

# Glycogen Synthase Kinase-3 $\beta$ Mutagenesis Identifies a Common Binding Domain for GBP and Axin\*

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**Glycogen synthase kinase-3 $\beta$  (GSK-3) is a key downstream target of Wnt signaling and is regulated by its interactions with activating and inhibitory proteins. We and others have shown that GSK-3 activity toward non-primed substrates is regulated in part through a competition between its activating (Axin) and inhibitory (GBP/FRAT) binding partners. Here we use a reverse two-hybrid screen to identify mutations in GSK-3 that alter binding to GBP and Axin. We find that these mutations overlap and propose that GBP and Axin compete for binding to the same region of GSK-3. We use these mutations to examine the ability of GSK-3 to block eye development in *Xenopus* embryos and suggest that GSK-3 regulates eye development through a non-Wnt pathway.**

GSK-3<sup>1</sup> is a constitutively active kinase that participates in multiple signaling pathways, including growth factor, insulin, and Wnt signaling. While activation of each of these pathways results in GSK-3 inhibition, its activity is regulated by different mechanisms in the different pathways (1–4). Growth factor and insulin signaling inhibit the ability of GSK-3 to act on pre-phosphorylated (primed) substrates by phosphorylating serine 9 of GSK-3 (5–11), which then blocks the interaction of GSK-3 with the phosphate group on primed substrates (12, 13). However, the activity of GSK-3 toward non-primed substrates is not affected by these pathways. Conversely, Wnt signaling blocks GSK-3s activity toward non-primed substrates without affecting its activity toward primed substrates.

In the absence of a Wnt signal, GSK-3 is part of a multiprotein complex that includes the proteins Axin, APC, and  $\beta$ -catenin (14–25). These proteins help GSK-3 to efficiently phosphorylate the signaling molecule  $\beta$ -catenin, thus targeting it for ubiquitination and subsequent proteosomal degradation (26, 27). Axin acts as a scaffolding protein in this complex, binding both GSK-3 and  $\beta$ -catenin in a manner that brings them into close proximity, thus allowing GSK-3 to phosphorylate  $\beta$ -catenin (15, 19, 21, 28). Axin therefore acts as a GSK-3 activating protein. Another GSK-3 interacting molecule, GBP (GSK-3 binding protein), and its mammalian homologue FRAT, binds to GSK-3 and inhibits its phosphorylation of non-primed GSK-3 substrates, including  $\beta$ -catenin (29, 30). We and others

have shown that GSK-3 regulation occurs in part through a competition between its activating partner (Axin) and its inhibiting partner (GBP/FRAT) (30–32).

We wished to better understand the nature of this competition and hypothesized that GBP and Axin might compete for GSK-3 binding by sharing overlapping binding sites on GSK-3. To identify the residues of GSK-3 important for binding to each, and to develop useful reagents for analyzing the roles of GSK-3 *in vivo*, we performed a reverse two-hybrid screen in yeast. We have identified mutations that show large alterations in binding to Axin or GBP, and we show that a cluster of mutations that diminishes GSK-3 binding to Axin overlaps a cluster of mutations that alters binding to GBP. We therefore propose that GBP/FRAT and Axin can compete for binding to GSK-3 to differentially regulate its activity because they share overlapping binding sites on GSK-3. We have used our GSK-3 mutants to examine the previously reported role of GSK-3 in eye development (33) and show that the ability of GSK-3 to suppress eye development is not dependent upon its interactions with Axin or GBP. Furthermore, we show that a mutation in GSK-3 that selectively inhibits its ability to phosphorylate primed substrates has no effect on eye development. These findings suggest that GSK-3 alters eye development by targeting a primed substrate and not by affecting the  $\beta$ -catenin pathway.

## EXPERIMENTAL PROCEDURES

**Reverse Two-hybrid Screen**—We followed the general procedure of Inouye *et al.* (34). The complete *Xenopus* GSK-3 (Xgsk-3) coding region was inserted into the f1-VP16 vector (35) to produce XGV16, which puts a VP16 transcriptional activating domain on Xgsk-3. The GSK-3-binding region of mouse Axin was inserted into MA424 (36) to produce Axin-Gal4, which puts a Gal4 DNA-binding region on Axin. The complete GBP coding region was inserted into BTM116 (37) to produce BP-lexA, which puts a lexA DNA-binding region on GBP. Xgsk-3 was mutagenized by PCR amplification following a published procedure (38) using one primer in the *LEU2* gene and one primer after the transcriptional stop sequence. The amplified product was combined with an *AflIII*-*Bam*HI fragment of XGV16, which lacks the GSK-3 coding region and part of the *LEU2* gene. This was transfected into strain YCJ4, which has Gal4-binding sites in front of *URA3* and LexA-binding sites in front of *LacZ* (39). Approximately 12,000 transfectants were plated and colonies were selected that were either URA+ or, using 5-fluorouracil, that were URA-. These colonies were then screened using a filter assay for  $\beta$ -galactosidase activity, to find white, URA+ colonies and blue, URA- colonies. The mutant XGV16 plasmids were isolated from these colonies and retransfected into YCJ4 and retested for growth on URA and  $\beta$ -galactosidase activity.

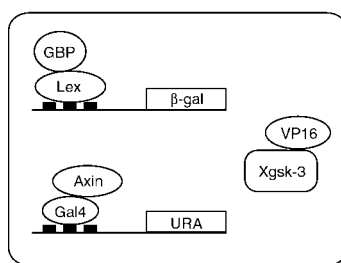
**RNA Expression Constructs**—To create an amino-terminal Myc epitope-tagged *Xenopus* GSK-3 (XG220), the coding region of wild-type *Xgsk-3* was inserted into the *Bgl*II site of CS3MT (D. Turner, University of Michigan). For analysis in *Xenopus* embryos, each of the GSK-3 mutants was inserted into CS3MT. The GSK-3 single mutants Xgsk-CS8-N, Xgsk-CS8-C, Xgsk-CS116-N, Xgsk-CS116-C, and Xgsk-R96A were created in XG220 using the QuikChange site-directed mutagenesis system (Stratagene). Mutations were confirmed by DNA sequencing.

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<sup>1</sup> The abbreviations used are: GSK-3, glycogen synthase kinase-3 $\beta$ ; CREB, cAMP response-element binding protein.

FIG. 1. **Diagram of the reverse two-hybrid screen.** The yeast strain used for the selection is shown on the left side and the predicted Xgsk-3 mutants are shown on the right. Whereas wild-type Xgsk-3 will produce colonies that are blue and URA<sup>+</sup>, Xgsk-3 mutants that lack either Axin or GBP binding will lose one of these markers. Mutants with premature stop codons or mutants that are degraded or misfolded would be white and URA<sup>-</sup>, and these were not picked in the screen.



	wild-type Xgsk-3	Axin mutants	GBP mutants
Colonies:	blue, URA <sup>+</sup>	blue, URA <sup>-</sup>	white, URA <sup>+</sup>
Xgsk-3 binds:	Axin, GBP	GBP only	Axin only

GBP-HA (GBPCS2+HA) (31) and Xaxin-HA (Xaxin/CS2HA) (20) were described previously.

**Embryos and Microinjections**—Embryos were obtained as previously described (40) and were microinjected (41) with RNA synthesized from CS2<sup>+</sup>-derived constructs (42) linearized with *NotI* using the mMessage mMachine kit (Ambion) according to the manufacturer's directions.

**RNA Injections and Immunoprecipitations**—For eye development experiments, each of the two dorsal blastomeres of 4-cell stage embryos were injected at the marginal zone with 2 ng of RNA encoding wild-type or mutant GSK-3s. Embryos were allowed to develop to tadpole stages before scoring eye development. For immunoprecipitation experiments, 1–2 ng of each RNA, alone or in combination, was injected into the animal pole of 4-cell stage embryos in a single 10-nl injection. Immunoprecipitations and Western blotting were performed as previously described (29). Anti-Myc and anti-HA monoclonal antibodies were purchased from Covance.

**Kinase Assays**—Injections and immunoprecipitations were performed as previously described (29). The CREB peptide kinase assay was performed as previously described (31) and <sup>32</sup>P incorporation was measured by liquid scintillation. All values were normalized to the amount of GSK-3 protein expressed in each sample, as determined by Western blot, and then scaled to set wild-type GSK-3 activity at 100% in each trial. Activity in uninjected samples was always less than 0.5%. Assays were performed in duplicate. Tau phosphorylation assays were performed in the same manner, using 10 μM Tau protein, purchased from Calbiochem. Reactions were stopped after 20 min by the addition of SDS sample buffer, boiled, and separated by SDS-polyacrylamide gel electrophoresis. Tau phosphorylation was quantitated using the Storm Imaging system (Molecular Dynamics). Levels of Tau phosphorylation were normalized to the amount of GSK-3 protein expressed in each sample. Wild-type GSK-3 activity was set at 100% in each trial.

## RESULTS

**GBP- and Axin-binding Sites Overlap**—To identify the GBP and Axin-binding sites on GSK-3 we performed a yeast “reverse two-hybrid” screen, as shown in Fig. 1, that allowed us to select GSK-3 mutants that bound either GBP or Axin, but not both. The advantage of this type of screen is that it allowed us to avoid GSK-3 mutants that were simply unstable or misfolded, since one positive binding selection was always present. From this screen, eight mutants that lack GBP binding (Fig. 2A) and seven mutants that lack Axin binding (Fig. 2B), as indicated by the yeast assay, were chosen for further analysis.

DNA sequence analysis revealed that while many of the GSK-3 mutants contain more than one amino acid change, each contains at least one mutation that clusters in a region in the carboxyl terminus (Fig. 2). Strikingly, the cluster of residues that appear to be important for GBP binding overlaps the region containing the cluster of residues important for Axin binding, suggesting that GBP and Axin share overlapping binding sites on GSK-3. The binding domains defined by the mutational clusters also overlap in the carboxyl-terminal lobe when mapped onto the three-dimensional structure of GSK-3 (Fig. 3, A–C).

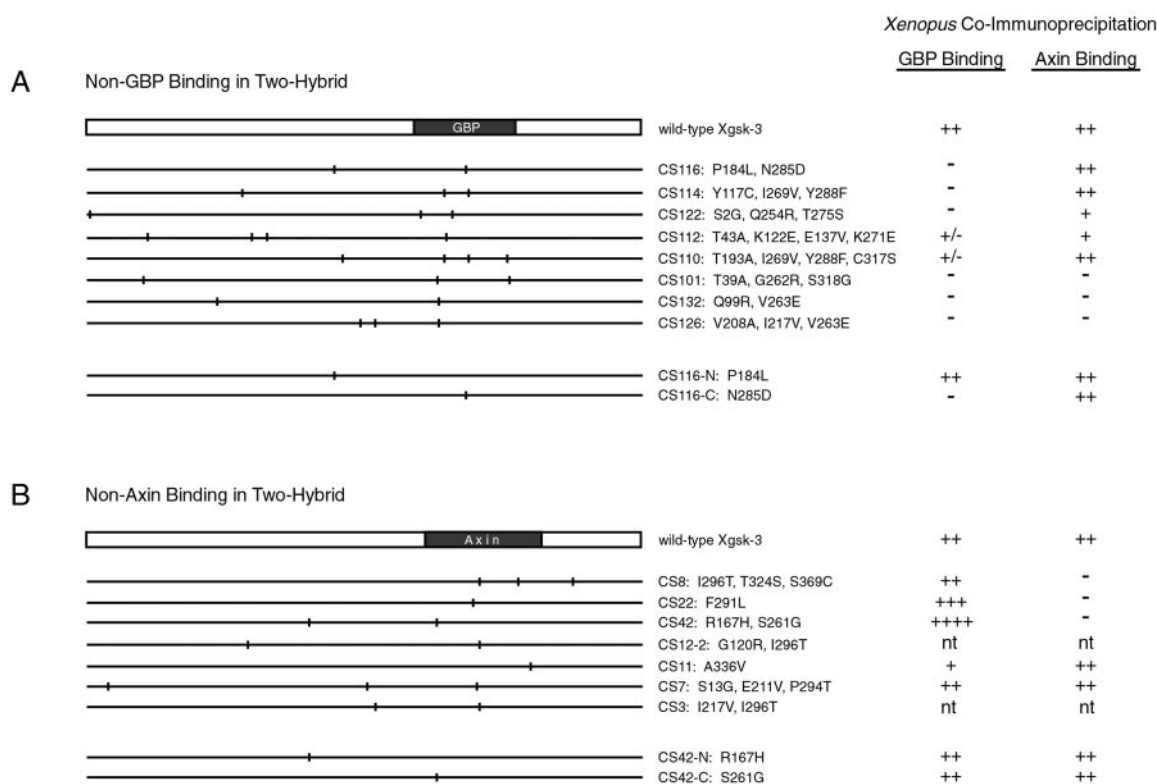
Each of the GSK-3 mutants was also tested for its ability to bind Axin and GBP when injected into *Xenopus* embryos. mRNA encoding Myc-tagged wild-type or one of the mutant GSK-3s was co-injected into *Xenopus* embryos with mRNA encoding Xaxin-HA and GBP-HA. Proteins were immunoprecipitated with anti-Myc antibodies and analyzed by Western blot using anti-Myc and anti-HA antibodies. The binding re-

sults are summarized in Fig. 2. In this assay some of the “non-Axin binding” mutants identified in yeast bound to Axin (Fig. 2B), perhaps because only the GSK-3-binding region of Axin was used in the two-hybrid screen, whereas full-length Axin was tested in the *Xenopus* co-immunoprecipitation assay. Full-length Axin may provide additional contacts that help to stabilize its interaction with GSK-3. Surprisingly, some of the mutants that bind Axin, but not GBP, in yeast do not interact with either in *Xenopus*, suggesting a different stringency of binding between the yeast and *Xenopus* assays (Fig. 2). In the yeast assay, a GSK-3 mutant that has decreased binding to Axin or GBP can presumably still activate expression of the reporter gene, although the interaction is not strong enough in *Xenopus* embryos to be seen by co-immunoprecipitation. We also noticed that GBP levels were sometimes lower in the presence of GSK-3 mutants that cannot bind GBP, suggesting that GBP may be stabilized by binding to GSK-3 (data not shown).

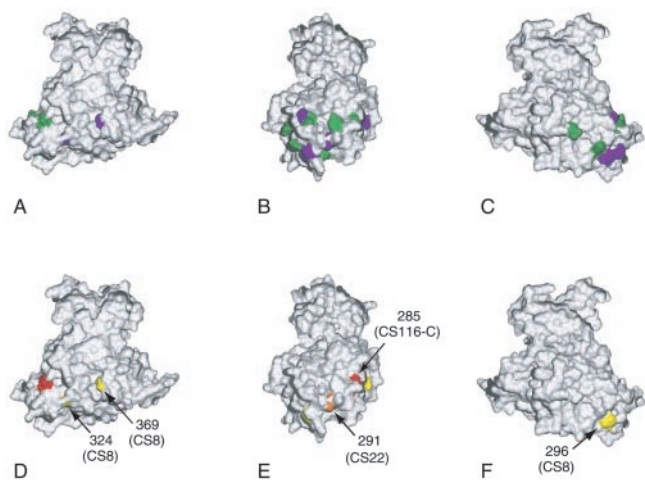
From this analysis we identified three interesting mutants that showed strong differential binding effects in both yeast and *Xenopus*. These results are summarized in Fig. 2 and the *Xenopus* binding data is shown in Fig. 4. As predicted from the yeast assay, Xgsk-CS116 does not bind GBP but binds Axin at wild-type levels, and Xgsk-CS8 binds GBP but not Axin. While Xgsk-CS22 has lost most Axin binding, it has acquired the novel feature of being a GBP “super-binder.” The single point mutation in Xgsk-CS22 and the carboxyl-terminal mutations in Xgsk-CS116 and Xgsk-CS8 are in very close proximity on the GSK-3 structure (Fig. 3, D–F).

Since only one of the two changes in Xgsk-CS116 and Xgsk-CS42, a GBP super-binder that does not bind Axin, lies in the carboxyl-terminal mutation cluster (Fig. 2), we wished to determine whether either mutation alone was sufficient to affect GBP or Axin binding. While the amino-terminal mutation in Xgsk-CS116 (Xgsk-CS116-N) did not disrupt binding to GBP-HA, the carboxyl-terminal mutation, which changes residue 285 from asparagine to aspartate (Xgsk-CS116-C), was sufficient to disrupt binding (Fig. 2A). In the case of Xgsk-CS42, however, neither single mutation was sufficient to disrupt binding to Xaxin-HA or to enhance binding to GBP-HA (Fig. 2B).

**GBP and Axin Binding Mutants Retain Kinase Activity**—To determine whether the GSK-3 mutations affected GSK-3 catalytic activity as well as binding to GBP and Axin, mutants Xgsk-CS116, Xgsk-CS8, and Xgsk-CS22 were compared with wild-type GSK-3 for their ability to phosphorylate two different substrates. GSK-3s activity toward primed substrates (pre-phosphorylated at the +4 position) is not regulated by Wnt signaling components (1, 12, 13, 30, 31, 43). We therefore compared the ability of Xgsk-CS116, Xgsk-CS8, Xgsk-CS22, and wild-type GSK-3 to phosphorylate P-CREB, a primed peptide substrate. While the ability of Xgsk-CS8 and Xgsk-22 to phosphorylate P-CREB was near wild-type, Xgsk-CS116 consistently had somewhat lower activity (Fig. 5A). Statistical analysis using the Student's *t* test revealed that only Xgsk-CS116 had a statistically significant decrease in catalytic activity toward P-CREB ( $p \leq 0.05$ ).

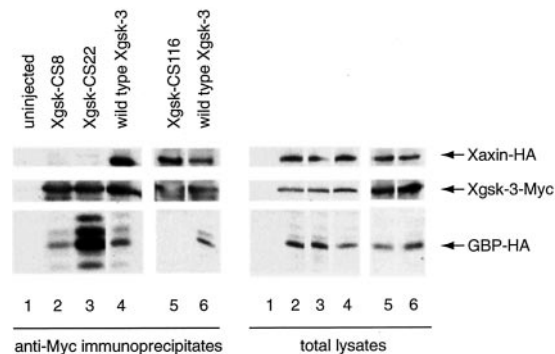


**FIG. 2. Diagram of GSK-3 mutants.** GSK-3 mutants that did not bind GBP (A) or Axin (B), in the yeast reverse two-hybrid screen are shown. Each mutant contains at least one mutation in a region in the carboxyl terminus, indicated by the shaded boxes labeled *GBP* and *Axin*. mRNA for each mutant was also injected into *Xenopus* to test its binding to GBP and Axin by co-immunoprecipitation and Western blot analysis. Levels of binding in *Xenopus* are shown on the right (nt, not tested). The mutants shown in the top part of each panel are those isolated from the yeast screen. The mutants in the bottom part of each panel were constructed.



**FIG. 3. GSK-3 mutations on the three-dimensional surface of GSK-3.** A–C, all amino acid changes within the carboxyl-terminal GBP and Axin boxes shown in Fig. 2 were mapped onto the surface of GSK-3 and seem to cluster and overlap on the carboxyl-terminal lobe. Green indicates residues that disrupt binding to GBP. Purple indicates residues that disrupt binding to Axin. Front view (B) is looking toward the catalytic cleft. Side views are rotated 90° from the front. A, left side view. C, right side view. D–F, specific amino acid changes. Yellow, residues 296, 324, and 369 (in Xgsk-CS8); orange, residue 291 (in Xgsk-CS22); and red, residue 285 (in Xgsk-CS116-C). D, left side view. E, front view. F, right side view.

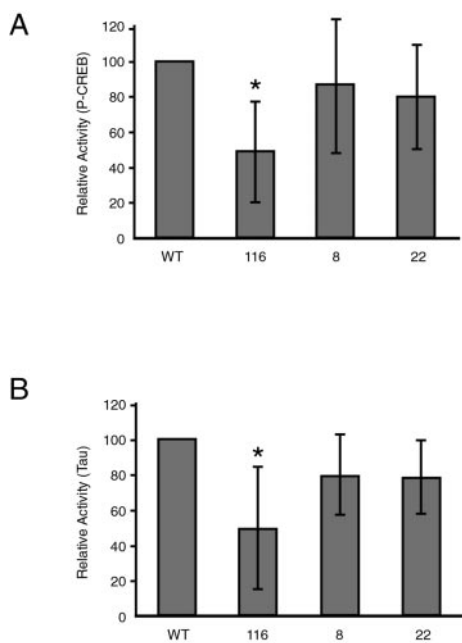
Tau protein is a GSK-3 substrate that does not require pre-phosphorylation. GBP and the GSK-3-binding region of FRAT (FRATtide) can inhibit GSK-3 phosphorylation of Tau (29, 30). We therefore compared the ability the GSK-3 mutants to phosphorylate Tau protein. Wild-type GSK-3, Xgsk-CS8, and Xgsk-CS22 all efficiently phosphorylate Tau protein (Fig. 5B).



**FIG. 4. GSK-3 mutants that alter binding to GBP or Axin.** A, mRNA encoding Myc-tagged wild-type or mutant GSK-3 was co-injected into *Xenopus* embryos along with *Xaxin-HA* and *GBP-HA* mRNA. Protein complexes were immunoprecipitated with anti-Myc antibodies, separated by polyacrylamide gel electrophoresis, and detected by Western blot using anti-Myc and anti-HA antibodies. Xgsk-CS116 binds Axin but not GBP (lane 5), Xgsk-CS8 binds GBP but not Axin (lane 2), and Xgsk-CS22 does not bind Axin but binds GBP at elevated levels (lane 3).

As was observed in the P-CREB assay, the activity of Xgsk-CS116 on Tau is also reduced, although quite variable. In three out of four experiments, the activity was less than 40% of wild type. Statistical analysis revealed that only Xgsk-CS116 had a statistically significant decrease in activity toward Tau ( $p \leq 0.05$ ). Together, these results demonstrate that the mutants Xgsk-CS8 and Xgsk-CS22 retain relatively normal levels of GSK-3 catalytic activity on both primed and non-primed substrates, while Xgsk-CS116 has a reduction in overall activity compared with wild-type GSK-3.

*Wild-type GSK-3 and the Axin/GBP Binding Mutants Affect Eye Development*—Ectopic dorsal expression of GSK-3 causes



**FIG. 5. GSK-3 kinase activity.** A, kinase activity on P-CREB. mRNA encoding wild-type or mutant *GSK-3* was injected into *Xenopus* embryos. Proteins were immunoprecipitated with anti-Myc antibodies and used to phosphorylate P-CREB or CREB using [ $\gamma$ - $^{32}$ P]ATP.  $^{32}$ P incorporation was normalized to the amount of protein expressed in each sample. Wild-type GSK-3 activity ( $^{32}$ P incorporation of P-CREB/CREB) was set at 100% in each trial and the activity of each mutant is expressed relative to wild-type GSK-3. The average of three independent experiments, each performed in duplicate, is shown. \*, statistically significant decrease in activity,  $p \leq 0.05$ . B, kinase activity on Tau.  $^{32}$ P incorporation on Tau protein was measured using the Storm Imaging system (Molecular Dynamics). Levels of Tau phosphorylation were normalized to the amount of GSK-3 protein expressed in each sample. Wild-type GSK-3 activity was set at 100% in each trial and the activity of each mutant is expressed relative to wild-type GSK-3. The average of four independent experiments is shown. \*, statistically significant decrease in activity,  $p \leq 0.05$ .

the reduction or loss of eye structures in *Xenopus* embryos (33). This loss is similar to that seen by dorsal overexpression of a dominant-negative *Xenopus* Frizzled-3, whereas ectopic Frizzled-3 induces the development of ectopic eyes (44). Furthermore, zebrafish homozygous for a mutation in *axin* develop with reduced or missing eyes (45, 46). These results suggested a role for Wnt signaling in eye development. The mutations described here allowed us to test whether the effects of GSK-3 overexpression were due to alterations in the Wnt pathway. If this were the case, we would expect that mutations in GSK-3 that lack either Axin or GBP binding would show an altered effect on eye development.

We therefore injected mRNA encoding the GSK-3 mutants into *Xenopus* embryos to analyze their effect *in vivo*. As previously described (33), dorsal expression of GSK-3 resulted in a high percentage of embryos with missing or reduced eyes (Fig. 6, A and C–F). While the effects of Xgsk-CS8 and Xgsk-CS22 were similar to wild-type GSK-3, the effects of Xgsk-CS116 were not as strong (Fig. 6A). Our results suggested that the effects of GSK-3 on eye development were not due to effects on the Wnt pathway since all of the mutants tested perturbed eye development. However, while the somewhat reduced effects of Xgsk-CS116 could be explained by its reduced kinase activity instead of its inability to bind GBP (Fig. 5), we wanted more direct evidence that the effects of GSK-3 were on a non-Wnt pathway.

Mutation of residue 96 from arginine to alanine was previously reported to selectively inhibit human GSK-3 phosphorylation of primed substrates, but not non-primed substrates such

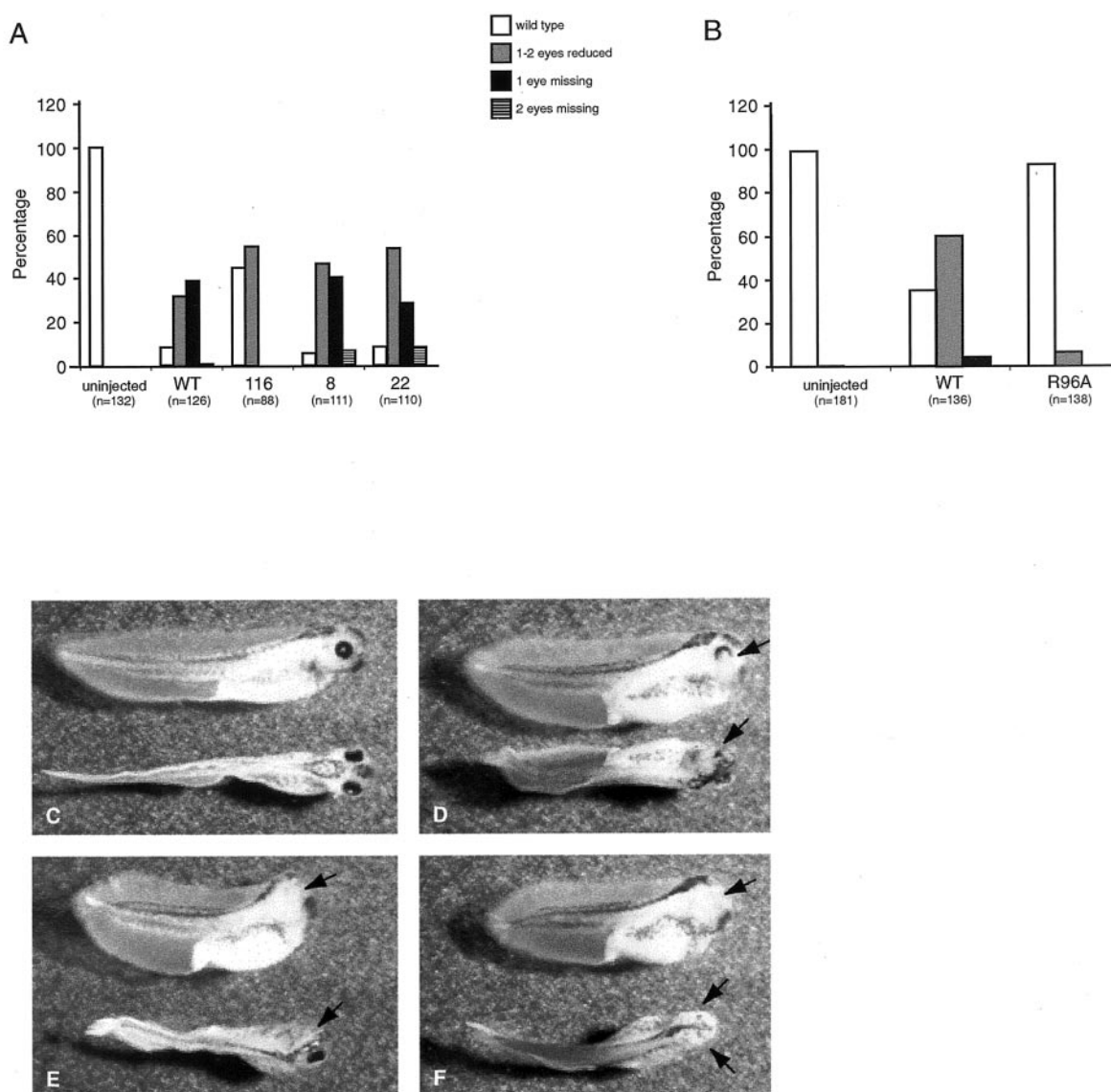
as  $\beta$ -catenin (12). We made the same mutation in *Xenopus* GSK-3 (Xgsk-R96A) and found that it did not efficiently phosphorylate primed substrates (data not shown) as previously shown for human GSK-3 (12). As was shown for other non-primed substrates with this mutation in human GSK-3 (12), Xgsk-R96A phosphorylated Tau protein more efficiently than wild-type GSK-3, and we found that it efficiently bound both GBP and Axin (data not shown). When injected dorsally, Xgsk-R96A had almost no effect on eye development (Fig. 6B). Since this GSK-3 mutant is unable to phosphorylate primed substrates and has lost the ability to block eye development, combined with the data from our other GSK-3 mutants, our results suggest that the ability of GSK-3 to affect eye formation involves regulation of a non-Wnt pathway.

#### DISCUSSION

An important mode of GSK-3 regulation occurs through its interactions with multiple binding partners. GBP/FRAT can compete with Axin for binding to GSK-3, resulting in GSK-3 inhibition (29, 30, 32). It has not been clear how the competition between GBP/FRAT and Axin occurs. We have identified mutations in GSK-3 that suggest GBP and Axin share overlapping binding sites on the carboxyl-terminal lobe of GSK-3 (Figs. 2 and 3). However, it is likely that multiple residues are involved in the association of GSK-3 with each, as we identified several different residues that affect GBP and Axin binding.

Most of the GSK-3 mutants identified in our screen also contain at least one additional mutation amino-terminal to the overlapping mutational clusters. While neither of the Xgsk-CS42 mutations was sufficient to disrupt binding to Axin when tested alone, the single change in Xgsk-CS22 prevented Axin binding, suggesting that different residues contribute differently to Axin binding. Interestingly, the single change in Xgsk-CS22 not only abolished the GSK-3-Axin interaction, but it greatly enhanced the ability of GSK-3 to bind GBP. Since a single amino acid change differentially affects GSK-3s interactions with both GBP and Axin, it strongly suggests that the two binding sites on GSK-3 are intimately associated. In addition, it demonstrates that the binding of GBP to GSK-3 has not evolved to be maximally strong, most likely to allow highly dynamic interactions between GSK-3 and its binding partners.

**Comparison with Other GSK-3 Mutations**—While this article was in preparation, Fraser *et al.* (47) reported that FRATtide and the GSK-3-binding region of Axin (Axin-GID) can both disrupt GSK-3 dimers, supporting the hypothesis that GBP/FRAT and Axin share overlapping binding sites on GSK-3. Additionally, using surface scanning mutagenesis of GSK-3, they identify five mutants that have differential effects on Axin-GID and FRATtide binding. These results nicely complement ours, although there are some interesting differences. While Fraser *et al.* (47) found four mutants that do not bind Axin-GID, all of them also have reduced FRATtide binding (20–80% of wild-type binding). Perhaps this is due to the fact that Fraser *et al.* (47) did not use full-length proteins as were used here. The Axin-GID corresponds to 59 amino acids of Axin, whereas FRATtide corresponds to only 38 amino acids in the carboxyl-terminal half of FRAT. They also identified one mutant that reduced FRATtide binding 3-fold, but did not find a GSK-3 mutant that selectively eliminated FRATtide binding. Additionally, whereas we found that a single mutation at residue 285 (N285D) is sufficient to eliminate binding to GBP without affecting Axin binding, Fraser *et al.* (47) found that a similar change (N285E) reduced, but did not eliminate, binding to both FRATtide and Axin-GID. The different results could be due to species differences (human *versus Xenopus* GSK-3), or because the bulkier side chain of glutamic acid in the Fraser *et al.* (47) mutant allows it to affect binding to both FRATtide and



**FIG. 6. Alteration of eye development by wild-type GSK-3 and GSK-3 mutants.** A, mRNA encoding wild-type or mutant GSK-3 was injected medially into the two dorsal blastomeres of 4-cell stage embryos. Eye development was scored at tadpole stages. The results shown are from two independent experiments.  $n$  = the total number of embryos injected with each mRNA. Wild-type GSK-3, Xgsk-CS8, and Xgsk-CS22 all block eye development to a similar degree. The effect of Xgsk-CS116 is less potent. B, the GSK-3 mutant Xgsk-R96A does not affect eye development. The results shown are from three independent experiments. C–F, lateral and dorsal views of (C) uninjected tadpoles and embryos injected with mRNA encoding wild-type GSK-3 showing representative tadpoles with reduced eyes (D), one eye missing (E), or both eyes missing (F).

Axin-GID, whereas the aspartate in our mutant interferes only with GBP binding.

Fraser *et al.* (47) report a mutant that does not bind Axin-GID (V267G/E268R), but still binds FRATtide (80% of wild-type). While our screen did not identify these residues, they clearly lie within our proposed Axin-binding domain, and are near to residue 261, which we find to be important for Axin binding (in combination with residue 167 in Xgsk-CS42). We also found a unique single mutation (F291L in Xgsk-CS22) that not only eliminates binding to Axin, but also greatly enhances GBP binding, thus affecting both interactions in an opposite manner. This residue lies between two mutations that Fraser *et al.* (47) identify as decreasing binding to both Axin-GID and FRATtide (Y288R and F293Q).

Bax *et al.* (48) have also just reported the structure of GSK-3 bound to FRATtide (residues 188–226 of FRAT1). Consistent with our mutagenesis studies using GSK-3 and GBP, they show that FRATtide binds to the carboxyl-terminal lobe of GSK-3. Significantly, they show that the side chains of Tyr<sup>288</sup> and Glu<sup>290</sup> of GSK-3 make important contacts with Lys<sup>214</sup> of

FRATtide. We previously identified the same lysine in GBP as being necessary for GBP/GSK-3 binding (29). Both of our GSK-3 mutants that affect GBP binding contain changes very near to these important residues: N285D in Xgsk-CS116C and F291L in Xgsk-CS22. It is likely that our changes affect the conformation at nearby residues (288/290) that are required for interaction with GBP/FRATtide. Interestingly, Bax *et al.* (48) also show that Ser<sup>261</sup> makes contacts with FRATtide, while we find that a mutation at this residue, in combination with Arg<sup>167</sup> in Xgsk-CS42, eliminates Axin binding and enhances GBP binding.

The reverse two-hybrid screen described here provides a good compliment to the structure based mutations of Fraser *et al.* (47), identifying a mutation that blocks GBP binding, which was not found from the structure based approach, and identifying a unique mutation (F291L) that has opposite effects on GBP and Axin binding. Due to the importance of GSK-3 in many biological processes, it will be valuable to assemble a collection of different GSK-3 mutants for *in vivo* analysis.

**GSK-3 and Eye Development**—We utilized the observation

that dorsal overexpression of GSK-3 causes alterations in eye development as the first *in vivo* test of the GSK-3 mutants. Because of the early role for GSK-3 in patterning the dorsal-ventral axis in *Xenopus* embryos (49–51), dorsal overexpression of wild-type GSK-3 might have been expected to produce ventralized embryos. However, this was not observed. While overexpression of Axin can cause effects on the dorsal-ventral axis, GSK-3, unlike Axin, does not appear to be present in limiting amounts (52). The mutant GSK-3s also did not affect the dorsal-ventral axis, likely because the injections cannot be done early enough to disrupt the endogenous Axin-GSK-3 protein complexes and thus affect the embryonic axis.

All three mutants tested altered eye development, as did wild-type GSK-3. The non-GBP binding mutant, Xgsk-CS116, was less effective than wild-type GSK-3, which might be due to its decreased catalytic activity. To test the hypothesis that the effects of GSK-3 were not on the Wnt pathway, but were due to its effects on primed substrates, we created the mutant Xgsk-R96A, which is defective only in phosphorylating primed substrates. Xgsk-R96A, like human GSK-3(R96A) (12), phosphorylates primed substrates at only 20% of wild-type levels. As shown previously for human GSK-3(R96A) phosphorylation of Axin and  $\beta$ -catenin (12), we found that Xgsk-R96A phosphorylates the non-primed substrate Tau more efficiently than wild-type GSK-3. Moreover, Xgsk-R96A retained binding to both Axin and GBP, but did not have a major effect on eye development. Together with our analysis of the GSK-3 mutants that do not bind Axin or GBP, our results indicate that the effects of GSK-3 on the eyes occurs through a non-Wnt pathway. Given the large repertoire of transcription factors now implicated in eye development (53, 54), it will be very interesting to determine whether any might be direct targets for GSK-3 phosphorylation and regulation.

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**Addendum**—In the online “Papers in Press” version of this manuscript, the amino acid changes in Xgsk-CS8 and Xgsk-CS42 were inverted. We apologize for any inconvenience caused by this error.

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