Supplementary Information

Oct-3/4 regulates stem cell identity and cell fate decisions by modulating Wnt/β-Catenin signaling

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Supplementary Materials and Methods

Nuclear/cytoplasmic fractionation

For nuclear and cytoplasmic separation, cells were washed, harvested with ice-cold PBS, and centrifuged at 960 x *g* for 5 minutes at 4°C. The pellet was suspended in 2 volumes of ice-cold, low-salt buffer [10 mM Tris (pH 7.6), 0.15 mM spermine, 0.75 mM spermidine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.1% NP40 and 2mM DTT] supplemented with protease inhibitor cocktail (Sigma) and incubated on ice for 30 minutes. Following 10 minutes of centrifugation at 10,600 x *g*, the supernatants were frozen as cytoplasmic extracts. Nuclei were extracted with 2 volumes of ice-cold, high-salt buffer [20 mM Tris (pH 7.6), 0.15 mM spermine, 0.75 mM spermidine, 0.2 mM EDTA, 2 mM EGTA, 420 mM NaCl and 25% glycerol] and incubated at 4°C for 40 minutes. Nuclear extracts were cleared by centrifugation at 20,800 x *g* for 15 minutes.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described above. Complementary OCTA oligonucleotide probes (5'GATCCGTACTAATTTGCATTTCTA3') were synthesized. Oligonucleotides were annealed by heating to 95°C in Tris-EDTA buffer and cooling slowly to room temperature. The double-stranded probes were labeled with [α-^{32}P] dCTP using Amersham labling kit. 0.3 ng of labeled probes were incubated with nuclear extracts, 2 µg of poly (deoxyinosinic-deoxycytidylic acid), and 1x binding buffer [binding buffer: 100 mM Tris-HCl (pH 8.0), 200 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, 10 mM DTT, 40% glycerol] at room temperature for 20 min.

The samples were loaded on a prerun (100 V for 1 h at room temperature) 4% polyacrylamide gel. The gel then was dried and exposed to X-ray films.

RT-PCR and Real-Time PCR

RNA was prepared with total Versagene RNA tissue kit (Gentra Systems). Total RNA was converted to cDNA with M-MLV reverse transcriptase (Promega). RT-PCR and Real-time PCR was performed with the following primers: *Xnr-3* 5'-CCGAAATCCATTCACCTTGT-3' and 5'- GTCTTGGCCAGAAGCTGTTC- 3'; *Goosecoid* 5'- ATCCAGCTATCCCAATGTGC-3' and 5'-CGAGCAGGCTAAAAGTGACC-3'; *Siamois* 5'- ATTTGAAGGGTTCGGAGCTT-3' and 5'- ATCTGCCAAGAGCAACCACT-3'; GAPDH 5'-GCTCCTCTCGCAAAGGTCAT-3' and 5'- GGGCCATCCACTGTCTTCTG-3'; Histone H4 5'- CGGGATAACATTCAGGGTATCACT-3' and 5'-ATCCATGGCGGTAACTGTCTTCCT-3'; UBC 5'-CAGCCGTATATCTTCCCAGACT-3' and 5'-CTCAGAGGGATGCCAGTAATCTA-3'; Gapdh 5'-CCTGGAGAAACCTGCCAAG-3', and 5'- CAACCTGGTCCTCAGTGTAGC-3'; N-cadherin 5'-GCCTATGAAGGAACCACATGAA-3' and 5'-GGCCCAGTGACGCTGTATC-3'; Fn1 5'-ATGTGGACCCCTCCTGATAGT-3' and 5'-GCCCAGTGATTTCAGCAAAGG-3'; Vim 5'-CTTGAACGGAAAGTGGAATCC-3' and 5'-GTCAGGCTTGGAAACGTCC-3'; Epha7 5'-TGACCCTGAAACCTATGAGGA-3' and 5'-ATTCTCCTGCACCAATCACAC-3'; Gli 5'-TACATCCGCTCATTGCACTG-3' and 5'-GCAGAGCCATCTGGTGATAGT-3'; Axin2 5'-TAGGCGGAATGAAGATGGAC-3' and 5'-CTGGTCACCCAACAAGGAGT-3'; Cyclin D2 5'-CACCGACAACTCTGTGAAGC and 5'-

TGCTCAATGAAGTCGTGAGG-3'; Myc 5'-TGAGCCCCTAGTGCTGCAT-3' and 5'-AGCCCGACTCCGACCTCTT-3'; MixL-1 5'-AGTTGCTGGAGCTCGTCTTCCG-3' and 5'-CTCTGAGAACCAGATGTGCAGACG-3'; Hand1 5'-CCTGGCATCGGGACCATA-3' and 5'-GGCAGCTACGCACATCATCA-3'; Pou5f1 5'-TCTCCAGAGGATGGCTGAGT-3' and 5'- CACCGGACACCTCACAAAC-3'

In vitro-transcribed RNAs

Capped RNA for microinjection was prepared by *in vitro* transcription, adding cap analog (GE Healthcare) at a ratio of 1:5 (GTP:cap analog) (Levy et al., 2002). Oct-3/4 RNA was prepared from pCS2-Oct-3/4. xWnt-8 RNA was prepared from pCS2-Wnt-8 and xSiamois mRNA was prepared from pCS2-Siamois.

Luciferase reporter assay

For the luciferase reporter assay in ES cells, TOP-Flash or FOP-Flash (1.7 μg) were co-transfected with 20 pg of pRL-CMV at time 0. Luciferase activity was measured 16, 30 and 52 hours after transfection using the dual luciferase assay system (Promega) as previously described (Otero et al., 2004). For the luciferase reporter assay in embryos, 10 ng of Xnr-3-luc in combination with Oct-3/4 RNA, Oct-25MO or Oct-60MO was injected into the embryos and luciferase activity was measured at the indicated time points.

Alkaline phosphatase (ALP) staining

ALP activity was measured according to the protocol described by the commercially available Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories). Briefly, the cells were washed twice with PBS. The buffer was prepared using reagents provided in the kit and adjusted to ensure pH in the range of pH 8.2–8.5. Sufficient buffer was added to cover the cells, which were incubated for 1 h at room temperature. Bright field images were then taken using an IX70 Olympus microscope at 100X magnification. Positive staining is defined as a red precipitate.

Matrigel invasion assay

Blind well chemotaxis chambers with 13-mm-diameter filters were used for this assay. Polyvinylpyrrolidone-free polycarbonate filters, 8-µm pore size (Costar Scientific Co., Cambridge, MA), were coated with basement membrane Matrigel (25 µg per filter) as previously described (Bellacosa et al., 1991). Briefly, the Matrigel was diluted to the desired final concentration with cold distilled water, applied to the filters, and dried under a hood. Cells (2-3 x 10^5) suspended in DMEM containing 0.1% bovine serum albumin were added to the upper chamber. Conditioned medium of 3T3 fibroblasts was applied as a chemoattractant and placed in the lower compartment of the Boyden chamber. Assays were carried out at 37°C in 5% CO_2. The chambers were incubated for 20 hr. At the end of the incubation, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The filters were fixed and stained with Dif Quick System (Dade Behring, Inc., Newark, DE). Cells on the lower surface were counted in 10 random fields of 100x magnification, and each assay was done in duplicate.

Phalloidin immunoflourescence and confocal microscopy

Cell monolayers were grown on sterile glass coverslips, and, after the treatments, were washed in PBS, fixed in 4% paraformaldehyde, and incubated in NH_4Cl for 10 min at room temperature. Afterward, the cells were permeabilized with 0.5% Triton X-100 for 5 min and blocked with 3% bovine serum albumin in PBS for 2 h, subsequently incubated with 500 ng/ml TRITC-phalloidin and 0.1 μg/ml 4'6-diamidino-2-phenylindole (DAPI, Sigma) for 30 min at room temperature. After washing, stained monolayers were analyzed using Nikon 90i confocal microscope and photographed with Nikon D-eclipse C1 camera.

Supplementary Legends

Supplementary figure 1. *Xenopus* Oct-25 assists Oct-3/4 in reducing β-catenin levels in a dose-dependent manner.

293T cells were co-transfected with expression vectors for Oct-3/4 (1 ug) and increasing amounts of Oct-25, as indicated. Nuclear β-catenin levels were analyzed by WB using the indicated antibodies. IκB and Fibrillarin were used as cytoplasmic and nuclear control markers, respectively.

Supplementary figure 2. Oct-3/4 associates with Axin1, nuclear β-catenin and promotes its phosphorylation.

A. 293T cells were co-transfected with expression vectors for β-catenin together with Flag-tagged-Oct-3/4 and Myc-Axin1, as indicated. Cell extracts were subjected to immunoprecipitation using either an antibody directed against the Flag-epitope or a control antibody, and the precipitated material was subjected to WB analysis with antibodies directed against the Myc- and Flag-epitope.

B. 293T cells were transfected with expression vectors for Flag-tagged-β-catenin and, Oct-3/4 as indicated. Cytoplasmic and nuclear extracts were prepared and subjected to immunoprecipitation using an antibody directed against the Flag-epitope, or a control antibody, and the precipitated material was subjected to WB analysis with antibodies directed against Oct-3/4 and the Flag-epitope. IκB and Fibrillarin were used as cytoplasmic and nuclear control markers, respectively.

C. ZHBTc4 and ZHBTc4/DN2-5 (expressing a dominant negative Tcf3) ES cells were either treated or not with 1 ug/ml of dox for two days and cytosolic and nuclear extracts were prepared. The levels of β-catenin and Oct-3/4, were analyzed by WB analysis using the indicated antibodies. IκB and Fibrillarin were used as cytoplasmic

and nuclear control markers, respectively. The experiment was replicated and a relative quantification of β-catenin levels is shown. Arrow bars depict +/- StDV.

D. 293T cells were co-transfected with expression vectors for β-catenin and Oct-3/4, as indicated. The phosphorylation level of nuclear β-catenin was analyzed by WB using an antibody that recognizes the phosphorylated residues S33 and S37. IκB and Fibrillarin were used as cytoplasmic and nuclear control markers, respectively.

Supplementary figure 3. Oct-3/4 antagonizes Wnt/β-catenin signaling in *Xenopus* embryos.

A. qRT-PCR analysis of Xnr-3, Siamois (Sia) and Goosecoid (Gsc) mRNA levels in un-injected embryos and embryos injected with Oct-3/4 mRNA. Histone H4 served as a loading control.

B. mRNA levels were analyzed using real-time quantitative PCR. Un-injected embryos served as controls.

Supplementary figure 4. Oct-25, 60 antagonize Wnt/β-catenin signaling in *Xenopus* embryos.

A. *Xenopus* embryos were injected with Oct-25-myc or Oct-60-myc expression vectors, as well as increasing amounts of morpholino oligos (MO) against Oct-25 and Oct-60, respectively. Proteins levels were measured by WB using antibodies directed against myc. α-tubulin served as a loading control.

B. *Xenopus* embryos were injected with MO against Oct-25 and or Oct-60. RNA was extracted at stage 9 and Xnr-3 and Siamois (Sia) expression levels were analyzed using quantitative real-time PCR. Data were normalized to un-injected control embryos.

C. *Xenopus* embryos were injected with a luciferase reporter gene regulated by the Xnr-3 promoter region. Embryos were injected with Oct-3/4 mRNA and MO against Oct-25 and Oct-60 as indicated, and was normalized to embryos injected with only the luciferase reporter plasmid (Relative expression). The p value for the indicated injections was < 0.02.

D. *Xenopus* embryos were injected with MO against Oct-25 and Oct-60. RNA was extracted at different stages as indicated. Xnr-3 expression levels were analyzed using quantitative real-time PCR. Data were normalized to un-injected control embryos.

E. *Xenopus* embryos were injected with TOPFlash and MO against β-catenin, Oct-25 and Oct-60 as indicated. Luciferase activity was assayed at stage 9.

Supplementary figure 5. Oct-3/4 repression induces cellular motility in ES cells *via* activation of the Wnt signaling pathway.

A. ZHBTc4 ES cells were transduced with lentiviral vectors encoding shRNA directed against mouse β-catenin (shβ-catenin) or Scrambled (shScr) as control. The cells were either treated or not with dox for two days. β-catenin protein levels were determined in cell extracts using WB analysis. β-actin was used as a loading control.

B. cDNA was prepared from the transduced cells in A and mRNA levels of β-catenin, Axin2 and fibronectin (FN1) were determined using real quantitative real-time PCR.

C. The indicated ES cells were analyzed for cellular motility in the presence of LIF, treated or untreated with dox. Data represent the average number of migrating cells, counting in magnification of 100x.

Supplementary References

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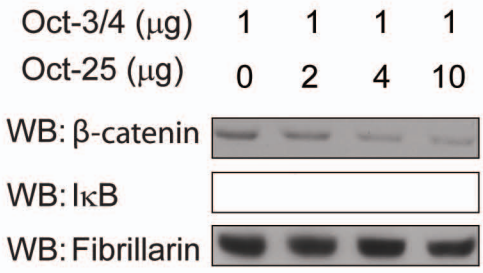
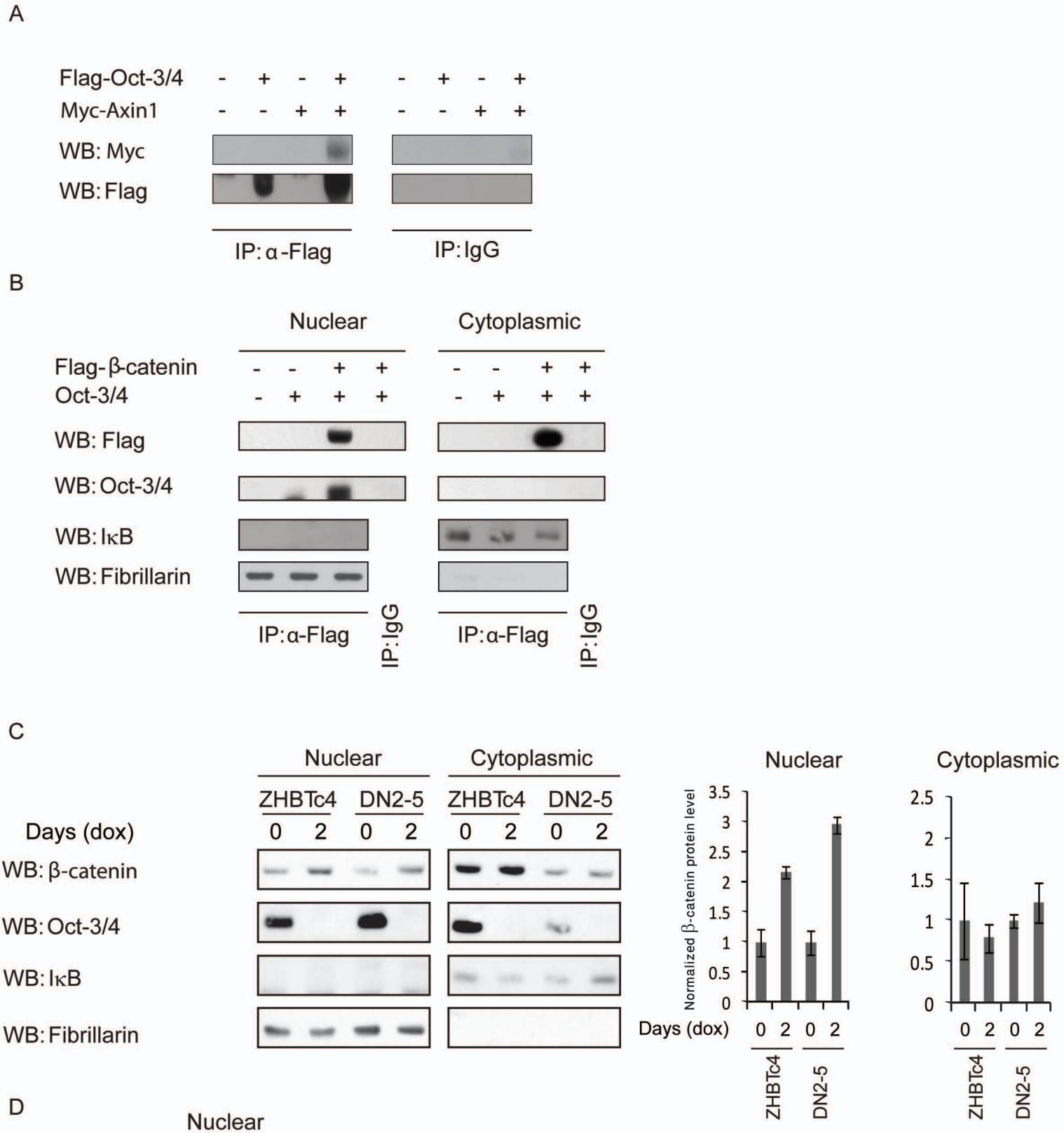
Supplementary Figure 1



Supplementary Figure 2



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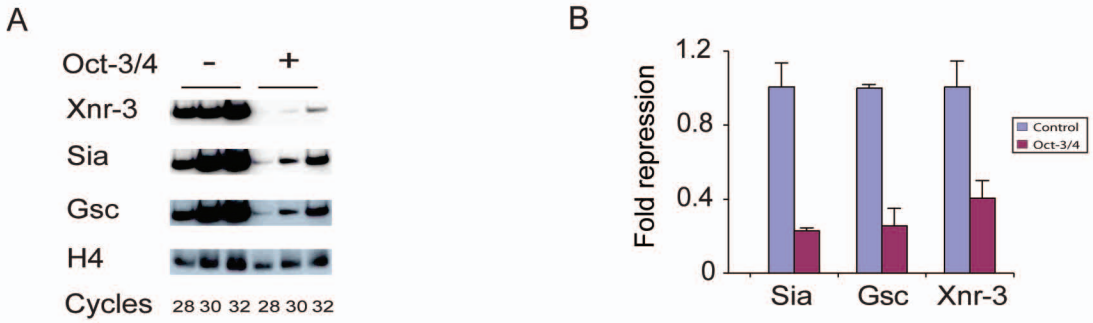

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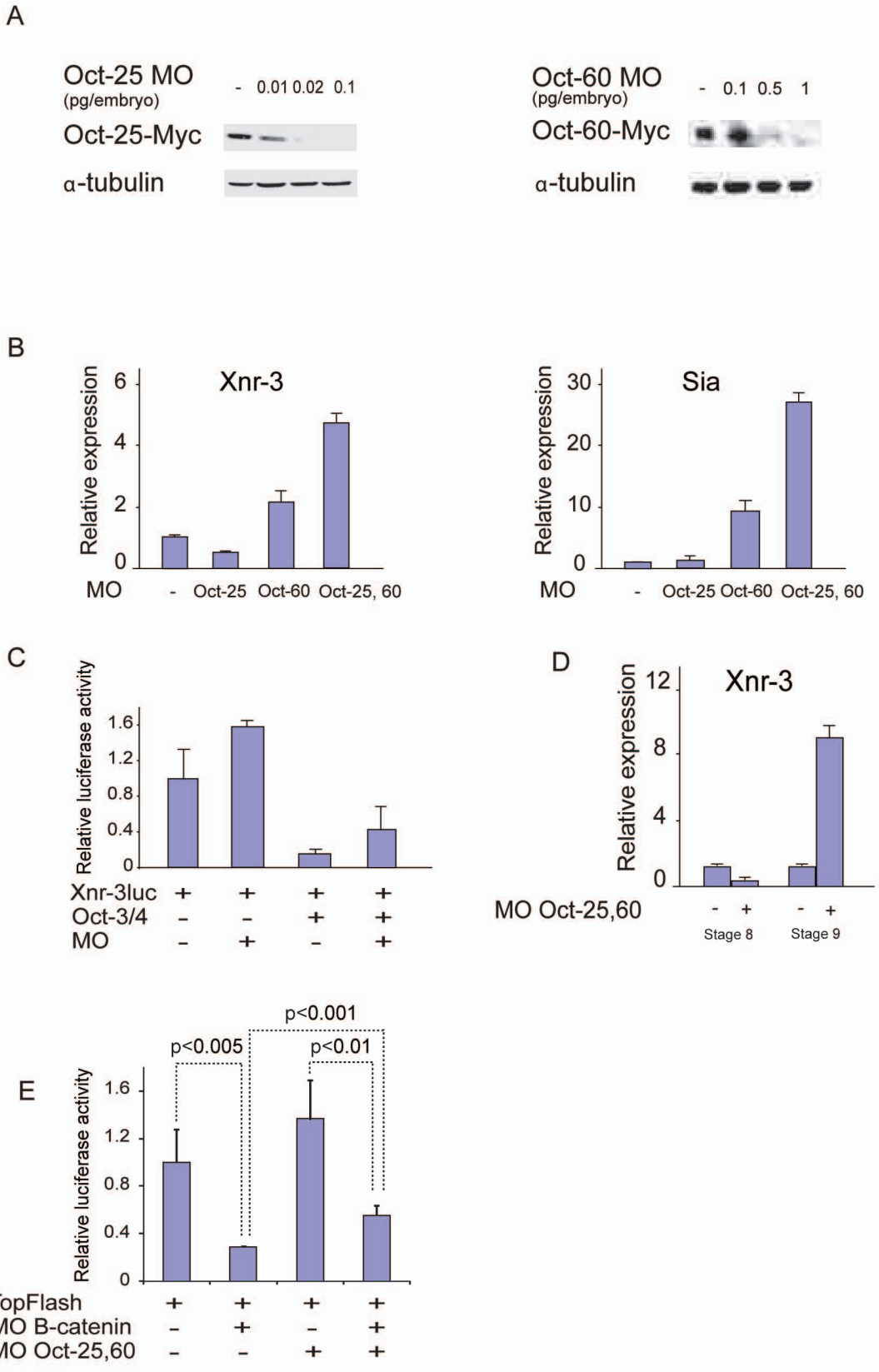
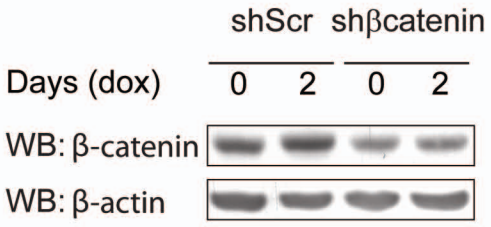


Supplementary Figure 5

A



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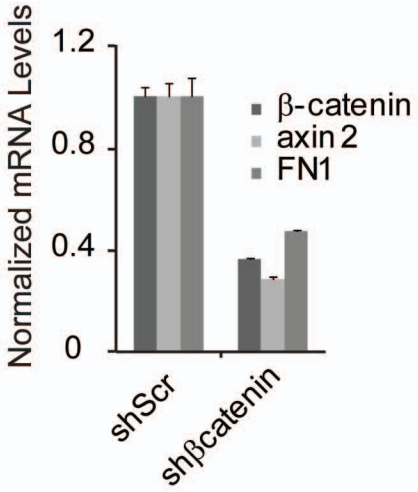

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