteins were detected by immunoblotting with anti-FLAG antibodies (bottom panel) and Xgsk-3-myc with anti-myc antibodies (top panel). The positions of Xgsk-3-myc and N-, C-, and GBP-FLAG are marked. Each lane represents 15 embryos. In this experiment, the FLAG tags were placed at the N terminus of all GBP constructs.

amino acids constitute the critical region for GBP activity, we sought to mutate GBP in this region without perturbing the overall structure of the protein. Based on structure prediction analysis of GBP, domain III forms an α helix. We replaced the amino acids at positions

139 and 140, which are solvent exposed, with two residues that are predicted to maintain the integrity of the helix and thus the overall structure of the protein (Byströff and Baker, 1998). To test the activity of this mutant, GBP(139A140Q), we compared its binding properties and axis-inducing activities to those of wild-type GBP. Western analysis showed that although both wild-type and mutant GBP proteins were produced at comparable levels in the embryo (Figure 6A; lanes 2 and 3), GBP(139A140Q) was not coimmunoprecipitated by Xgsk-3 (Figure 6A; compare lanes 5 and 6) and did not effectively precipitate Xgsk-3 (Figure 6A; compare lanes 11 and 12). In the UV axis rescue assay, 1 ng of GBP RNA caused significant axis rescue, whereas 1 or 2 ng of GBP(139A140Q) had no effect (Figure 6B). These results demonstrate that the mutation present in GBP(139A140Q) abolishes GSK-3 binding and axis-inducing activity, indicating that binding of Xgsk-3 through conserved domain III is required for the function of GBP and presumably the Frat proteins.

GBP Inhibits Xgsk-3 Phosphorylation of a Protein Substrate

Our work demonstrating that β -catenin is stabilized when the GSK-3 phosphorylation site is mutated (Yost et al., 1996) suggests that GBP stabilizes β -catenin by preventing its phosphorylation by Xgsk-3. An excellent *in vivo* GSK-3 assay has recently been developed by Klein and colleagues (Hedgepeth et al., 1997). This assay takes advantage of the observation that the microtubule-binding protein, tau, is a GSK-3 substrate (Hanger

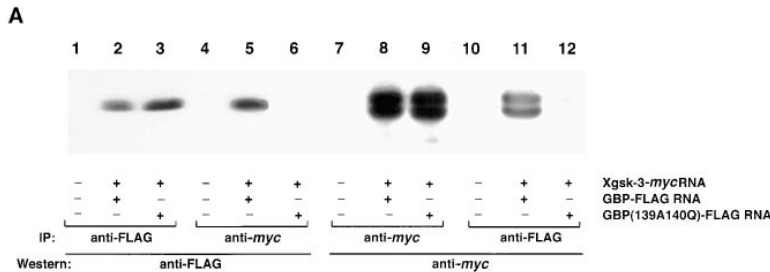
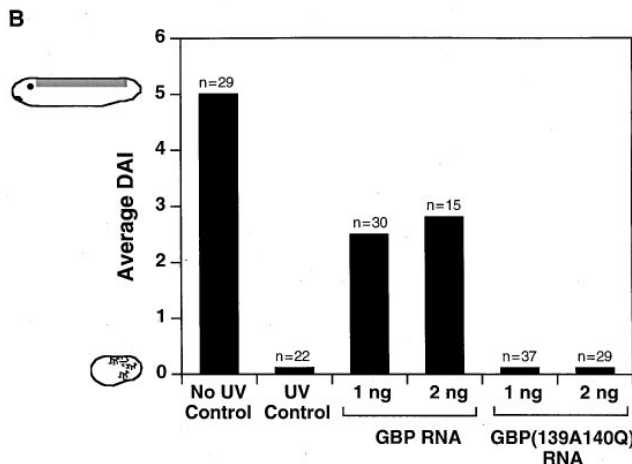


Figure 6. The Point Mutant GBP(139A140Q) Lacks Xgsk-3-Binding and Axis-Inducing Activity

(A) Embryos were injected in the marginal zone at the 2- to 8-cell stage with 2 ng of RNA encoding GBP(139A140Q)-FLAG or GBP-FLAG with or without 2 ng of Xgsk-3-myc RNA, as indicated. After 4 hr, proteins were isolated by immunoprecipitation with antibodies to the myc or FLAG epitope, as indicated, and run on a 10% polyacrylamide gel. Xgsk-3-myc and the FLAG-tagged proteins were detected by immunoblotting with anti-FLAG (left side) and anti-myc (right side) antibodies.

(B) Fertilized eggs were UV-irradiated and injected with GBP or GBP(139A140Q) RNA at the doses indicated, and the DAI was scored at 3 days. UV control embryos were uninjected.



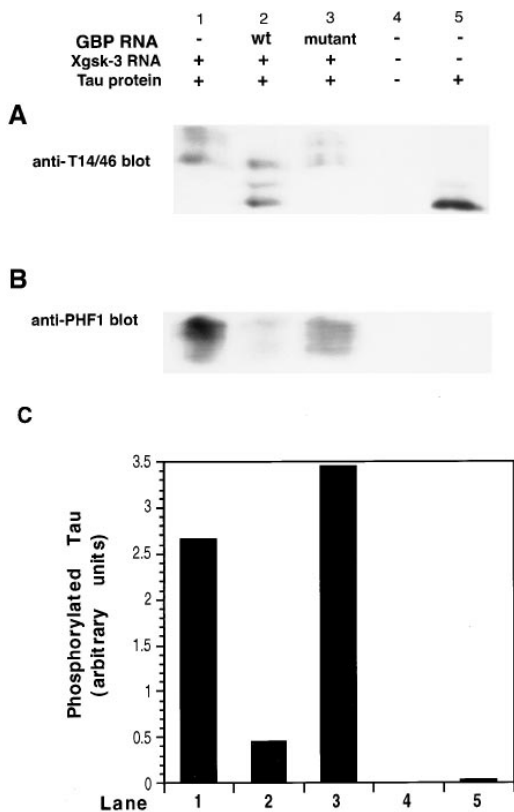


Figure 7. tau Phosphorylation by Xgsk-3 Is Blocked by GBP
Oocytes were injected with 1 ng of RNA encoding GSK-3-myc together with 10 ng of either a control RNA (Xgsk-3FS; lane 1), wild-type GBP-FLAG RNA (lane 2), or GBP(139A140Q)-FLAG RNA (lane 3). Following an overnight incubation, the oocytes were injected with 10 ng of recombinant tau protein. Two hours after tau injection, extracts from oocytes were analyzed by Western blotting. Lane 4 is from uninjected oocytes and lane 5 from oocytes injected with tau alone. (A) Total tau protein was detected using a combination of anti-T14 and T46 antibodies. (B) Anti-PHF1 antibodies were used to detect tau phosphorylated by GSK-3. (C) The samples in (A) and (B) were quantified by densitometry and the levels of phosphorylated tau protein are depicted as a fraction of the total tau in (A). In two other experiments tau phosphorylation was reduced 3- and 7-fold in the presence of wild-type GBP.

et al., 1992; Mandelkew et al., 1992), and antibodies specific for the GSK-3-phosphorylated form of tau are available (Greenburg et al., 1992; Otvos et al., 1994).

To measure Xgsk-3 activity, oocytes were injected with Xgsk-3-myc RNA together with either a control RNA (Xgsk-3FS), wild-type GBP-FLAG RNA, or RNA encoding the GBP mutant, GBP(139A140Q)-FLAG. Following an overnight incubation, the oocytes were injected with recombinant tau protein, and 2 hr later, extracts from oocytes were analyzed by Western blotting using either anti-tau antibodies T14/46 (Kosik et al., 1988) that recognize all forms of tau (Figure 7A) or an antibody specific for the GSK-3 phosphorylated form of tau (Figure 7B; Greenburg et al., 1992; Otvos et al., 1994). In the presence of Xgsk-3, tau migrated as a series of higher molecular weight forms that were absent when Xgsk-3 was not present (Figure 7A; compare lanes 1 and 5). The slower migrating forms are due to phosphorylation by

Xgsk-3, as shown with the antibody specific for GSK-3 phosphorylated tau (Figure 7B; lanes 1 and 5). Coinjection of GBP eliminated the highest molecular weight forms of tau (Figure 7A, lane 2) and reduced the level of GSK-3-specific phosphorylation 5-fold (Figure 7B, lane 2; Figure 7C), whereas the GBP mutant had no effect, either on the mobility or GSK-3-specific phosphorylation of tau (Figures 7A and 7B, lane 3; Figure 7C). These results demonstrate that GBP functions to inhibit the ability of Xgsk-3 to phosphorylate protein substrates and that this inhibition requires binding to Xgsk-3.

GBP Is Required for Dorsal-Ventral Axis Formation in *Xenopus*

To determine whether GBP is required for normal *Xenopus* development, we depleted the maternal GBP RNA using the procedure of Heasman and coworkers (1991). We tested 11 oligodeoxynucleotides that spanned the GBP coding region and found two adjacent oligonucleotides, as34 and as35 (Figure 4A), that effectively decreased the level of the GBP mRNA, with higher doses producing a stronger effect (data not shown). We found that injection of 6 ng of as34 per oocyte, which is well below the recommended maximum of 10 ng (J. Heasman, personal communication), depleted GBP mRNA (Figure 8A, compare lanes 1 and 3), whereas a control oligonucleotide had no effect (Figure 8A, lane 2).

Oocytes were injected with as34 or a control oligonucleotide and implanted into host mothers, along with uninjected oocytes, after the oocytes were matured in vitro. Both the uninjected (n = 76) and control-injected (n = 31; Figure 8B) embryos from these experiments developed normally, whereas the as34-injected oocytes were ventralized (n = 83; Figure 8B). To measure the extent of ventralization, the dorso-anterior index (DAI) was used. Whereas 90% of the uninjected and control-injected oocytes were normal (Figures 8C and 8D), as34-injected oocytes showed a full range of ventralized phenotypes (Figures 8B and 8E). To ensure the effects we observed were specific for GBP, we also examined oocytes injected with as35 and observed a similar range of phenotypes to that seen with as34 (data not shown). These results demonstrate that maternal GBP RNA is required for the normal formation of the dorsal-ventral axis in *Xenopus*.

Discussion

To account for the localized stabilization of β -catenin in early *Xenopus* embryos, we previously proposed that Xgsk-3 is inhibited within the future dorsal region during the early cleavage stages (Pierce and Kimelman, 1995; Moon and Kimelman, 1998). While GSK-3 can be inhibited by phosphorylation at a specific amino-terminal serine residue in several signaling pathways (reviewed in Yost et al., 1997), our studies with a mutant lacking this serine suggested that Xgsk-3 is regulated by an alternative mechanism in the early *Xenopus* embryo (S. B. P. and D. K., unpublished results). These results, combined with other experiments indicating that the normal Wnt signaling pathway is not used to specify the initial steps of axis formation (Hoppler et al., 1996; Sokol,

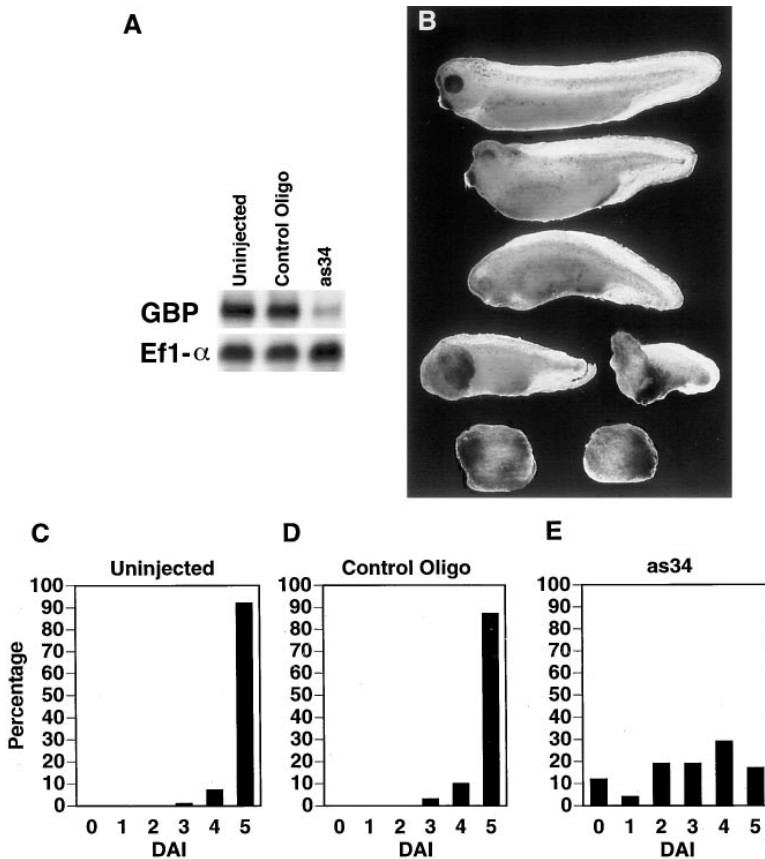


Figure 8. GBP Depletion Causes Ventralization
Stage VI oocytes were either left uninjected or injected with 6 ng of control or as34 oligonucleotides, matured in vitro, and transplanted into host mothers. To verify GBP RNA depletion, RNA from 10 oocytes was pooled and used in an RNase protection assay (A) in which GBP and Ef1- α were used as probes. (B) Representative embryos from control oligonucleotide injected (top embryo) or as34-injected (bottom six embryos) oocytes. Embryos from two experiments were assigned DAI scores, and the percentage of embryos at each DAI was graphed. (C) Uninjected oocytes, n = 76; (D) control oligonucleotide, n = 31; (E) as34, n = 83.

1996), led us to search for an alternative mechanism of GSK-3 regulation. We have identified here an essential maternal protein that binds Xgsk-3 and inhibits its function in *Xenopus* embryos. GBP is now the most upstream member of the maternal Wnt pathway that has been shown to be required for axis formation in *Xenopus*.

GBP Belongs to a Family of GSK-3-Binding Proteins

Two recently identified proteins, Frat1/FRAT1 and FRAT2 (Jonkers et al., 1997; our results), have provided important insight into the structure and function of GBP. The Frat proteins share three well-conserved regions with GBP that we predict are important for the activity and regulation of the Frats and GBP. Our studies demonstrate that region III, which is the most highly conserved domain, contains the GSK-3 binding and inhibitory activities. A C-terminal fragment of GBP, which lacks domains I and II, binds Xgsk-3 and induces an ectopic axis, and replacement of two adjacent residues in region III abrogates both Xgsk-3 binding and axis induction. Importantly, a fragment of FRAT2 containing domains II and III binds Xgsk-3 in vivo and induces an ectopic axis, demonstrating that FRAT2 and GBP share the same function, which is most likely due to the conserved amino acids in domain III. While we have not tested the activity or GSK-3 binding ability of FRAT1, which is much closer in sequence to FRAT2 than is GBP, we propose that all of these proteins form a family of GSK-3-binding proteins that interact with GSK-3 via domain III. Although

the identification of GBP from two-hybrid studies supports the model that GBP directly binds GSK-3, it is possible that this interaction is mediated by a linker protein. We are currently developing the tools to distinguish between these possibilities.

GBP Inhibits Phosphorylation by Xgsk-3

To test whether GBP functions by inhibiting the ability of Xgsk-3 to phosphorylate substrates, we used a recently developed in vivo assay that measures the GSK-3-dependent phosphorylation of tau protein in *Xenopus* oocytes (Hedgepeth et al., 1997). tau is a natural GSK-3 substrate for which antibodies have been made that specifically recognize the GSK-3-phosphorylated form. The tau assay has been used to demonstrate that lithium inhibits the kinase activity of Xgsk-3 (Hedgepeth et al., 1997), and we have shown the same results with GBP. We found on average a 5-fold reduction in Xgsk-3 activity in the presence of wild-type versus mutant GBP in multiple experiments. These observations support a mechanism in which GBP binding to Xgsk-3 prevents substrate phosphorylation.

GBP and Dorsal-Ventral Axis Establishment in *Xenopus*

With the emerging understanding of the role of the Wnt pathway in *Xenopus* development (reviewed in Moon and Kimelman, 1998), we can suggest how GBP functions in regulating axis formation. When bound to axin,

Xgsk-3 phosphorylates β -catenin (Peifer et al., 1994a; Yost et al., 1996; Ikeda et al., 1998), leading to the degradation of β -catenin by the ubiquitin-proteasome pathway (Aberle et al., 1997). We propose that GBP functions to locally inhibit Xgsk-3, leading to an elevation in β -catenin levels on the future dorsal side of the embryo, which results in the activation of *siamois* and the ultimate formation of the body axes (Moon and Kimelman, 1998).

Our finding that the *Xenopus* embryo expresses a maternal cytoplasmic GSK-3 inhibitory protein suggests a mechanism by which Xgsk-3 can be inhibited without involving the upstream members of the Wnt pathway. The early cortical rotation has been proposed to move an activator of the Wnt pathway from the vegetal pole to the dorsal region of the embryo (reviewed in Moon and Kimelman, 1998). GBP might be this factor, or it may be regulated posttranslationally by a translocated factor through conserved domains I and II. While we have shown that GBP can inhibit Xgsk-3 by ectopically expressing only domain III, the activation of the other domains through a posttranslational mechanism could greatly enhance the affinity of GBP for Xgsk-3, which may be critical at the GBP levels that are normally present in the embryo. These other domains could also be important for the regulation of the mammalian members of this family (see below). It is also possible that GBP is a novel member of the intracellular pathway through which Wnt stabilizes β -catenin and is not part of an alternate pathway. In this view, the cortical activity would regulate GBP posttranslationally, using the same mechanism as Wnt signaling.

A Role for the GBP/Frat Family in Cell Proliferation

Understanding the normal cellular functions of key regulatory proteins is critical for the study of cancer, in which growth control mechanisms are impaired or have been bypassed. Lacking the restraints present in normal cells, a number of components of signal transduction pathways have been identified as protooncogenes. *Frat1* was identified in a screen designed to identify cooperating oncogenes responsible for accelerating late-stage tumor development in mice that produce a high level of T cell lymphomas due to the presence of another oncogene (Jonkers et al., 1997). Our studies with GBP provide a basis for understanding the proliferation of lymphoma cells overexpressing *Frat1*. Because *Frat1* contains the conserved domain III, it is likely to bind and inhibit GSK-3, and we predict that it stimulates T cell lymphoma proliferation by decreasing GSK-3 function. In support of this, GSK-3 has been shown to act as a negative regulator of NF-ATc (nuclear factor activated in T cells; Beals et al., 1997), which is a direct activator of the T cell-specific cytokine IL-2 gene expression (Rooney et al., 1995). We suggest that inhibition of GSK-3 by *Frat1* in the lymphoma cells overexpressing *Frat1* causes NF-AT to induce IL-2 production, enhancing proliferation. In support of this, lithium, which inhibits GSK-3, has been shown to enhance the production of IL-2 (Kucharz et al., 1988; Wu and Yang, 1991) and cause T cell proliferation (Kucharz et al., 1988).

Experimental Procedures

Isolation of GBP cDNAs

A two-hybrid library was constructed essentially as described (Hollenberg et al., 1995) from oocyte and egg poly(A)⁺ RNA and 5×10^6 library transformants were screened with a Xgsk-3-LexA bait plasmid (MM-XGSK3; details upon request) as described (Hollenberg et al., 1995). Histidine-positive colonies were assayed for β -galactosidase activity using a filter assay (Durfee et al., 1993). Thirteen clones containing the same 274 bp insert were isolated and used to screen a *Xenopus* oocyte cDNA library (Rebagliati et al., 1985). Sequence analysis of the 6 cDNAs isolated revealed that the GBP transcript is approximately 4 kb.

RNA Expression Vectors

C-terminal myc epitope-tagged wild-type (XG134) and dominant-negative (XG137) forms of Xgsk-3 were generated using PCR and introduced between the BamHI and ClaI sites of CS2⁺MT (Turner and Weintraub, 1994). Frameshift GSK-3 (XG92) was previously described (Pierce and Kimelman, 1995). A GBP expression vector (GSKBP-1-CS) was produced by inserting the GBP coding region into CS2⁺ (Turner and Weintraub, 1994). N-terminal FLAG epitope-tagged forms of full-length GBP (BP22), the N- (BP23) and C- (BP24) terminal halves of GBP, and a C-terminal FLAG form of full-length GBP (BP20) were produced by PCR (details upon request). A mutant form of GBP with (BP139) or without a FLAG tag (BP139-CS) was constructed by oligonucleotide-mediated site-directed mutagenesis (Kunkel et al., 1987) to mutate residues 139 and 140 of GBP (139K>A and 140E>Q), using the oligonucleotide 5'-CGGGAAACCT CATTGCGCAGGCGGT CAGGA-3'. All RNA was synthesized from the CS2⁺ derived vectors, linearized with either Asp718 or NotI, using the SP6 mMESSAGE mMACHINE kit (Ambion) as described (Yost et al., 1996).

RNA Isolation and RNase Protection

Total RNA was prepared using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) and analyzed with the RNase protection assay (Melton et al., 1984). Antisense probe was generated by linearizing pBPCSK, which contains the 3' half of the GBP coding region in pBluescriptII SK⁺ (Stratagene), with BamHI and transcribing with T7 RNA polymerase. For Figure 1, duplicate RNA samples were hybridized with a *Xenopus* FGF receptor probe (Musci et al., 1990). Probes were hybridized with 12 μ g of RNA. For Figure 8, an E11- α probe was used as described (Cornell and Kimelman, 1994). For each sample, RNA was isolated from ten matured oocytes at the time when siblings were implanted into host females. One-third of the RNA was then used in a single RNase protection reaction. All samples were treated with 200 U/ml RNase T₁ (Sigma) for 1 hr at room temperature.

Embryos and Oocytes

Fertilized embryos were obtained as previously described (Newport and Kirschner, 1982). Ovaries were surgically removed from female frogs and the oocytes defolliculated manually. Oligonucleotide depletion of maternal mRNA was performed as previously described (Heasman et al., 1991). Six nanograms of either as34 (5'-CCCCATCC CCTGCCGCAC-3') or control oligo (5'-CGGCTCCCGTCCCGCAC-3') was injected into stage VI oocytes.

Immunoprecipitation, Western Blotting, and Kinase Labeling

For immunoprecipitations, embryos were lysed in Triton X-100 lysis buffer (Rubinfeld et al., 1993) without Pefabloc, and half of the clear supernatant was retained after centrifugation. Proteins were immunoprecipitated at 4°C with mouse anti-myc monoclonal (Oncogene Science) or rabbit anti-FLAG polyclonal antibody (Santa Cruz Biotechnology) and protein G-Sepharose beads (Sigma). For detection by Western blotting or kinase labeling, the beads were washed with buffer B (Rubinfeld et al., 1993) and boiled in SDS-PAGE sample buffer or labeled with ³²P using protein kinase A (Boehringer Mannheim; Zeng et al., 1996). Proteins were detected using the above primary antibodies followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Zymed), and blots were developed using enhanced chemiluminescence (Amersham).

GSK-3 tau Kinase Assay

The tau assay was performed essentially as described (Hedgepeth et al., 1997). In our experiments oocytes were manually defolliculated and injected with 10 ng of either BP20, XG92, or BP139 RNA together with 1 ng of XG134 RNA. Total and phosphorylated tau protein was detected using anti-T14/46 (a gift from V. M.-Y. Lee; Kosik et al., 1988; Hong and Lee, 1997) and anti-PHF1 antibodies (a gift from P. Davies; Greenburg et al., 1992), respectively.

In-Gel Protease Mapping

In-gel protease mapping was performed by a modification of the published procedure (Cleveland et al., 1977). Buffers were as described, with the addition of 2.5 mM DTT, and the protease dilution buffer included 0.1% bromophenol blue. Proteins in wet unfixed gel slices were digested with 1 mg *Staphylococcus aureus* V8 protease (Sigma).

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GenBank Accession Numbers

The GBP sequence is available as Genbank AF062738, and the corrected FRAT2 sequence is AF062739.