

# Dorsal downregulation of GSK3 $\beta$ by a non-Wnt-like mechanism is an early molecular consequence of cortical rotation in early *Xenopus* embryos

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## SUMMARY

Cortical rotation and concomitant dorsal translocation of cytoplasmic determinants are the earliest events known to be necessary for dorsoventral patterning in *Xenopus* embryos. The earliest known molecular target is  $\beta$ -catenin, which is essential for dorsal development and becomes dorsally enriched shortly after cortical rotation. In mammalian cells cytoplasmic accumulation of  $\beta$ -catenin follows reduction of the specific activity of glycogen synthase kinase 3-beta (GSK3 $\beta$ ). In *Xenopus* embryos, exogenous GSK3 $\beta$  suppresses dorsal development as predicted and GSK3 $\beta$  dominant negative (kinase dead) mutants cause ectopic axis formation. However, endogenous GSK3 $\beta$  regulation is poorly characterized. Here we demonstrate two modes of GSK3 $\beta$  regulation in *Xenopus*. Endogenous mechanisms cause depletion of GSK3 $\beta$  protein on the dorsal side of the embryo. The

timing, location and magnitude of the depletion correspond to those of endogenous  $\beta$ -catenin accumulation. UV and D<sub>2</sub>O treatments that abolish and enhance dorsal character of the embryo, respectively, correspondingly abolish and enhance GSK3 $\beta$  depletion. A candidate regulator of GSK3 $\beta$ , GSK3-binding protein (GBP), known to be essential for axis formation, also induces depletion of GSK3 $\beta$ . Depletion of GSK3 $\beta$  is a previously undescribed mode of regulation of this signal transducer. The other mode of regulation is observed in response to Wnt and dishevelled expression. Neither Wnt nor dishevelled causes depletion but instead they reduce GSK3 $\beta$ -specific activity. Thus, Wnt/Dsh and GBP appear to effect two biochemically distinct modes of GSK3 $\beta$  regulation.

Key words: GSK3 $\beta$ , Wnt, GBP,  $\beta$ -catenin, *Xenopus*, Dorsal axis

## INTRODUCTION

Glycogen synthase kinase 3-beta (GSK3 $\beta$ ) is an intracellular serine-threonine kinase implicated in vertebrate axis formation. Overexpressing GSK3 $\beta$  on the dorsal side of *Xenopus* embryos causes suppression of axis formation while overexpressing a dominant negative mutant GSK3 $\beta$  is sufficient to cause formation of a secondary, ectopic axis (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995). A causal role for GSK3 $\beta$  activity modulation in *Xenopus* axis formation is also implied by its ability to regulate the stability of  $\beta$ -catenin, an essential component of axis formation. GSK3 $\beta$  phosphorylates  $\beta$ -catenin protein and thereby triggers  $\beta$ -catenin degradation (Aberle et al., 1997; Pai et al., 1997; Yost et al., 1996). Evidence for a role for endogenous  $\beta$ -catenin in axis formation is very good. Overexpression of  $\beta$ -catenin induces axis formation (Guger and Gumbiner, 1995), while depletion of maternal mRNA encoding  $\beta$ -catenin results in embryos that lack a dorsal axis (Heasman et al., 1994). Furthermore, dorsal cortical enrichment of  $\beta$ -catenin protein from the 2-cell stage has been demonstrated biochemically and by immunofluorescent staining (Larabell et al., 1997). Thus  $\beta$ -catenin has been shown to be sufficient, necessary and present in the right place and time to initiate the dorsal axis.  $\beta$ -catenin

can enter the nucleus (Funayama et al., 1995) and activate or de-repress transcription of a number of target genes, including known dorsal-specific transcription factors (McKendry et al., 1997; Brannon et al., 1997). Wild-type GSK3 $\beta$  and dominant-negative mutant GSK3 $\beta$  also have the predicted effects on  $\beta$ -catenin levels, namely suppression and enrichment, respectively (Larabell et al., 1997).

GSK3 $\beta$  and  $\beta$ -catenin are both parts of the canonical Wnt signalling pathway. Wnt proteins are a family of intercellular signalling molecules known to be involved in many processes in animal development and in human cancers (Nusse and Varmus, 1992). The Wnt pathway minimally includes Wnt itself, frizzled (a membrane-spanning Wnt receptor), dishevelled (a transducer of unknown activity), GSK3 $\beta$ ,  $\beta$ -catenin and transcriptional co-factors of the TCF/LEF family. Proteins of the axin family and APC are also involved. Axin and its close relatives are sometimes referred to as scaffold proteins, most likely involved in establishing or maintaining the other components in transducing complexes (see Peifer, 1999 for review). The Adenomatous Polyposis Coli (APC) protein is also involved, though its exact function in the normal pathway is unclear (Polakis, 1997). The Wnt pathway has long been thought to be involved in *Xenopus* dorsal axis formation because several Wnt proteins are potent axis inducers

(McMahon and Moon, 1989). Overexpression of dishevelled also induces axis formation (Sokol et al., 1995). Dishevelled protein has recently been shown to be moved from a vegetal position to the dorsal side of the embryo during cortical rotation (Miller et al., 1999), the event known to establish dorsoventral asymmetry. This is consistent with dishevelled being the dorsal determination molecule of *Xenopus* embryos. However, as the authors of that study point out, in the absence of a functional test, one must be cautious about the role of the dishevelled protein. Studies have been carried out to test whether dishevelled or Wnt are actually necessary endogenously, as well as sufficient experimentally, for axis generation. Unexpectedly, expression in embryos of dominant negative (interfering) dishevelled and Wnt mutants do not suppress axis formation (Sokol, 1996; Hoppler et al., 1996). This suggests that, although Wnt and Dsh are experimentally sufficient for axis induction, they may not be endogenously necessary. However, such mutant experiments rely for their interpretation on a negative result, namely failure to ventralize the embryo. Thus, while they are suggestive, their interpretation is always vulnerable to relatively trivial complications such as inadequate expression, inappropriate mutant isoform, etc. (Miller et al., 1999).

Given that the roles of GSK3 $\beta$  and  $\beta$ -catenin in axis formation are well established but those of Wnt and dishevelled are in some doubt, it remains an open question how GSK3 $\beta$  is regulated in vivo. There has not hitherto been a direct biochemical study of endogenous GSK3 $\beta$ . In this paper, we carry out such a study. We demonstrate that, while exogenous Wnt and dishevelled can reduce GSK3 $\beta$ -specific activity as expected, there is no detectable reduction of GSK3 $\beta$ -specific activity on the dorsal side of the embryo. Instead, endogenous GSK3 $\beta$  is downregulated by a depletion of the protein. Furthermore, the time, place and magnitude of the dorsal depletion of GSK3 $\beta$  correspond precisely with the accumulation of endogenous  $\beta$ -catenin. UV and deuterium oxide treatments that are known to prevent or enhance the distribution of dorsal determinants from the vegetal pole of the embryo (Scharf and Gerhart, 1983; Scharf et al., 1989; Holowacz and Elinson, 1993; Rowning et al., 1997) also prevent and enhance GSK3 $\beta$  protein depletion. We go on to investigate the role of GBP, a GSK3 $\beta$ -binding protein that has recently been shown to be sufficient and necessary for dorsal axis formation in *Xenopus* (Yost et al., 1998). We find that ectopic GBP can mimic the endogenous depletion of GSK3 $\beta$ . This effect of GBP identifies it as part of a new, apparently Wnt-independent signalling pathway for GSK3 $\beta$  regulation.

## MATERIALS AND METHODS

### Embryo manipulation

In vitro fertilization and embryo culture were carried out in 0.1 $\times$  MMR as described (Peng and Kay, 1991). Staging was according to Nieuwkoop and Faber (1967). For RNA microinjections, embryos were injected at stage 2 or 3 in 2% Ficoll (Pharmacia), 0.5 $\times$  MMR and injected with in vitro-transcribed XWnt-8 (2 pg), XDsh (0.7 ng) and GBP (0.5 ng) (Christian et al., 1991; Sokol et al., 1995; Yost et al., 1998). Embryos were bisected with an eyelash knife at various stages. UV-ventralization and D<sub>2</sub>O dorsalization were carried out as described (Scharf and Gerhart, 1983; Scharf et al., 1989) with an egg batch-by-batch titration for UV or D<sub>2</sub>O efficacy.

### Embryonic extractions and immunoprecipitations

For activity measurements, embryo halves were homogenized in lysis buffer (1% Triton X-100, 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 10 mM NaF, 1 mM vanadate 25 mM  $\beta$ -glycerophosphate, 10  $\mu$ g/ml of leupeptin, aprotinin and pepstatin, 1 mM PMSF). Lysates were cleared by centrifugation at 14,000 g for 20 minutes. Cleared lysates were immunoprecipitated with anti-GSK3 $\beta$  antibody (Transduction Laboratories, no. G22320) for 2 hours at 4°C, then incubated for an additional 30 minutes with protein A-agarose. Beads were washed three times with lysis buffer and twice with kinase buffer (30 mM TrisHCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 mM DTT). For protein abundance measurements, cleared lysates or embryo halves homogenized in SDS-PAGE sample buffer without fractionation were loaded directly on gels.

### Kinase assays

Immune complex kinase assays were carried out in the presence of 25  $\mu$ M [<sup>32</sup>P]ATP, (4 Bq/pmol) and 10  $\mu$ M peptide substrate ('2B-(S)' of Welsh et al. (1997)), RRAAEELDSRAGS\*PQL (where S is the target serine and S\* is pre-phosphorylated). Control peptide (2B-(A)), RRAAEELDSRAGAPQL was also used in some experiments. One fifth of each reaction mixture was subjected to SDS/15% PAGE followed by autoradiography. Peptide bands from the gel were excised and counted in a scintillation counter. Immunoprecipitates were also subject to 8% SDS-PAGE followed by western blotting and densitometry to determine the amounts of GSK3 $\beta$  immunoprecipitated in each sample.

### Quantitative western assays

For western analysis, one quarter embryo equivalent was loaded per lane and electrophoresed on an SDS/8% polyacrylamide gel and electroblotted onto PVDF membranes (Millipore). The membrane was blocked with 10% goat serum, and then incubated with anti-GSK3 $\beta$  antibody (either Transduction Laboratories, no. G22320 as above or Santa Cruz Biotechnology no. 0011-A: sc-7291), anti-spectrin (Sigma) or anti-tubulin (Sigma) followed by horseradish peroxidase-conjugated secondary IgG antibodies (Jackson ImmunoResearch). Note that the anti-GSK3 $\beta$  antibodies recognize different epitopes since the Transduction Laboratories antibody does not recognize mammalian GSK3-alpha, while the Santa Cruz antibody does (manufacturers' data). Quantitation in *Xenopus* was based exclusively on the GSK3 $\beta$  band. (In mammalian cells and *Xenopus*, GSK3-alpha runs at a higher apparent molecular weight (I. D., unpublished data). Incubations were 1 hour each, separated by three 15 minute washes with phosphate-buffered saline (PBS), 0.05% Tween 20. Peroxidase activity was visualized with an ECL system (Amersham) and preflashed film (Kodak). Exposed films were scanned with a flatbed scanner and quantitated using NIH image. For each band, integrated pixel density minus local background density was determined. Incubation times, substrate and enzyme concentrations, gel loading and densitometry were optimised to be within the linear range of assay (see Fig. 1).

### Immunostaining

Embryos were fixed exactly according to the protocol of Larabell et al. (1997) at the 4- to 8-cell stage and left intact or bisected with a razor blade in the horizontal plane prior to antibody incubations. Fixation was carried out overnight at room temperature with the embryos animal pole uppermost and without agitation. The same anti-GSK3 $\beta$  mouse monoclonal antibody as used for western blots (Transduction Laboratories) was used at a dilution of 1:25 or 1:50 with the same result. No additional non-GSK3 $\beta$  bands are seen at this concentration compared to lower antibody concentrations in western blots (data not shown). Anti- $\beta$ -catenin rabbit polyclonal antibody kindly provided by Dr B. Gumbiner (McCrea et al., 1993) was used at a dilution of 1:250. Secondary antibodies were diluted to 1:500. Three 1 hour washes at room temperature and one overnight wash at 4°C overnight with nutation (rocking) after antibody incubations were

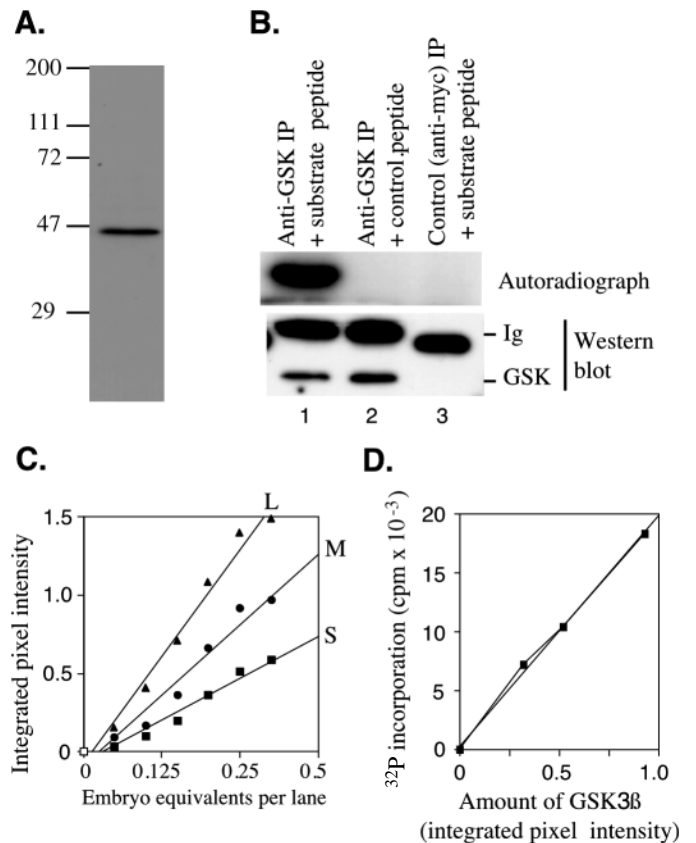
carried out in Superblock (Pierce). The secondary antibodies used were Cy-3 and Cy-5 conjugates (Jackson ImmunoResearch), which fluoresce in the rhodamine and far-red channels, respectively. Stained specimens were cleared and mounted between coverslips. Optical sections were taken with a Zeiss inverted laser scanning confocal microscope. Channels were analyzed in series (i.e. not simultaneously) to avoid any 'bleed through' between them. Autofluorescence of yolk cytoplasm in the fluorescein channel was used to reveal the extent of the embryo.

## RESULTS

### Endogenous GSK3 $\beta$ abundance but not specific activity is lower dorsally

We set out to determine whether GSK3 $\beta$  is downregulated endogenously on the dorsal side of the *Xenopus* embryo (and if so, when and how) by directly measuring GSK3 $\beta$  activity. Embryos were cut into dorsal and ventral halves (as evaluated by sperm entry point and pigmentation differences), homogenized and subjected to immunoprecipitation with a specific anti-GSK3 $\beta$  antibody (see Methods). This antibody is specific in *Xenopus* to GSK3 $\beta$ , marking a single band both in western blot and immunoprecipitation (Fig. 1A,B). Western blots show that GSK3 $\beta$  protein is present maternally and throughout all the stages analyzed in this study (data not shown). Precipitated GSK3 $\beta$  was assayed for kinase activity by incubation with radiolabelled ATP and a synthetic peptide known to be a sensitive, specific substrate (Welsh et al., 1997). A control peptide, substituted at a critical prephosphorylation site, was not phosphorylated in our assays (Fig. 1B; see Methods for details of peptides), ruling out contaminating non-GSK3 $\beta$  kinase activity. After incubation, enzyme and phosphorylated substrate were separated by gel electrophoresis. Phosphorylated substrate was excised and quantitated in a scintillation counter. GSK3 $\beta$  was detected by western blotting and quantitated densitometrically. Rigorous quantitative controls established the linearity of the measurements (Fig. 1C,D).

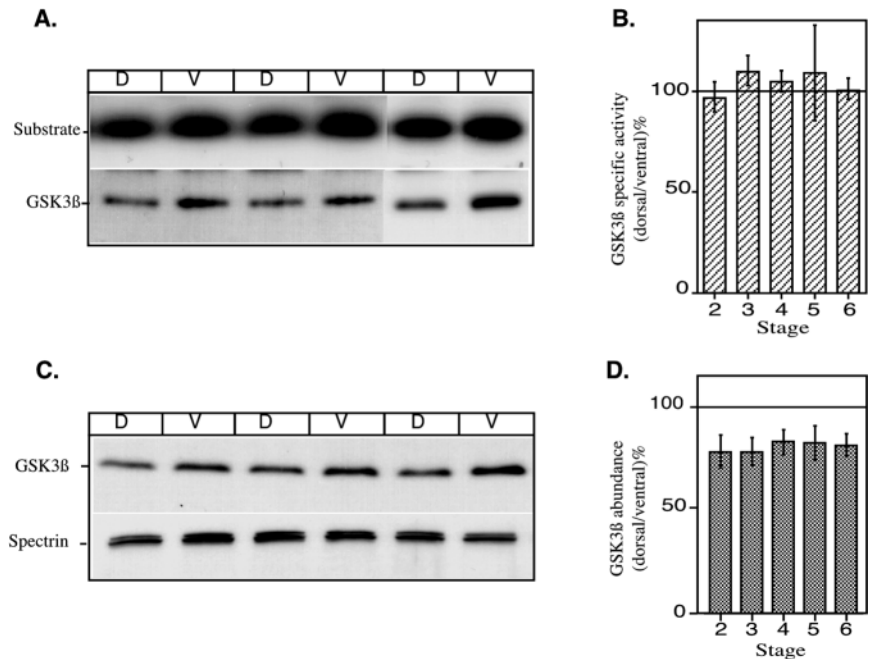
We measured GSK3 $\beta$ -specific activity during the 2-cell to 32-cell (stage 6) period. This period was chosen because by stage 2 (2-cell stage) dorsal enrichment of  $\beta$ -catenin has been observed by immunofluorescence (Larabell et al., 1997) while, by stage 6 (32-cell stage), a dorsal enrichment of  $\beta$ -catenin about 30% has been measured biochemically (Larabell et al., 1997). Surprisingly, we found no significant differences in the *in vivo* specific activity of GSK3 $\beta$  between dorsal and ventral halves at any of the stages analyzed (Fig. 2A,B), even though a specific activity difference sufficient to generate an axis should have been detectable in our assay (see below). These data suggest that GSK3 $\beta$ -specific activity does not change dramatically during the establishment of the dorsoventral axis. However, in the course of the immunoprecipitation experiments, we noticed that dorsal yields of GSK3 $\beta$  consistently appeared lower than ventral yields (Fig. 2A). To control for the possibility that this was due to differential immunoprecipitation, we loaded cleared embryonic lysates equalized for total protein content onto gels and analyzed by western blotting and probing for GSK3 $\beta$ . Relative to total amount of protein, dorsal halves contained approximately 20% less GSK3 $\beta$  protein than ventral halves (data not shown, but see below). This difference was observed in all the stages studied and occurs at least as early as the earliest detectable dorsoventral



**Fig. 1.** Specificity, calibration and linearity tests for GSK3 $\beta$  abundance and specific activity assays. (A) The anti-GSK3 $\beta$  antibody recognizes a single band in western blots of *Xenopus* embryonic lysates. (B) In immune-precipitate kinase assays, substrate peptide (lane 1) is efficiently labelled, while a control peptide in which a prephosphorylated serine is replaced by an alanine (lane 2), has no detectable radiolabel (see Methods for peptide details). Immunoprecipitates made with a control antibody (anti-myc, 9E10) fail to incorporate label into substrate peptide (lane 3). (C,D) Western blot quantitation was in the linear range. Serial dilutions of embryonic lysates were assayed by western blot and scintillation counting of phosphorylated substrate as described in Methods. Integrated pixel intensity for GSK3 $\beta$  detection on western blots is plotted against amount loaded for short, medium and long ECL exposure times (S,M,L) corresponding to maximal raw pixel values of 170, 190 and 220 for the highest loadings. Linearity is maintained within the middle loadings for all three exposures (pixel intensities in the range 90-210). For subsequent assays, 0.25 embryo equivalents were loaded per lane and film exposures kept within the linear range. Loss of linearity outside of this range occurs for each of these exposures and is probably, therefore, a function of western blotting efficiency rather than the linearity of detection. This linearity test was repeated twice. In addition, for all experiments, quantitation was carried out at least two different exposures to eliminate film densitometry effects. (D) GSK3 $\beta$  kinase activity assays (scintillation counts) were linear for  $^{32}\text{P}$  incorporation versus amount of GSK3 $\beta$  protein (assayed by quantitative western blotting) showing reliable measurement of specific activity under the conditions used. This linearity test was repeated three times.

differences in  $\beta$ -catenin abundance (Larabell et al., 1997). This dorsal deficit in GSK3 $\beta$  abundance is thus consistent, both in timing and magnitude, with it being responsible for the

**Fig. 2.** GSK3 $\beta$  abundance, but not specific activity, is lower dorsally than ventrally in normal embryos. (A) Example lanes from typical western blots of GSK3 $\beta$  immunoprecipitates probed for GSK3 $\beta$  and the autoradiograph of corresponding phosphorylated substrate. Pairs of lanes are from stages 2,3 and 4 read from left to right. Both western and autoradiograph bands are less intense in dorsal (D) than ventral (V) lanes. (B) GSK3 $\beta$ -specific activity expressed as dorsal values relative to ventral (100%). Values represent the ratio of activity (counts in substrate bands in A) divided by corresponding GSK3 $\beta$  protein abundance (integrated intensity of bands in western blots, lower bands in A). Loading was normalized to total protein (Bradford assay). A total of 28 assays were carried out (2-10 for each stage) on pools of 5 embryo halves from eight different egg batches. Values are not significantly different from 100%, i.e. dorsal = ventral. (C) Example of a typical western blot of unfractionated embryo halves loaded directly onto SDS gels and probed for GSK3 $\beta$  and spectrin (loading control). GSK3 $\beta$  band is less intense in the dorsal sample than in ventral while the dorsal spectrin band is equal to or slightly more intense than the ventral band. (D) GSK3 $\beta$  abundance expressed as dorsal values relative to ventral (100%). Values represent the ratio GSK3 $\beta$  abundance (integrated intensity of upper bands in B) divided by the corresponding spectrin abundance (loading control). GSK3 $\beta$  abundance is consistently 20-30% lower ventrally than dorsally. The difference is statistically significant ( $P < 0.0001$ ; Wilcoxon Signed Rank Test). A total of 41 measurements were made from pools of 5 embryo halves from eight different egg batches. Error bars represent s.e.m. Quantitation using a second anti-GSK3 $\beta$  antibody for western detection that recognizes a different epitope (see Methods) gave indistinguishable results (not shown).



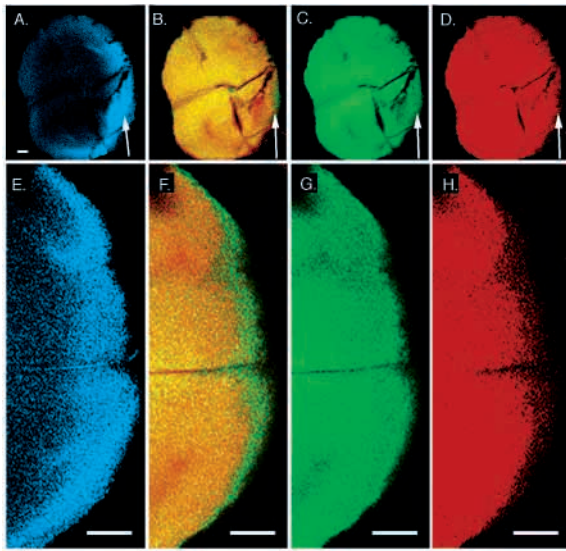
dorsoventral difference in  $\beta$ -catenin levels (Larabell et al., 1997) in the absence of a dorsoventral-specific activity difference.

One explanation for differential yields of GSK3 $\beta$  in embryonic extracts might be that dorsal GSK3 $\beta$  is somehow less extractable than ventral GSK3 $\beta$ . To bypass potential effects of differential extractability, embryo halves were lysed in sample loading buffer and the total unfractionated lysates loaded directly onto gels for western analysis. Abundance of GSK3 $\beta$  was normalized to that of the ubiquitous protein spectrin (Larabell et al., 1997). A 20-30% reduction of GSK3 $\beta$  on the dorsal side of the embryo was observed (Fig. 2C,D), consistent with the results above. Normalizing by a specific protein also rules out the possibility that the measured difference is merely a consequence of asymmetric distribution of bulk protein (mostly yolk) which is known to move with cortical rotation (Denegre and Danilchik, 1993). Similar results were obtained when we normalized to a second protein, tubulin or used a second anti-GSK3 $\beta$  antibody that recognizes a different epitope (data not shown; see Methods). To control for variability in identification of the dorsoventral axis (which is well but not completely correlated with the position of sperm entry), we also did measurements on embryos that had been held at an angle and stained at the uppermost point with Nile blue vital dye. This method forces the formation of the dorsal axis at the uppermost, marked meridian and makes dorsoventral dissection close to 100% reliable (Peng and Kay, 1991). An identical result was obtained (data included in Fig. 2D). Overall, the dorsoventral abundance difference was found to be statistically highly significant ( $P < 0.0001$  or 99.9999% confidence using either Wilcoxon signed rank test or Student's  $t$ -test). Thus, the lower abundance of GSK3 $\beta$  on the dorsal versus the ventral side of the embryo, whilst small, is consistent. This

observed dorsoventral difference in abundance and hence total activity quantitatively matches the concurrent accumulation of cytoplasmic  $\beta$ -catenin and consequent axis formation.

### Co-localization of $\beta$ -catenin enrichment with GSK3 $\beta$ depletion at the cortex

We reasoned that the 20-30% gross dorsoventral difference in GSK3 $\beta$  might disguise a much greater effective difference in a cortical domain. Early  $\beta$ -catenin enrichment is restricted to the most cortical dorsal region of the embryo (Larabell et al., 1997), which is known to be the location of dorsal determinants following cortical rotation in the first cell cycle (Fujisue et al., 1993; Kikkawa et al., 1996). We wished to know whether this spatial restriction also applied to GSK3 $\beta$  depletion. We performed in situ immunostaining according to the protocol of Larabell et al. (1997). Fig. 3 shows the results of staining from two different egg batches. Similar results were obtained from a total of sixteen embryos from five independent egg batches. We found that GSK3 $\beta$  is depleted in a narrow cortical fringe corresponding to the region of  $\beta$ -catenin enrichment. The region of GSK3 $\beta$  depletion usually appears to be more cortically restricted than that of  $\beta$ -catenin enrichment, although this difference seems to vary between batches. However, it is consistent with diffusion of hypo-phosphorylated  $\beta$ -catenin away from the region of GSK3 $\beta$  depletion. The dorsal restriction (as marked by  $\beta$ -catenin) confirms the biochemical data. The  $\beta$ -catenin also provides a useful technical control, since it shows that the cortical region is stainable and thus lack of GSK3 $\beta$  staining is not due to non-specific blocking of stainability or edge effects due to physical bisection or optical sectioning. The narrowness of this region explains the relative

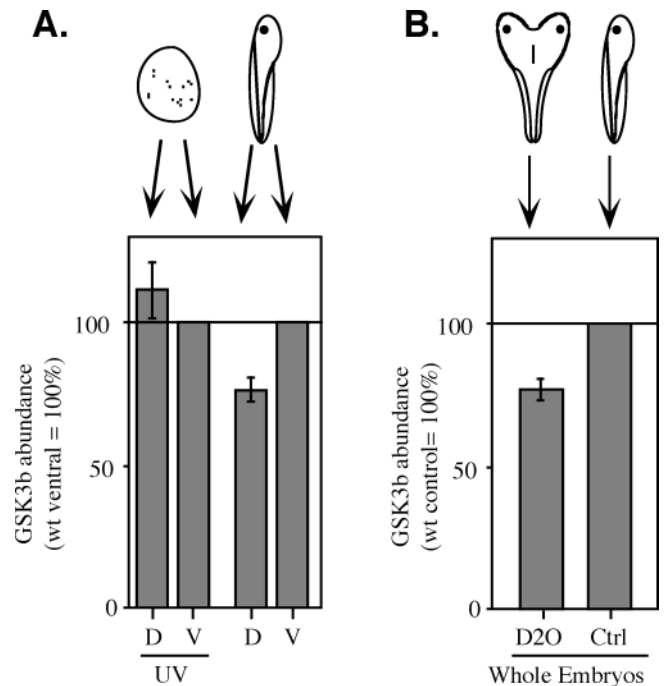


**Fig. 3.** Immunostaining of embryos showing cortical depletion of GSK3 $\beta$  coincident with enrichment of  $\beta$ -catenin. Anti-GSK3 $\beta$  (red channel, D, H) and anti- $\beta$ -catenin (blue channel, A, E) antibodies were used simultaneously. Autofluorescence of yolk protein (green channel, C, G) serves as a 'counterstain' to show cytoplasm. Horizontal confocal optical sections from two independent egg batches are shown (A-D, E-H). (A, E) Cortical (peripheral) enrichment of  $\beta$ -catenin. This enrichment marks the dorsal side of the embryo (Larabell et al., 1997) and provides a positive control for stainability of cortex. (B, F) Merged green and red images showing GSK3 $\beta$ -containing cytoplasm in yellow and GSK3 $\beta$ -depleted cytoplasm in green (arrows). Images were captured digitally on a laser scanning (confocal) microscope as optical sections. Contrast and brightness were adjusted for printing to equalize maximal intensities in each channel. Controls with transposed secondary antibodies gave similar results and controls without primary antibodies showed negligible background fluorescence (data not shown). Depletion coincides with the most cortical area of  $\beta$ -catenin enrichment. Scale bars, 100  $\mu$ m.

smallness of both  $\beta$ -catenin enrichment and the GSK3 $\beta$  depletion measured in homogenates of embryo halves ((Larabell et al., 1997) and Fig. 2D above). It suggests that locally the depletion of GSK3 $\beta$  may be close to 100%.

#### UV ventralization abolishes and D<sub>2</sub>O dorsalization enhances GSK3 $\beta$ depletion

Vegetal cortical cytoplasm has been shown to contain the endogenous determinants whose dorsal displacement during cortical rotation is both sufficient and necessary for normal axis formation (Yuge et al., 1990; Fujisue et al., 1993; Holowacz and Elinson, 1993; Kikkawa et al., 1996; Sakai, 1996). If dorsal GSK3 $\beta$  depletion is functionally significant, preventing dorsal axis initiation should prevent the dorsoventral difference from arising. To test this prediction, we analyzed GSK3 $\beta$  abundance in embryos ventralized by UV irradiation of the vegetal pole in the first cell cycle (Scharf and Gerhart, 1983). This treatment abolishes the cortical rotation that brings dorsal determinants from the vegetal pole to the dorsal side of the embryo opposite the sperm entry point (Kikkawa et al., 1996; Fujisue et al., 1993). As predicted, UV-ventralization results in equalization of GSK3 $\beta$  levels on 'ventral' (sperm entry point) and 'dorsal' (opposite) sides (Fig. 4A).



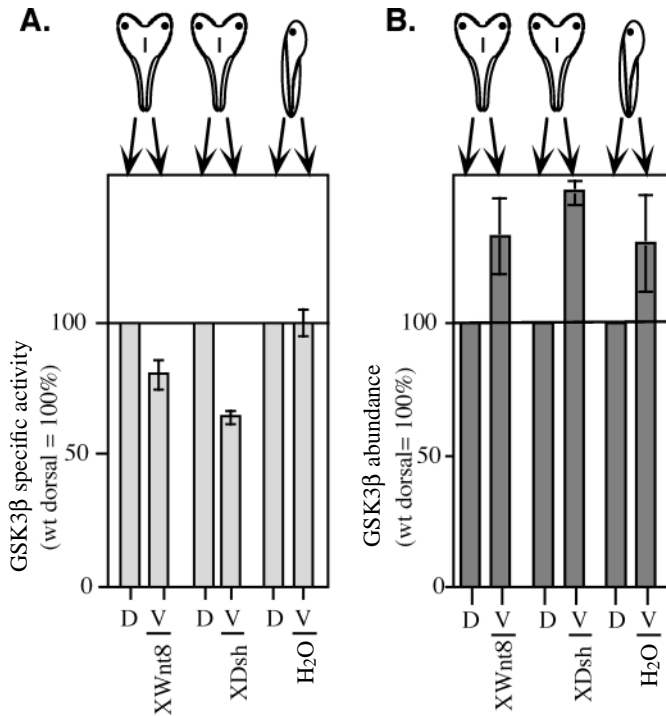
**Fig. 4.** Early dorsal axis perturbations cause corresponding changes in GSK3 $\beta$  abundance. Diagrams show tadpole stage phenotypes corresponding to protein measurements made at stage 6. (A) UV ventralization of embryos abolishes the GSK3 $\beta$  depletion normally found on the prospective dorsal side (opposite the sperm entry point). Quantitative western blots of halves of UV-treated and control embryos show that the GSK3 $\beta$  abundance difference is abolished. Average dorsoanterior index (DAI) for these experiments was 0.36 (zero is a completely ventralized embryo, 5 is wild type (Kao and Elinson, 1988)) ( $n=15$ ). (B) Deuterium (D<sub>2</sub>O) treatment, which causes dorsalization of embryos, also causes net depletion of GSK3 $\beta$  relative to control embryos. Average DAI was 7.0, which indicates predominantly twinned-axis embryos. GSK3 $\beta$  was depleted on average by 36% ( $n=19$ ) in D<sub>2</sub>O-treated whole embryos.

Another prediction is that dorsalization of embryos by deuterium (D<sub>2</sub>O) treatment should cause an increased depletion of GSK3 $\beta$ . D<sub>2</sub>O treatment is unlike lithium-dependent dorsalization in that it acts during the first cell cycle and usually causes axis duplication rather than radial dorsalization (Scharf et al., 1989). D<sub>2</sub>O causes chaotic polymerization of cortical microtubules, which disperses the spread of vegetal cortical materials (Scharf et al., 1989; Rowning et al., 1997). Fig. 4B shows that as predicted, D<sub>2</sub>O-induced double-axis embryos (DAI = 7.0, see Scharf et al., 1989) show roughly double the net depletion seen in single-axis controls. This result in whole embryos argues against translocation as a possible mechanism for dorsal GSK3 $\beta$  depletion since translocation would not result in a net disappearance of GSK3 $\beta$ .

#### Wnt and Dsh mimic GSK3 $\beta$ depletion quantitatively by specific activity reduction

Reduction in GSK3 $\beta$  activity is known to cause  $\beta$ -catenin enrichment and  $\beta$ -catenin enrichment is known to cause axis formation. This chain of causality is confirmed by the axis-forming effects of expression exogenous dominant negative mutant GSK3 $\beta$ . It might be argued, however, that the reduction



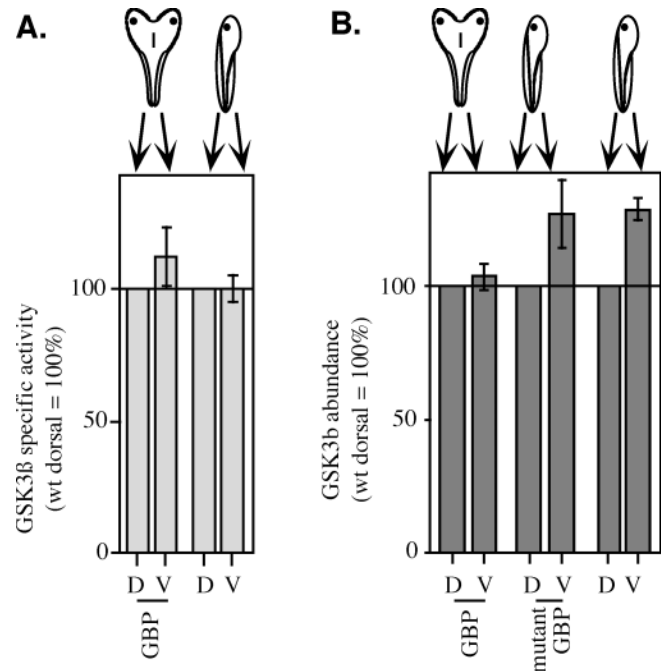


**Fig. 5.** XWnt8 and XDsh expression reduce GSK3 $\beta$ -specific activity but not abundance. RNA encoding XWnt8 or XDsh was injected on the ventral side of embryos in the minimum amounts sufficient to generate a complete ectopic axis in 80% of embryos. Diagrams show tadpole stage phenotypes corresponding to measurements made at stage 6. Injected RNA's reduce GSK3 $\beta$ -specific activity (A) and but do not reduce abundance (B). The endogenous dorsal depletion of GSK3 $\beta$  protein is matched in magnitude by the induced ventral reduction in GSK3 $\beta$ -specific activity. D, dorsal; V, ventral.

in endogenous GSK3 $\beta$  that we observed is too small to account for endogenous axis formation. To address this question, we used a biochemically well-characterized method for reducing GSK3 $\beta$  activity, namely Wnt8 signalling, to test how much of a reduction would be sufficient to generate an axis. Wnt8 and dishevelled regulate GSK3 $\beta$  by reduction of specific activity (Cook et al., 1996; Fisher et al., 1999). We therefore measured GSK3 $\beta$  abundance and specific activity in embryos injected ventrally with mRNA encoding XWnt8 or *Xenopus* dishevelled (XDsh), a transducer of Wnt signalling. We used the minimum amounts of RNA sufficient to induce complete ectopic (secondary) axes in 80% of recipient embryos. As shown in Fig. 5, the effect both of XWnt8 and of XDsh was a 25–40% reduction in ventral GSK3 $\beta$ -specific activity, with no observable change in abundance. This result shows that specific activity differences sufficient to generate an axis are measurable in this assay, an important positive control for the measurements showing no dorsoventral GSK3 $\beta$ -specific activity differences in normal embryos (Fig. 2A,C above). It also shows that protein depletion is not merely an effect (direct or indirect) of specific activity reduction by Wnt or dishevelled.

#### GBP causes GSK3 $\beta$ depletion, mimicking the endogenous axis formation mechanism

The above result constitutes biochemical evidence suggesting



**Fig. 6.** Effects of expression of GSK3 $\beta$ -binding protein (GBP) on GSK3 $\beta$ -specific activity and abundance. RNA encoding GSK3 $\beta$ -binding protein (GBP) was injected on the ventral side of embryos in the minimum amounts sufficient to generate a complete duplicate axis in 80% of embryos. Diagrams show tadpole-stage phenotypes corresponding to measurements made at stage 6. (A) Specific activity of GSK3 $\beta$  is not significantly affected by GBP overexpression. (B) Abundance of GSK3 $\beta$  is reduced on the ventral (GBP-injected) side of the embryo such that it is now equal to dorsal abundance. Injection of a control protein, a mutant form of GBP that does not bind to GSK3 $\beta$  (Yost et al., 1998) has no effect on GSK3 $\beta$  abundance. For each data point,  $n=10$  or greater. D, dorsal; V, ventral.

that Wnts are not the endogenous regulators of GSK3 $\beta$  in the early *Xenopus* embryo. We therefore decided to test a recently identified GSK3 $\beta$ -binding protein (GBP) as a candidate endogenous regulator. GBP has been shown not only to bind GSK3 $\beta$  but also to be required maternally for dorsal axis formation in *Xenopus* (Yost et al., 1998). Yost and colleagues have shown that when injected into oocytes, GBP RNA reduces the (total) activity of exogenous tagged GSK3 $\beta$ . The reported measurements did not distinguish between effects on specific activity or abundance and so we tested whether GBP reduces GSK3 $\beta$ -specific activity (as Wnt does) or amount of protein (as endogenous factors do). To demonstrate this effect in a normal physiological context and to correlate it with axis formation, we injected embryos on the ventral side with just sufficient GBP to make a duplicate axis. We then measured GSK3 $\beta$  activity and abundance as before. As a control, we used a GBP mutant that does not bind GSK3 $\beta$  (Yost et al., 1998). Fig. 6 shows that GBP reduced the ventral abundance of GSK3 $\beta$  to that of the dorsal side but did not reduce the GSK3 $\beta$ -specific activity. Thus, GBP can mimic the endogenous axis-forming process at a biochemical level making it a good candidate for the in vivo regulator of GSK3 $\beta$  and a critical component of the dorsal determinant.

## DISCUSSION

The above data show that endogenous GSK3 $\beta$  is downregulated on the prospective dorsal side of embryos as an early consequence of cortical rotation and that the mechanism is protein depletion rather than specific activity reduction. This is the first study to examine in detail the endogenous GSK3 $\beta$  protein rather than rely on overexpression of exogenous protein to elucidate mechanism. The weakness of simple measurement alone, of course, is that observations on unperturbed embryos cannot by themselves demonstrate mechanistic causation. Thus, a priori, the change could merely be a corollary or epiphenomenon of some other mechanism of axis formation. (This would fit with a view that a dorsal reduction of GSK3 $\beta$  would have no significant effect on dorsal  $\beta$ -catenin abundance or that dorsal  $\beta$ -catenin enrichment is not the mechanism of axis formation.) The ideal experiment to show causal significance would be restoration of equal GSK3 $\beta$  activity to the dorsal side with consequent loss of dorsal specification. This experiment is in fact fraught with complications. Since depletion appears to be highly restricted to a small cortical domain, exogenous enzyme would have to be delivered to this domain. This domain cannot be targeted quantitatively with microinjection because microneedles and fluid flow from them cannot be so accurately controlled. The only resort is to inject relatively large amounts of GSK3 $\beta$  mRNA or protein. In this crude form, this experiment has already been done. The result – that dorsal overexpression of GSK3 $\beta$  does indeed suppress not only axis formation, but also early dorsal gene transcription and dorsal  $\beta$ -catenin accumulation – is well documented (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995; Larabell et al., 1997). We have obtained similar results by injecting purified recombinant GSK3 $\beta$  protein (I. D., unpublished observations). However, even in this crude form, complete ventralization is rarely achieved because flooding the embryo with GSK3 $\beta$  is toxic to embryos by gastrula stages (our unpublished observations). This is not surprising, since GSK3 $\beta$  is a multifunctional kinase that would be expected to interfere with multiple pathways when overexpressed at high levels. Perhaps the best indication of the specificity of the GSK3 $\beta$  effect is the experiment of Larabell et al. (1997) in which dorsal overexpression of GSK3 $\beta$  suppresses the dorsal accumulation of  $\beta$ -catenin without affecting cell division or morphology. The dramatic effect of axis duplication elicited by ventral expression of a kinase-dead GSK3 $\beta$  (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995) is also highly suggestive of a role for the enzyme, although the mechanism of action of such a putative dominant negative mutant may be complex.

It might be argued that the ‘small’ dorsal depletion of GSK3 $\beta$  revealed by the data makes only a small contribution to dorsal character, and that the real mechanism involves some other process. However, the data show that a similarly ‘small’ reduction in GSK3 $\beta$ -specific activity caused by exogenous Wnt or dishevelled expression is quite sufficient to increase  $\beta$ -catenin abundance and generate a complete ectopic axis. (Incidentally, S6 protein kinase p90-rsk also reduces GSK3 $\beta$ -specific activity and increases  $\beta$ -catenin abundance, though it sends the  $\beta$ -catenin to the membrane where it does not act as a dorsalizing signal; Torres et al., 1999.) Comparison of our data with those of Larabell et al. (1997) show that the dorsoventral differences in GSK3 $\beta$  protein abundance are quantitatively

similar to ‘small’ differences observed in  $\beta$ -catenin abundance. In other words, if other mechanisms for regulating  $\beta$ -catenin abundance are operating, they are redundant. The ‘smallness’ of the changes is, in any case, an artefact of measuring abundance in a homogenized half embryo. The immunofluorescence data show that the depletion is cortically localized and may be close to 100% in the cortical region.

What is the likelihood that GSK3 $\beta$ -regulating mechanisms other than the depletion shown in this paper are important for axis formation? The experiment by Yost et al. (1998) in which depletion of maternal GBP leads to complete ventralization of the embryo would seem to show that all the important mechanisms require the action of endogenous GBP. Since exogenous GBP causes GSK3 $\beta$  depletion but apparently not specific activity reduction, it seems likely that depletion represents the predominant endogenous mechanism. It should be noted, however, that while we have shown that GBP causes depletion of GSK3 $\beta$ , we have not ruled out the possibility that this depletion is accompanied by some GBP-dependent change of the specific activity of GSK3 $\beta$  to some physiological substrate, such as an axin-APC- $\beta$ -catenin complex. However, such a downregulation, tightly coupled to disappearance, would not appear to be necessary and might be hard to demonstrate experimentally.

What we have also shown is that GBP’s action is mechanistically distinct from the actions of Wnt and dishevelled signalling. Specifically, GBP causes GSK3 $\beta$  to disappear while Wnt and dishevelled signalling do not. Conversely, Wnt and Dsh both alter specific activity of GSK3 $\beta$  (this study, Cook et al., 1996; Fisher et al., 1999), though there may be circumstances in which Wnt does not reduce GSK3 $\beta$  kinase activity (Yuan et al., 1999). The lack of effect on GSK3 $\beta$  abundance due to Wnt or Dsh suggests that decreased GSK3 $\beta$  abundance is not a consequence of Wnt or dishevelled signalling, either directly (e.g. protein modifications reduce both specific activity and stability) or via a feedback mechanism (e.g.  $\beta$ -catenin signals to destabilize GSK3 $\beta$ ). Furthermore, the sizes of endogenous and experimentally induced decreases in total GSK3 $\beta$  activity necessary to cause axis formation are similar. This implies that the dorsal decrease in endogenous GSK3 $\beta$  protein levels can account for dorsal axis formation without invoking some additional undetected Wnt- or Dishevelled-dependent specific activity change. These results provide direct biochemical evidence that either the Wnt signal transduction pathway is not the endogenous initiator of dorsal axis formation or that endogenous Wnt and dishevelled signalling is very different from signalling by exogenous Wnt or dishevelled. Later roles for Wnt and dishevelled in axis formation are also not ruled out. For example, Wnt or Dsh signalling could come into play after stage 6 (the latest stage analyzed in this study). Experiments with dominant negative mutants of XWnt8 and *Xenopus* dishevelled suggest a critical role for these molecules in morphogenesis of a fully extended dorsal axis (Hoppler et al., 1996; Sokol, 1996).

There are formally three possible explanations for dorsal depletion of GSK3 $\beta$ : translocation to the ventral side, reduced synthesis and increased degradation. Dorsal depletion by translocation towards the ventral side would appear to be ruled out by the D<sub>2</sub>O experiment. After D<sub>2</sub>O dorsalization of the embryo, a net decrease in GSK3 $\beta$  abundance in whole embryos is observed. Since dorsal and ventral halves are measured together in the same homogenate, translocation cannot explain this effect. However, although in the hyperdorsalized D<sub>2</sub>O embryo net loss

of GSK3 $\beta$  occurs, it does not directly exclude translocation as a mechanism in the normal embryo. Reduced synthesis is unlikely because depletion is caused by GBP (GSK-binding protein) which presumably does not bind GSK3 $\beta$  mRNA or the GSK3 $\beta$  gene promoter. However, some inhibition of synthesis by interaction with nascent GSK3 $\beta$  protein at the ribosome is not inconceivable. More indirect signalling from protein to mRNA is also possible. Thus, while the most plausible explanation for dorsal GSK3 $\beta$  depletion is cortically localized protein degradation, multiple mechanisms have not been ruled out.

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