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Supplemental Data

Article

Mutations in *Fbx4* Inhibit Dimerization of the SCF^{Fbx4} Ligase and Contribute to Cyclin D1 Overexpression in Human Cancer

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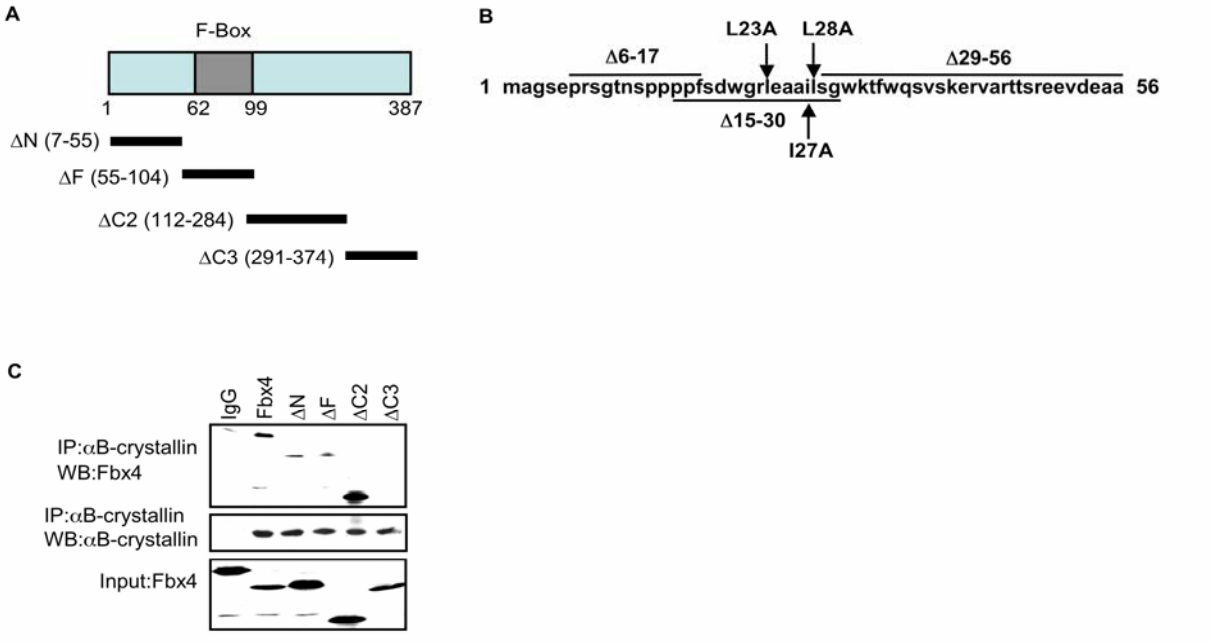
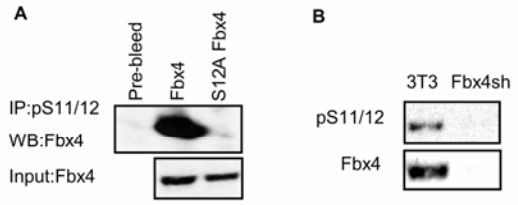
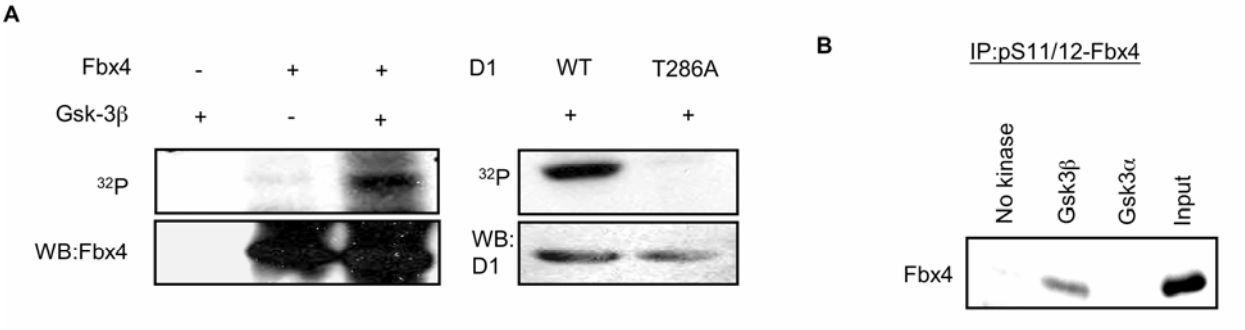


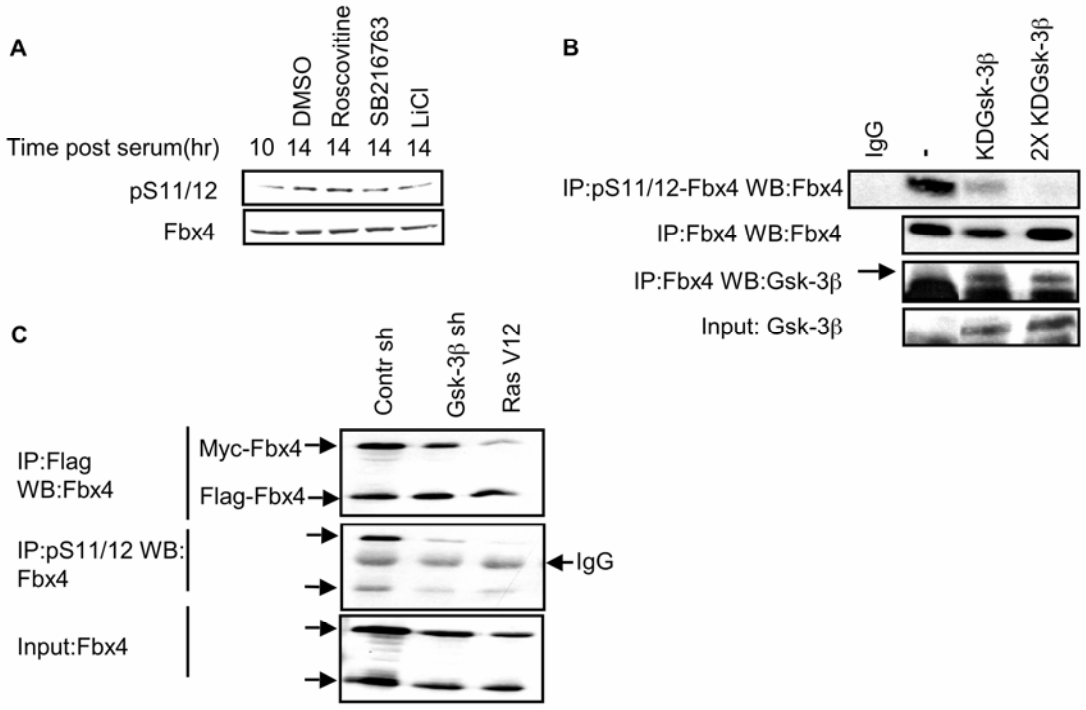
Supplemental figure 1. **A**. and **B.** Schematic representation of Fbx4 (human) deletions and mutants used in this study. **C.** Binding of Fbx4 deletion mutants to αB-crystallin was addressed by immunoprecipitation experiment using anti-αB crystallin antibody and western blotting with Fbx4 and αB-crystallin antibodies.



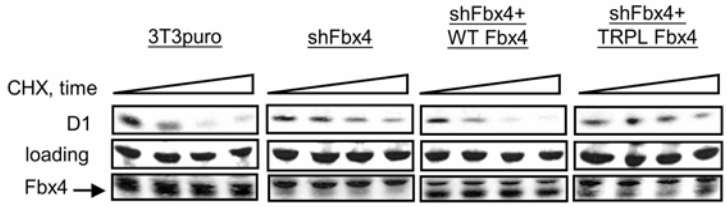
Supplemental figure 2. A. Lysates prepared from 293T cells expressing wild type Fbx4 or Fbx4S12A were subjected to precipitation with a pS11/12-Fbx4 antibody followed by Western blot with the Fbx4 antibody. **B.** Protein extracts from asynchronous NIH3T3 wt and shFbx4 cells were separated on SDS-PAGE gel followed by immunodetection with pS11/12-Fbx4 antibody.



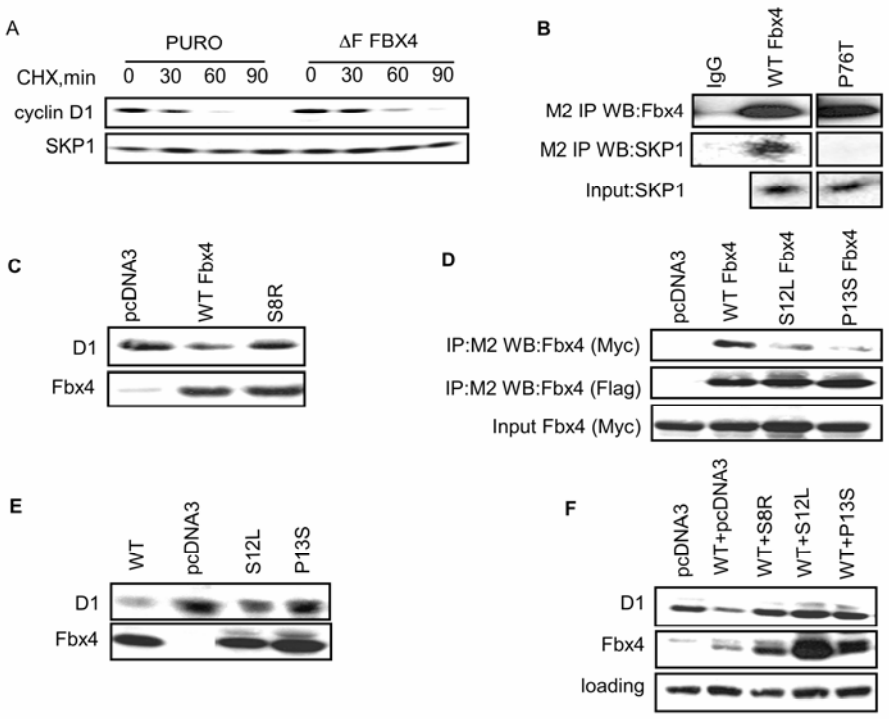
Supplemental figure 3. A. In vitro kinase assay performed using ^{32}P-ATP, purified Flag-Fbx4, GST-cyclin D1 or GST-D1T286A peptides and recombinant GSK3β. Reaction products were resolved by SDS-PAGE transferred to membrane and visualized by autoradiography or western analysis. **B.** Recombinant GST-Fbx4 was phosphorylated *in vitro* using indicated kinases. Reactions were precipitated using pS11/12-Fbx4 antibody followed by immunoblotting with Fbx4 antibody.



Supplemental figure 4. A. NIH3T3 cells were arrested in G0 phase by serum deprivation. 10 hrs following serum stimulation, cells were treated with indicated protein kinase inhibitors for 4 hrs. Both, Fbx4 phosphorylation and total protein levels were assessed by immunoblot. **B.** SF9 cells were infected with Fbx4 and kinase-defective GSK3β expressing baculoviruses. Fbx4 was precipitated using pS11/S12-Fbx4 (first panel) or total Fbx4 (second panel) antibodies. Fbx4 was detected by western blot using an antibody that recognizes total Fbx4 and associated GSK3β using an appropriate monoclonal antibody. An irrelevant lane was cropped between lanes two and three. **C.** 293T cells were co-transfected with Myc- and Flag-tagged Fbx4 constructs along with RasV12 or shGSK3β vectors. Fbx4 dimerization was analyzed by M2 IP followed by western blot with Fbx4 antibody.



Supplemental figure 5. NIH3T3 shFbx4 stable cell line was transfected with either wild-type or TRPL Fbx4. A cyclohexamide chase was performed, followed by analysis of cyclin D1 and Fbx4 levels by immunoblot.



Supplemental figure 6. A. Cyclin D1 half life experiment from cell lines used in mouse transplant experiment (Figure 4D). **B.** Cells were transfected with plasmids encoding either wild type Flag-Fbx4 or Flag-Fbx4P76T. Complexes were collected by M2 affinity chromatography and F bx4 or endogenous Skp1 detected by immunoblot. **C.** NIH3T3 shFbx4 cells were transfected with pcDNA3 as a control or Fbx4 wild type and S8R mutant. Asynchronous cells were harvested 48 hours after transfection and analyzed by immunoblot with cyclin D1 and Fbx4 antibodies. **D**. 293T cells were transfected with WT Myc-Fbx4 and Flag-tagged WT, S12L or P13S Fbx4. 24 hrs post transfection cells were lysed and protein extracts were precipitated with M2 agarose. Purified complexes were separated on SDS-PAGE followed by immunoblot with Fbx4 antibody. **E.** U2OS cells were transfected with indicated vectors and asynchronous cells were harvested 48 hrs post transfection, followed by immunoblot with cyclin D1 and Fbx4 antibodies. Irrelevant lanes were cropped between lanes 2 and 3. **F.** NIH3T3 cells were transfected with indicated vectors. Asynchronous cells were harvested 48 hrs after transfection and lysates were analyzed by immunoblotting with cyclin D1 and Fbx4 antibody.