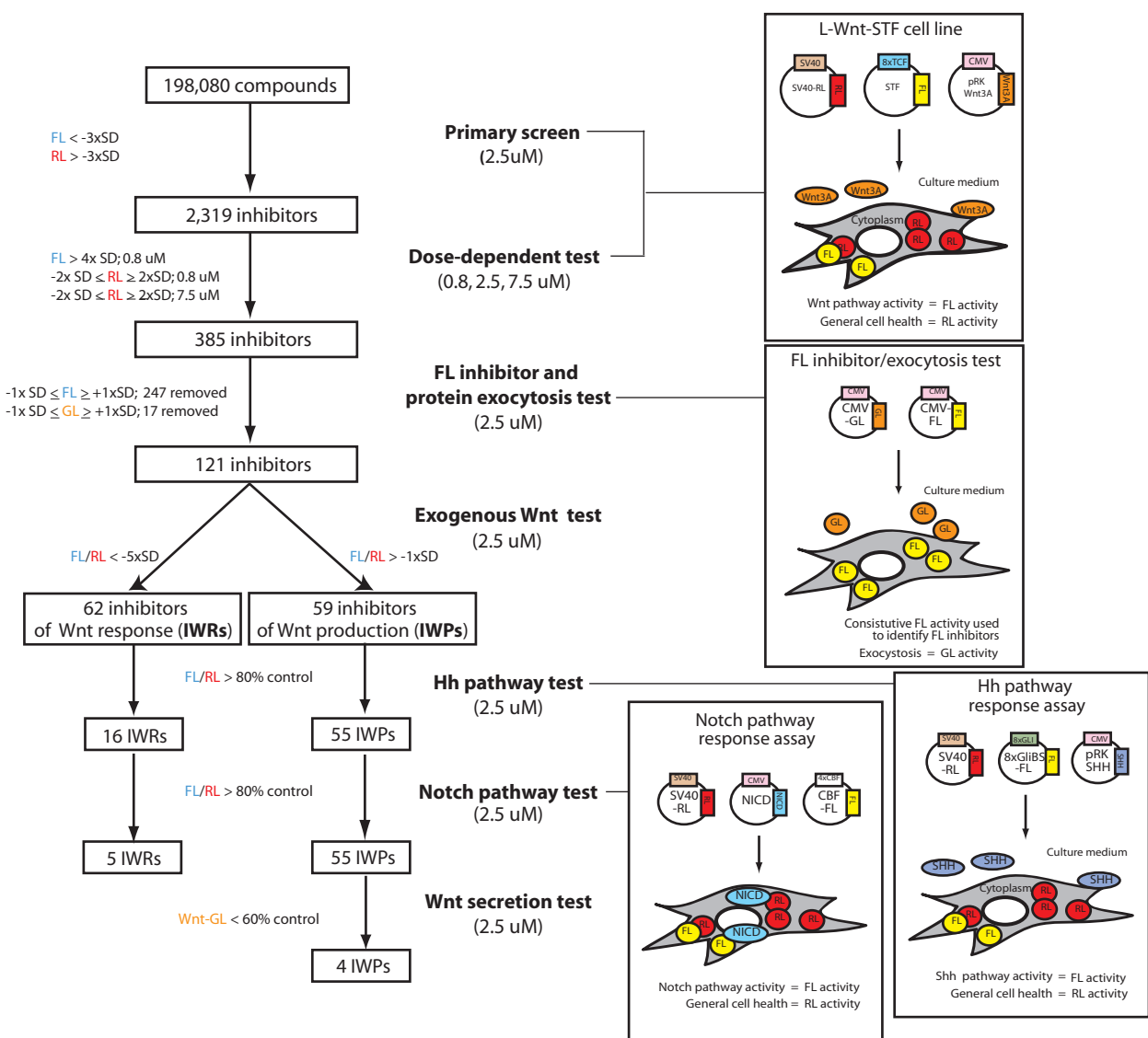


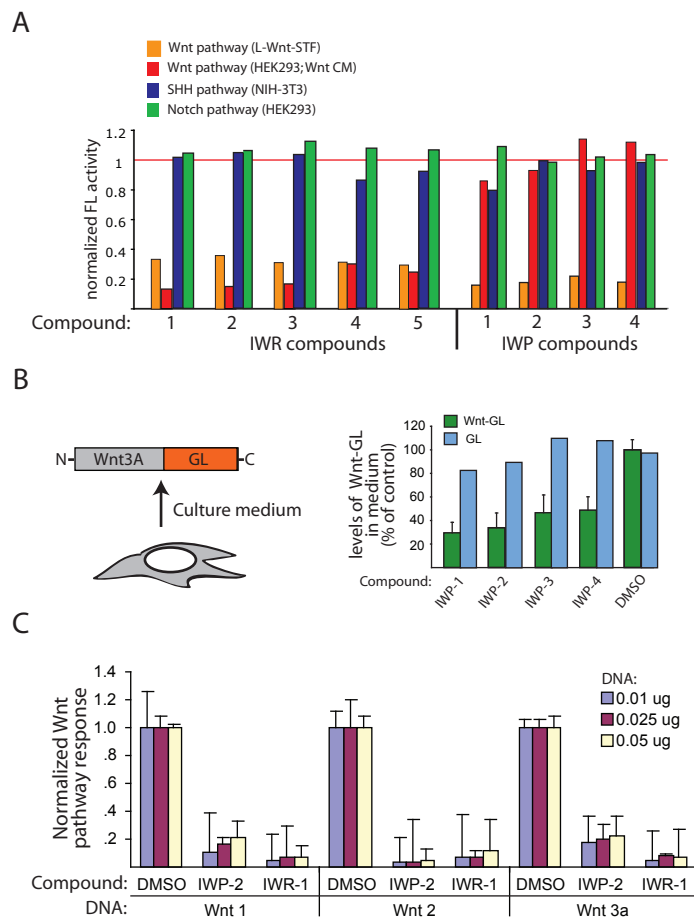
Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer

Supplementary Material

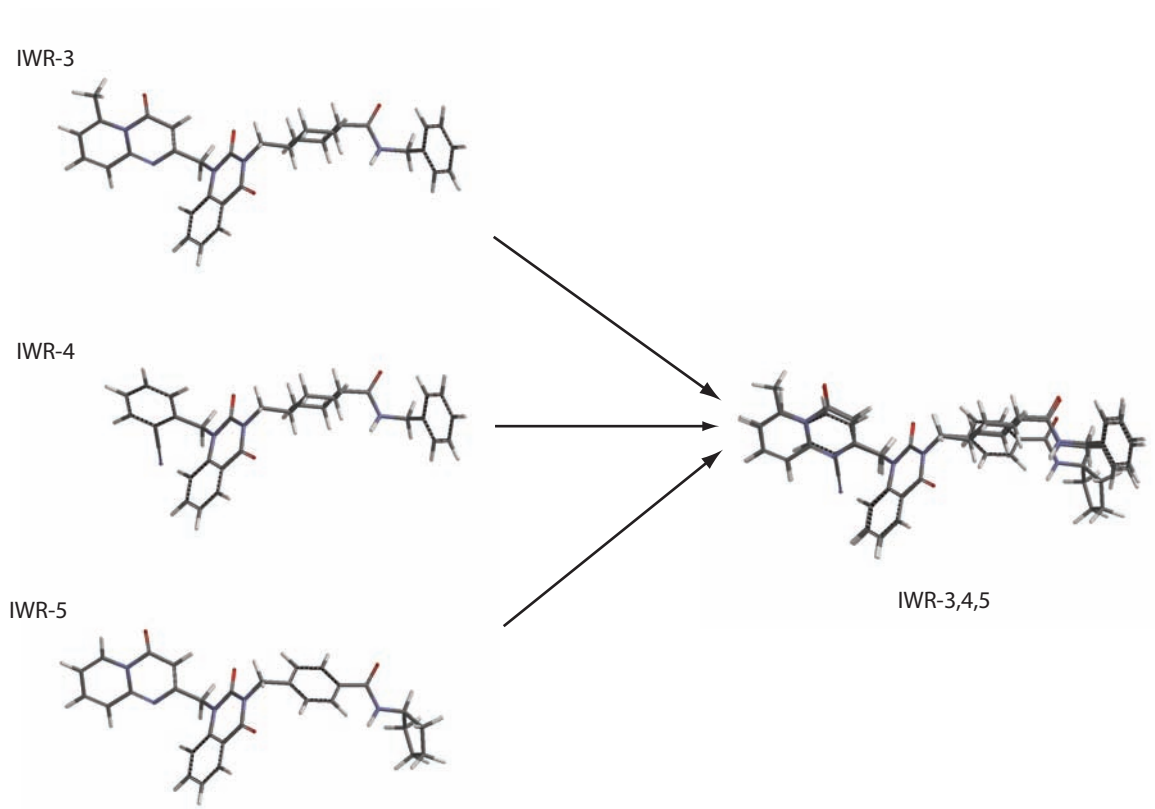
Baozhi Chen, Michael E. Dodge, Wei Tang, Jiangming Lu, Zhiqiang Ma, Chih-Wei Fan, Shuguang Wei, Wayne Hao, Jessica Kilgore, Noelle S. Williams, Michael G. Roth, James F. Amatruda, Chuo Chen, and Lawrence Lum



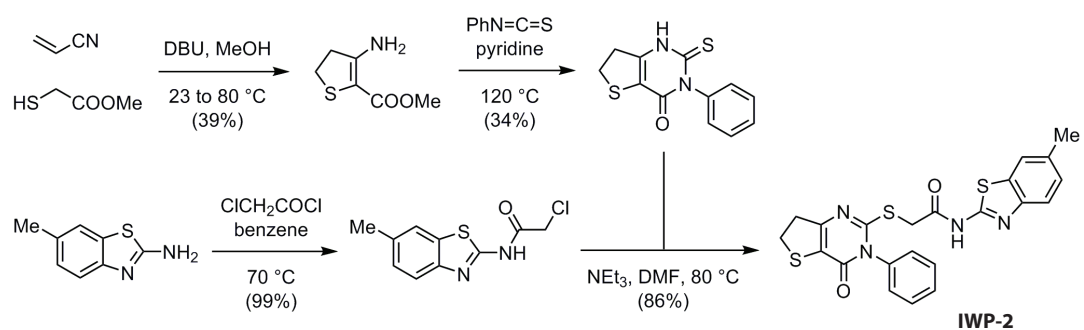
Supplemental Figure 1. Identification of small molecule antagonists of the Wnt/ β -catenin signal transduction pathway. The ~200K compound UTSouthwestern chemical library was screened using a cell line with constitutive Wnt/ β -catenin pathway activity maintained by cell-autonomous Wnt3A protein production (L-Wnt-STF cells; Primary screen). Potential Wnt/ β -catenin pathway antagonists were identified using a stably transfected Wnt-responsive firefly luciferase (FL) and control Renilla luciferase (RL) reporters. Approximately 1% of the compounds in the library that scored as hits was tested again in a dose-dependent manner to identify the most potent compounds with minimal cellular toxicity (Dose-dependent test). Compounds that abrogated FL activity by inhibition of FL activity, or that generally blocked cellular secretion of proteins were removed (FL inhibitor and exocytosis test). To separate compounds that either inhibit Wnt/ β -catenin pathway response or Wnt3A protein production, compounds were tested in HEK293 cells using the same assay as described in the Primary screen with the exception that exogenous Wnt3A protein (provided in conditioned medium) is used to stimulate pathway response (Exogenous Wnt test). Compounds that retained their anti-pathway activity in this test were considered Inhibitors of Wnt response (IWRs), whereas those that did not were considered Inhibitors of Wnt production (IWPs). Compounds from both categories were tested for effects on two other stem cell-associated signal transduction pathways (the Hh and Notch pathways) using cultured cell-based assays similar to those used to identify Wnt/ β -catenin pathway antagonists (Hh and Notch pathway tests). Hh and Notch pathways were stimulated using either Shh or an activated Notch (NICD) cDNA construct, respectively. Those compounds that minimally impacted these two pathways were considered to have specific activity for the Wnt/ β -catenin pathway. Lastly, IWPs were directly tested for their ability to inhibit Wnt3A protein secretion (Wnt secretion test; see Supp. Fig. 2b). Criteria for selecting hits are provided. In the end, five IWRs and four IWPs with high specificity for attacking the Wnt/ β -catenin pathway were selected for further analysis (see Fig. 1). Concentration of compounds used in each test is noted. Insets show schematics of assays used in the screen and secondary tests with the utility of each luciferase signal. Criteria used to identify compounds of interest are noted for each test.



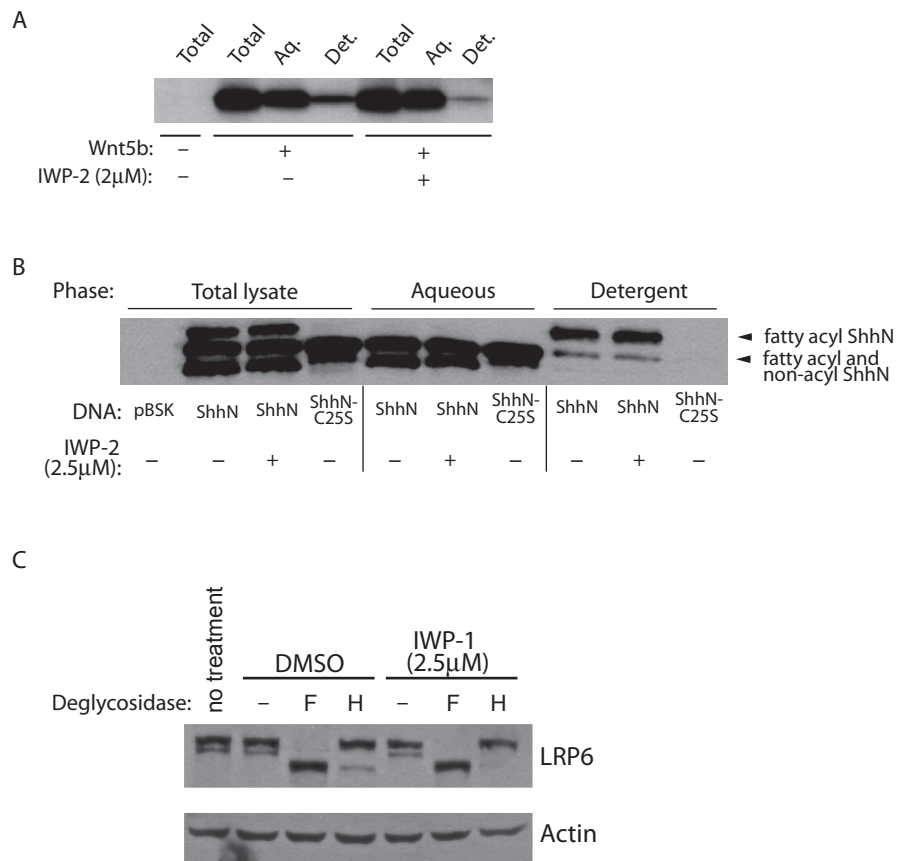
Supplemental Figure 2. IWR and IWP compounds specifically inhibit the Wnt/ β -catenin pathway. **(a)** Summary of results relating to IWR and IWP compounds from the screening process. Wnt pathway tests were performed in either cells responding to autonomously produced Wnt protein (L-Wnt-STF cells) or exogenously provided Wnt in conditioned medium (HEK293 cells). **(b)** IWP compounds inhibit Wnt3A secretion. Left: schematic of Wnt-Gaussia luciferase (Wnt-GL) fusion protein used to monitor levels of secreted Wnt protein in the cell medium. Right: levels of Wnt-GL but not GL secreted from cells treated with IWP compounds are decreased as compared to cells treated with carrier. The Wnt-GL protein elicits levels of Wnt/ β -catenin pathway response similar to that of Wnt3A protein (data not shown). **(c)** IWR and IWP compounds generally inhibit Wnt/ β -catenin pathway response induced by Wnt proteins. Pathway activity induced by Wnt1, Wnt2, or Wnt3A, and monitored using the STF reporter, is decreased in cells treated with either IWR-1 or IWP-2. FL activity was normalized to control RL activity as before.



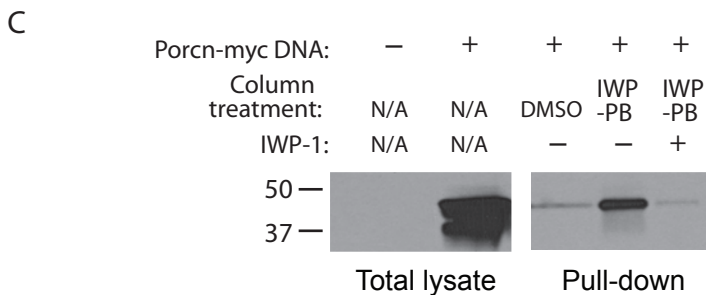
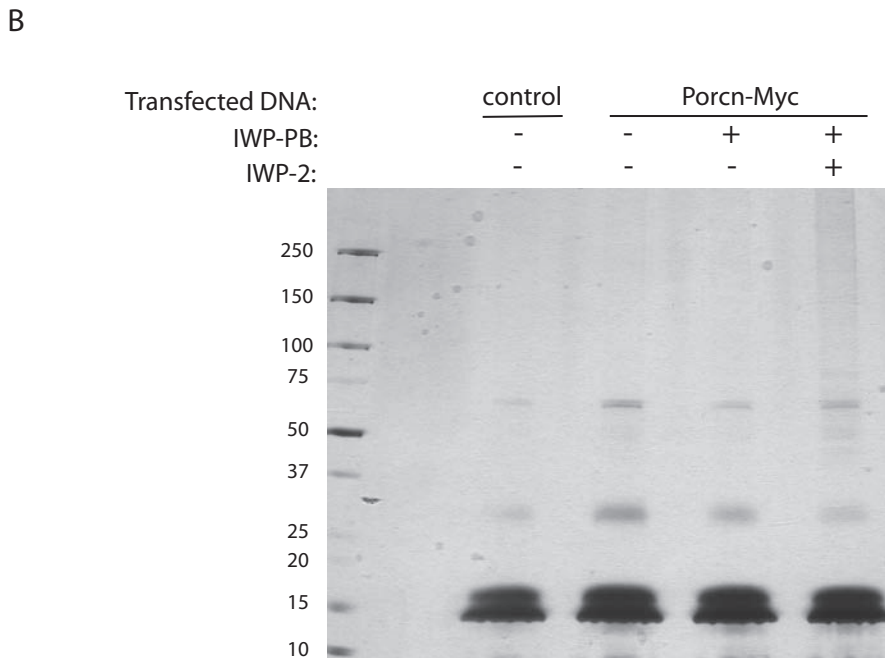
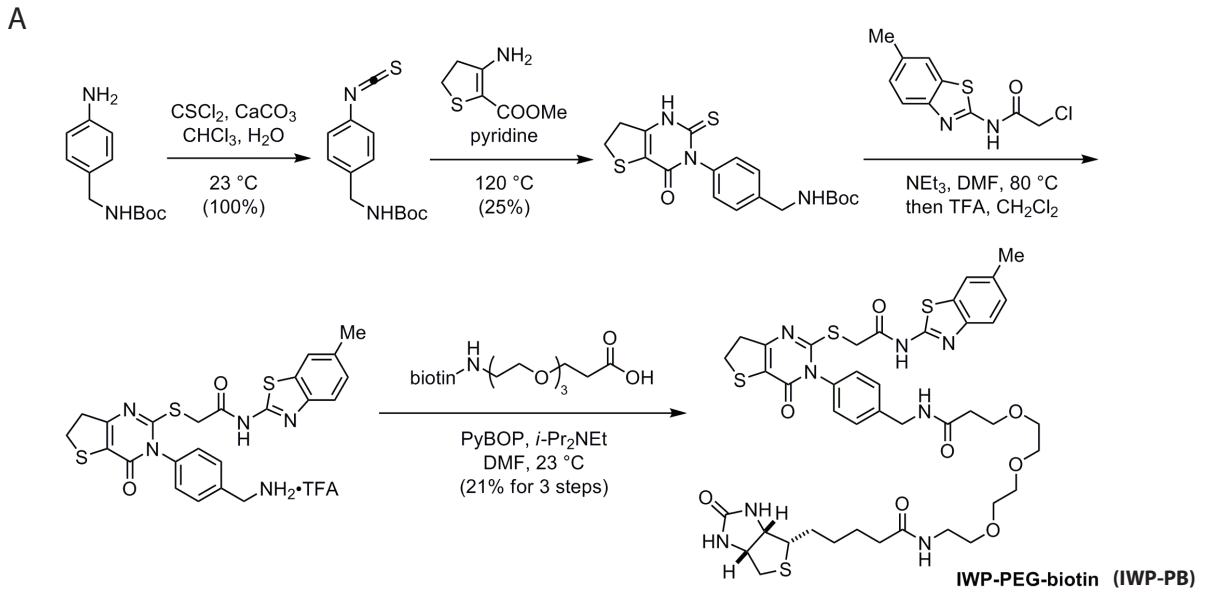
Supplemental Figure 3. IWR-3, -4, and -5 share structural similarity. Three-dimensional representation of IWR-3, -4, and -5 in equilibrium geometry using AM1 semi-empirical methods reveals similarities in structure. All three structures are superimposed on the right.



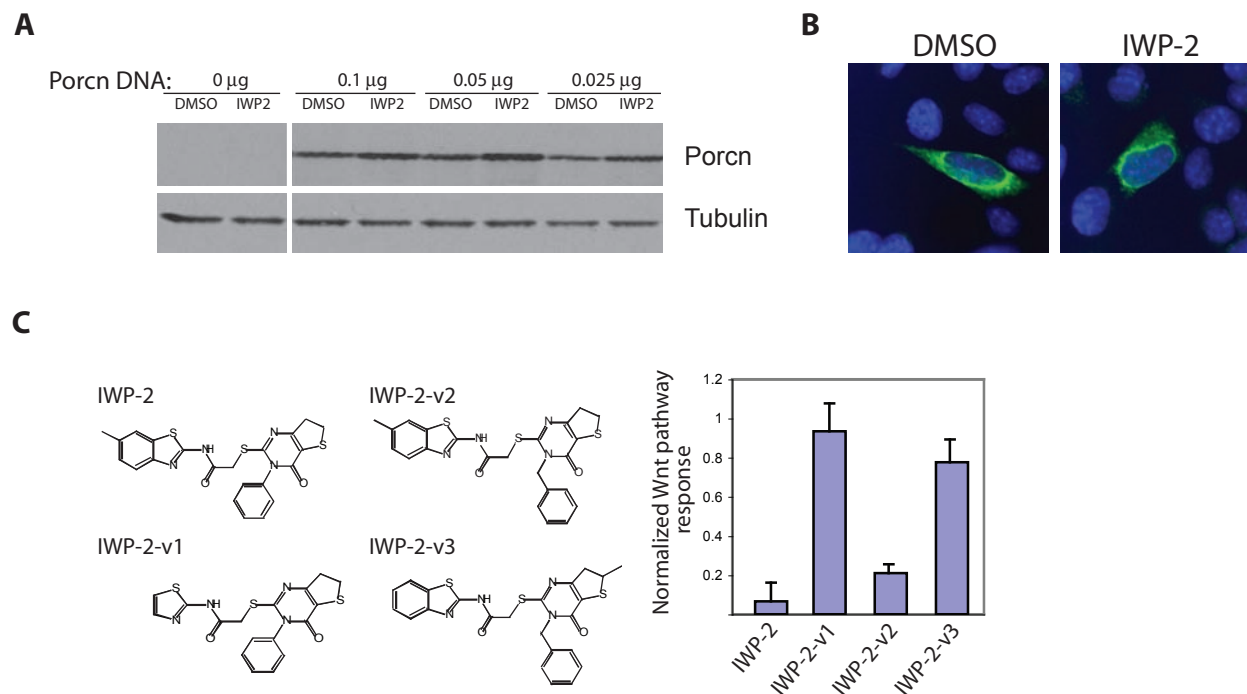
Supplemental Figure 4. Synthetic scheme for IWP-2. A similar synthetic route was taken to generate IWP-1.



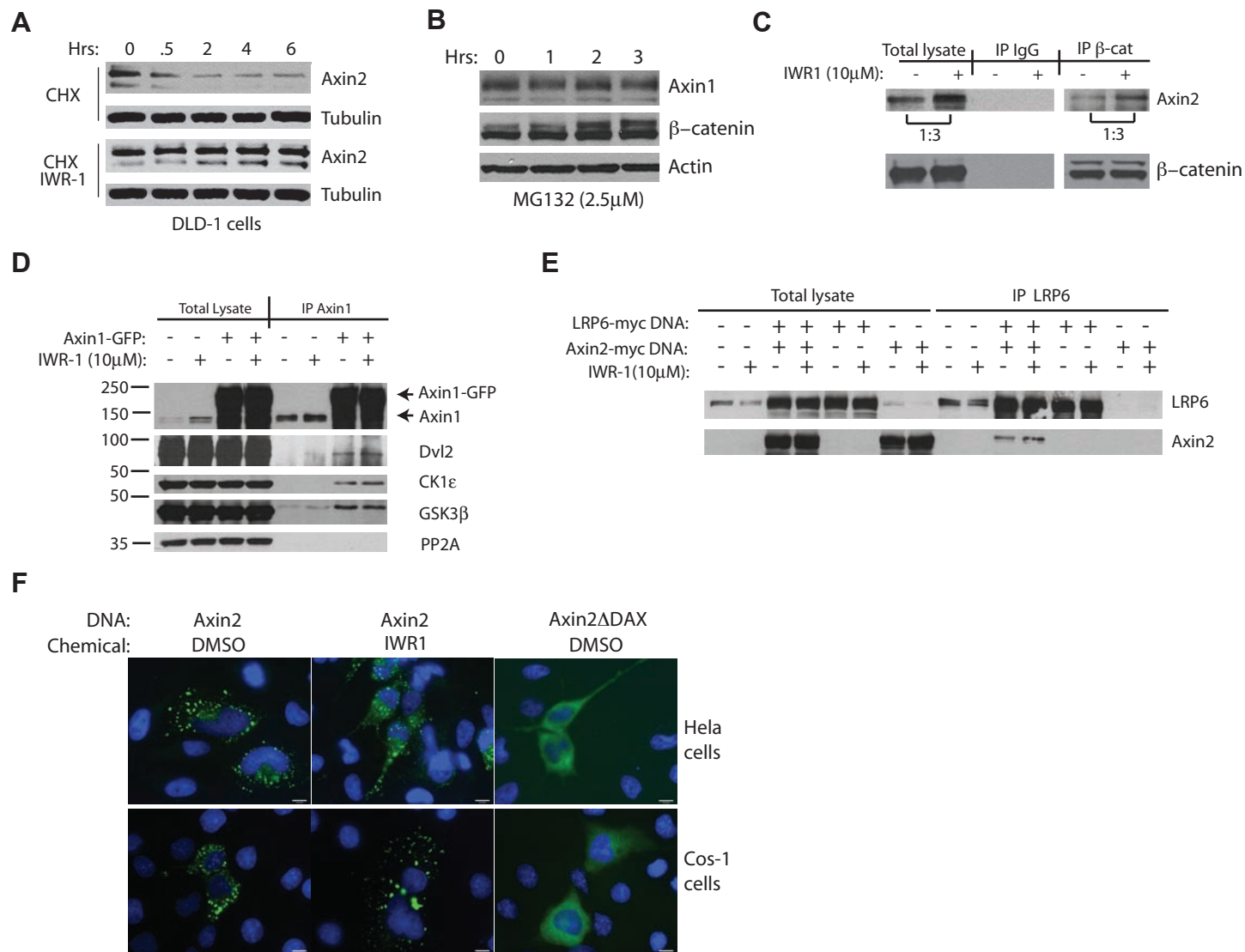
Supplemental Figure 5. Specificity tests for IWP compounds. **(a)** IWP compounds inhibit palmitoylation of Wnt5A, a Wnt protein that does not presumably activate β -catenin (so “non-canonical” Wnts). **(b)** IWP compounds do not affect maturation of LRP6, a process dependent upon palmitoyl modification of its cytotail domain. Lysate derived from DLD-1 cells treated with DMSO or IWP-1 was incubated with either Endo H (H) or PNGaseF (F), and then probed for LRP6 protein. IWP-1 does not alter LRP6 acquisition of EndoH resistance, suggesting it does not inhibit intracellular palmitoylation of LRP6. Sensitivity of LRP6 to PNGaseF confirms that LRP6 is glycosylated. **(c)** IWP on phase separation of the N-terminal signaling domain of the Shh protein (ShhN), a protein palmitoylated by Hhat/Rasp, another MBOAT family member.



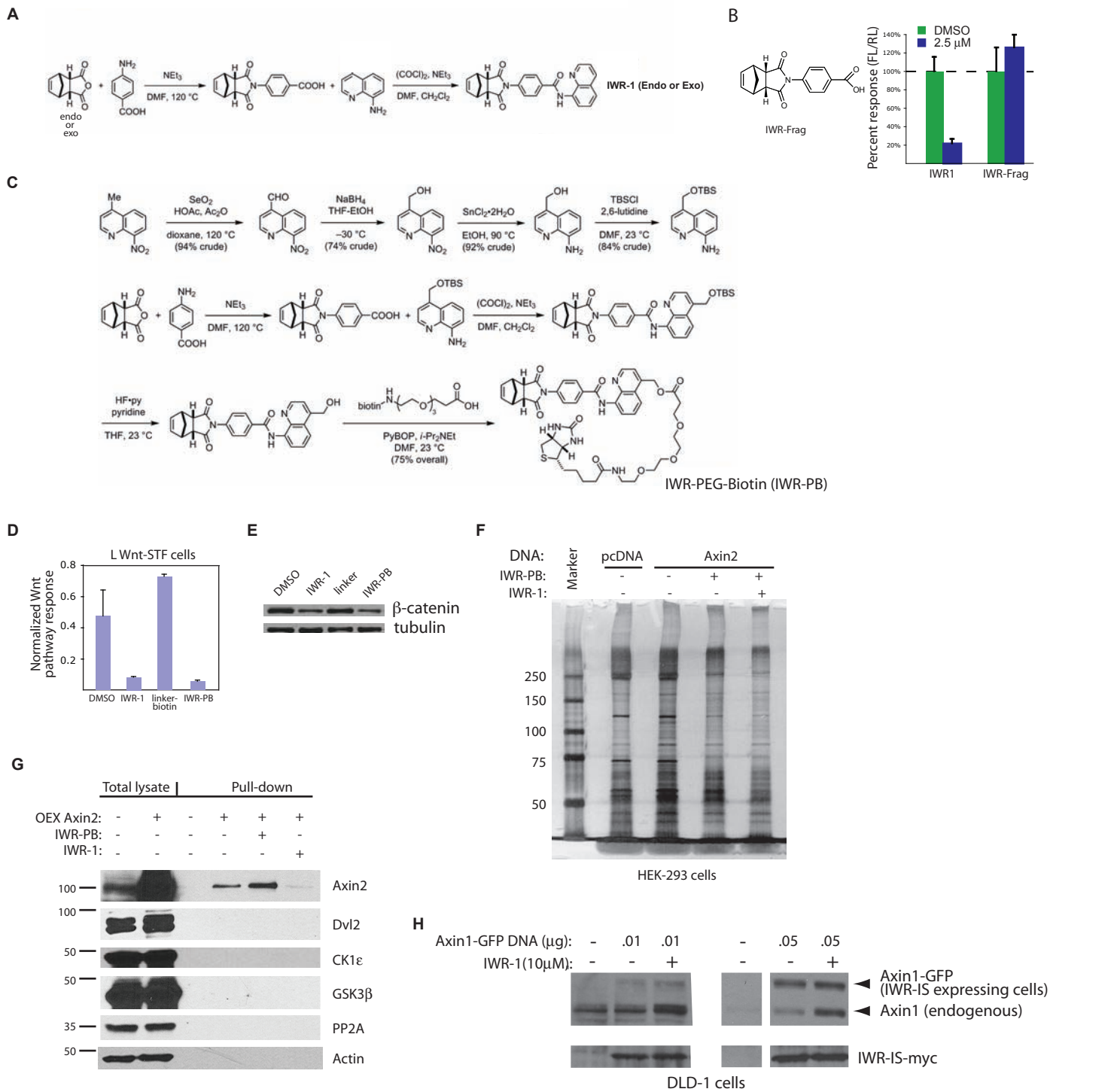
Supplemental Figure 6. Synthesis of IWP-PB and further characterization of its interaction with Porcn. (a) Synthetic route for a biotinylated IWP compound (IWP-PB). (b) Silver stain analysis of IWP-PB-bound material using identical conditions to those used in Fig. 3g. (c) IWP-1 can compete for Porcn binding to the structurally distinct IWP-2. A similar experiment as described in Fig. 3g except IWP-1 is used for competition.



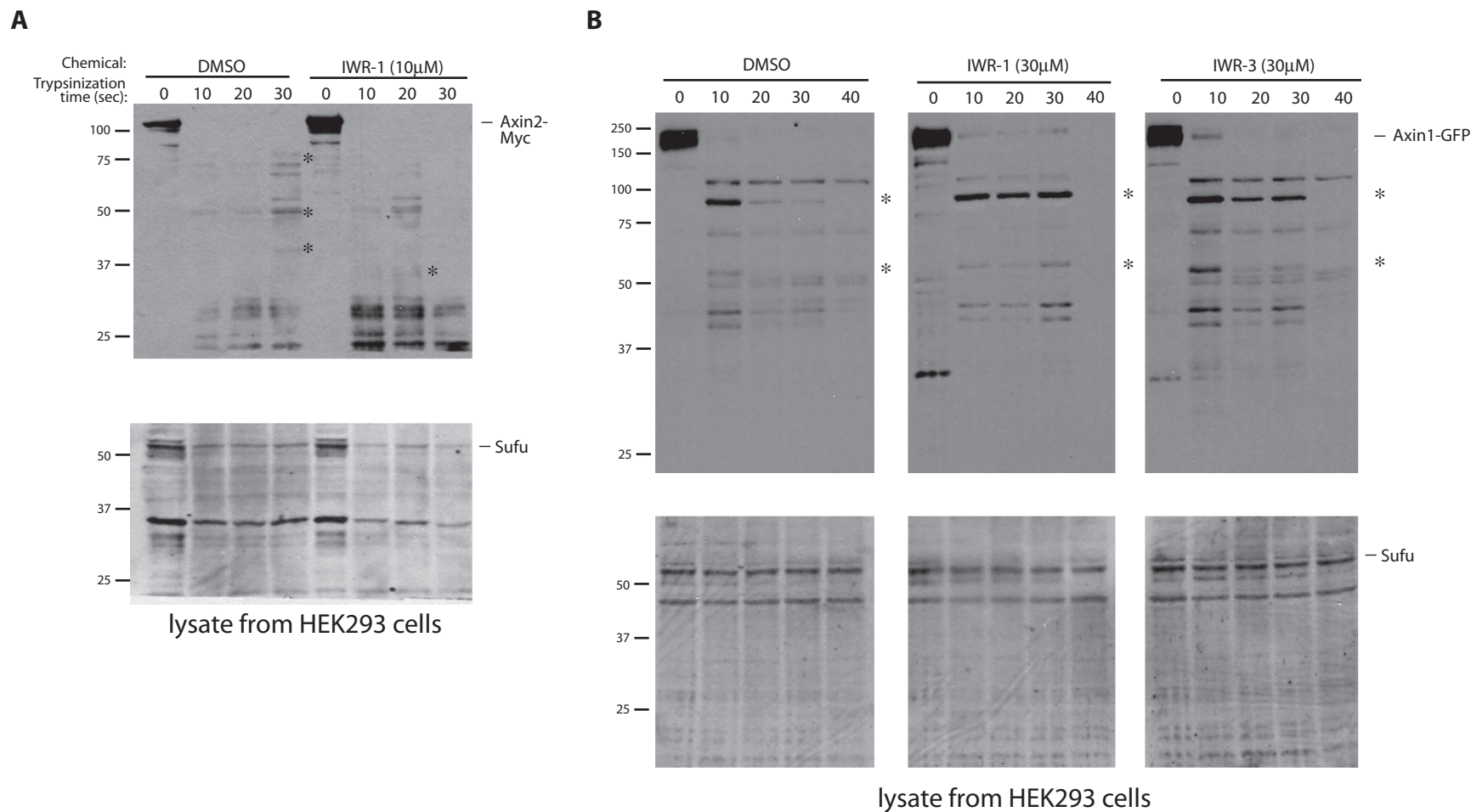
Supplemental Figure 7. Characterization of IWP action and specificity. **(a)** IWP-2 does not induce destruction of Porcn. Levels of overexpressed Porcn increase in the presence of IWP-2. **(b)** IWP-2 does not appear to alter localization of Porcn. **(c)** Chemical structure and activity of several compounds related to IWP-2. IWP-2-v2 retains activity against the Wnt/ β -catenin pathway as measured using the STF reporter (right), whereas IWP-2-v1 and -v3 do not, suggesting that the benzothiazole group is essential to IWP activity against Porcn.



Supplemental Figure 8. Characterization of IWR/Axin interactions. **(a)** IWR inhibits Axin2 protein destruction. Lysate from DLD-1 cells treated for with IWR-1 for increasing time durations in the presence or absence of cycloheximide (100μM) was subjected to Western blot analysis. In the absence of protein synthesis, IWR is able to block degradation of Axin2 protein. **(b)** Chemical inhibition of the proteasome does not phenocopy the effects of IWR-1 on Axin1 protein levels. Lysate from L-Wnt cells treated with either IWR-1 or the proteasome inhibitor MG132 for increasing time durations were subjected to Western blot analysis. Consistent with the inability of IWR-1 to inhibit proteasome function, we do not observe accumulation of β-catenin in cells treated with IWR compounds (see Fig. 2). **(c)** IWR does not alter the affinity between Axin2 and β-catenin. A similar ratio of Axin2 protein from cells treated with either DMSO or IWR-1 is observed from both β-catenin immunoprecipitation and total lysate, suggesting that IWR-1 induces little or no change in the affinity of β-catenin for Axin2. **(d)** IWR does not disrupt interaction of Axin1 with known binding partners. Lysate from HEK293 cells transfected with control or Axin-GFP and treated with or without IWR-1 was subjected with immunoprecipitation using an Axin1 antibody. Bound material was Western blotted for proteins previously shown to interact with Axin protein. We were unable to observe interaction between Axin and PP2A. **(e)** IWR does not disrupt LRP6-Axin interaction. Lysate from HEK293 cells transfected with LRP6-myc and/or Axin2-myc cDNAs and treated with or without IWR-1 was subjected to immunoprecipitation using an anti-LRP6 antibody. Precipitated material was subjected to Western blot analysis. **(f)** IWR compounds do not induce subcellular redistribution of Axin2 protein. HeLa or Cos1 cells transfected with Axin2 cDNA and treated either with DMSO or IWR-1, were immunostained with an anti-Axin2 antibody. Subcellular localization of Axin2 protein does not appear to be altered in the presence of IWR-1. Axin2 lacking the DAX domain, which has been previously shown to be important to normal Axin2 subcellular localization, is diffusely localized in the cytoplasm and is used here as a positive control.



Supplemental Figure 9. Synthetic scheme for IWR-1 and IWR-PB compounds. **(a)** Synthetic route for IWR-1. Endo and exo diastereomers result depending on the starting material. **(b)** A fragment of IWR-1 (IWR-Frag) is incapable of inhibiting Wnt pathway response in L-Wnt cells. **(c)** Synthetic scheme for a biotinylated IWR-1 (IWR-PB). **(d)** IWR-PB is active in cultured cells. **(e)** IWR-PB blocks accumulation of β -catenin in response in L-Wnt-STF cells. **(f)** Silver stain analysis of eluted proteins associated with IWR-PB/streptavidin agarose resin derived from HEK293 cells transfected with control or Axin2 expression constructs. The identical conditions were used in Western blot analysis of full-length Axin2 protein shown in Fig. 4f. **(g)** Axin2 interacts with IWR independently of other known Wnt pathway components. Material associated with IWR-PB under identical conditions used in Fig. 4f was probed by Western blot for various Wnt pathway components known to interact with Axin2. **(h)** IWR-IS abrogates IWR-induced Axin protein stabilization. Cells expressing IWR-IS exhibit mitigated Axin protein stabilization in response to IWR-1 as measured using Western blot analysis of a co-expressed Axin1-GFP protein. The IWR-1-dependent changes in endogenous Axin1 protein levels confirms the effectiveness of IWR-1 and reveals the low efficiency of DNA transfection in these experiments.



Supplemental Figure 10. Altered proteolytic sensitivity of Axin2 protein in cells treated with IWR. **(a)** Lysates derived from HEK293 cells expressing Axin2-myc protein treated with or without IWR-1 for 24 hrs were incubated with trypsin for indicated time periods in the presence or absence of additional IWR-1. Partial trypsinization of Axin2 (top) reveals IWR-1-sensitive cleavage sites and overall increased susceptibility to proteolysis. Re-probing of the same samples with an antibody recognizing an irrelevant protein [Suppressor of fused (Sufu)] reveals no IWR-dependent differences in the partial trypsinization profile. **(b)** Lysates derived from HEK293 cells expressing Axin1-GFP protein treated with IWR-1 or IWR-3 for 1 hr on ice prior to addition of trypsin for indicated time periods at room temperature. IWR-1 and IWR-3 are both able to alter the partial trypsinization profile of Axin1 (top), suggesting that their mechanism of action on Axin1 is similar to that in the case of Axin2. Sufu Western blot is a loading and trypsinization control (bottom). "*" denote Axin trypsin fragments with increased stability in the presence of IWR compounds.

Supplementary Methods

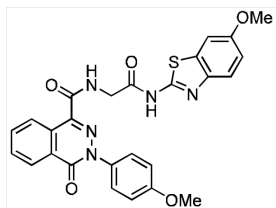
Reagents.

L-Wnt-STF cells were generated by transfecting L-Wnt cells (ATCC) with SuperTopFlash (STF; R. Moond) and SV-40 *Renilla* luciferase plasmids and selecting for clones resistant to G418 and Zeocin. The following expression constructs were generously provided by: P. Beachy (Shh and Wnt3A), R. Kopan (NICD), P.-T. Chuang (ShhN-C25S), and M. Brown and J. Goldstein (MBOAT family members). Wnt1, Wnt2, Wnt3, and Wnt5B expression constructs were purchased from OpenBiosystems. Notch reporter construct was provided by J. LaBorda. The Wnt-GL expression construct was generated by ligating Wnt3A coding sequence to GL (AA15-185) via an XbaI site. CMV-GL was generated by inserting the CMV promoter into the pGluc-Basic vector (New England Biolabs). Expression constructs for mPorcn-myc and hAxin2-myc constructs were engineered using PCR-based cloning and mutagenesis strategies. Axin-GFP construct was from M. Bienz. The source of primary antibodies used in this study are as follows: β -catenin, Kif3A, Actin, and β -tubulin (Sigma); p- β -catenin (Ser33/37/Thr41), GSK3 β , LRP6, P-Lrp6 (Ser1490), Dvl2, Shh, Axin1, and Axin2 (from Cell Signaling Technology); E-Cadherin and PP2A (BD Transduction Laboratories); Myc, Apc, and CK1 ϵ (Santa Cruz Biotechnology); and Gsk3 β (Stressgen). Pools of four pre-designed siRNA reagents used in RNAi experiments were purchased from either Qiagen (APC) or from Dharmacon (β -catenin). Axin2 primers used in RT-PCR experiments: 5'-AGCTCTGAGCCTTCAGCATC (reverse) and 5'-TCAGCAGAGGGACAGGAATC (forward).

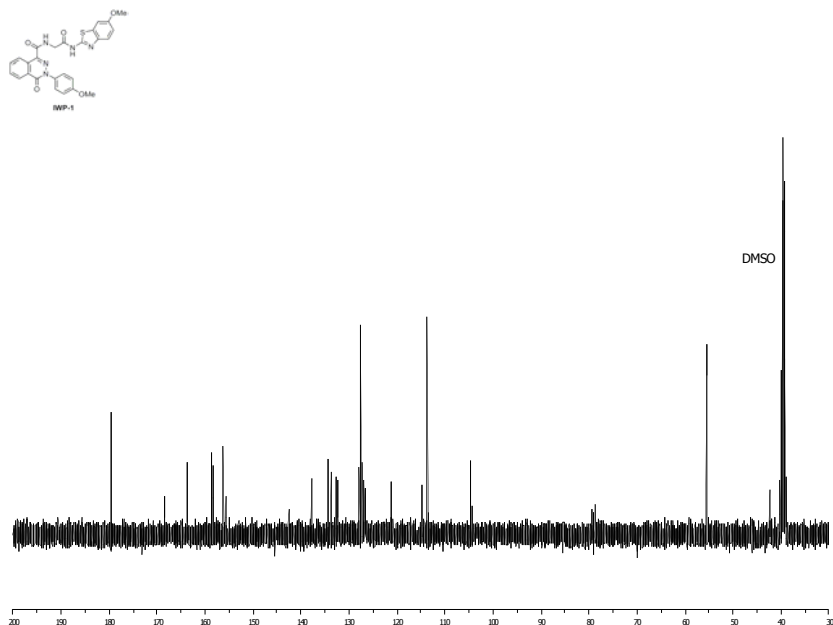
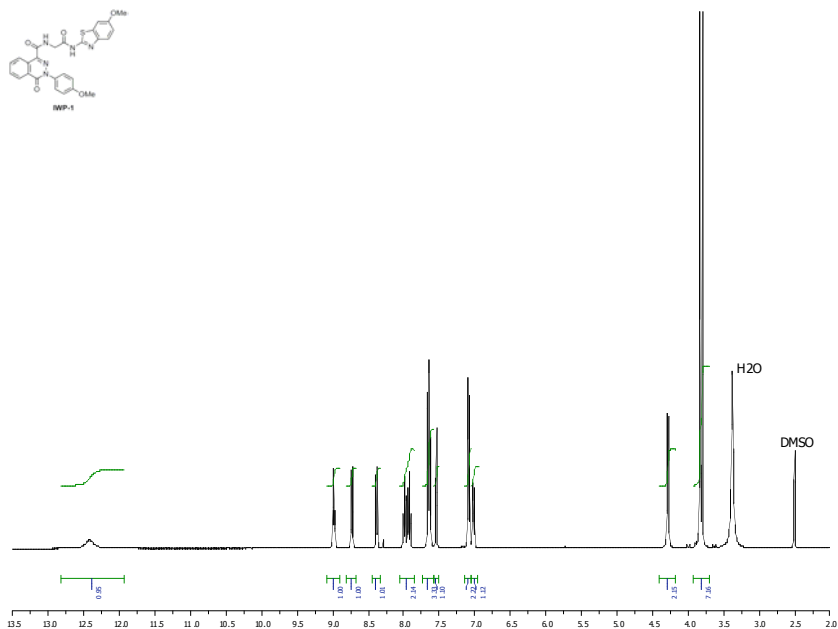
Radiolabeling experiments.

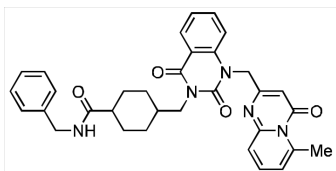
Wnt3A-myc and murine Porc expression constructs were transfected into Cos7 cells (6 well format, 150K cells/well) using Fugene6 transfection reagent (1:3 DNA:Fugene, Roche). After 48 hrs, the medium was replaced with DMEM (HyClone), 2.5% FBS and supplemented with 2.5 μ M IWP-2 (0.2% DMSO final) as appropriate. 37.5 μ Ci/mL H³-Palmitate (47.7 Ci/mmol, Perkin Elmer) was added to all wells, and media, compound, and radiation replaced after 2 hours. After 4 hrs total incubation time, cells were lysed (PBS/ 1% NP-40/ protein inhibitors) and Wnt3A-myc pulled down with anti-Myc monoclonal crosslinked to Protein G agarose. Precipitated material was run out on a 4-20% gradient gel (Biorad), transferred to nitrocellulose, and exposed through a LE Transcreen to film at -80°C for 14 days.

Chemical synthesis, continued.



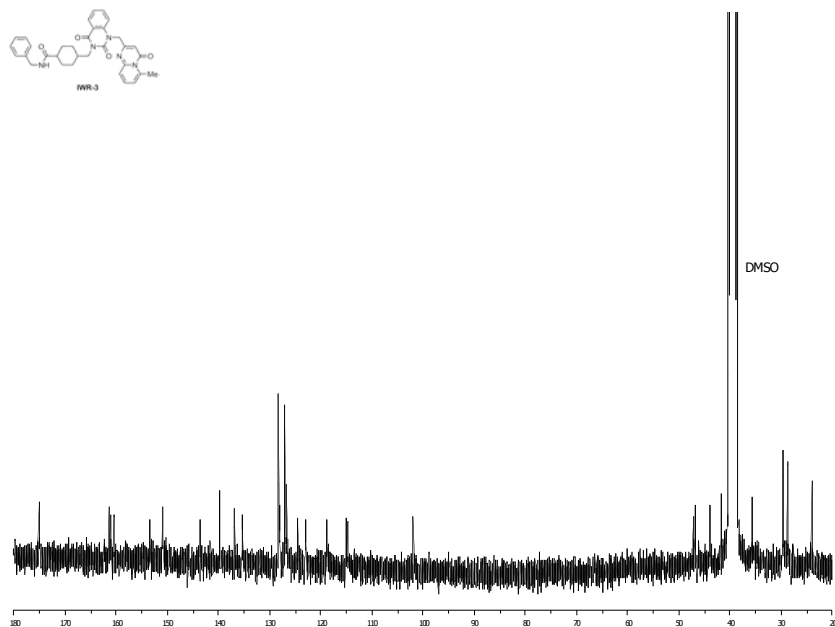
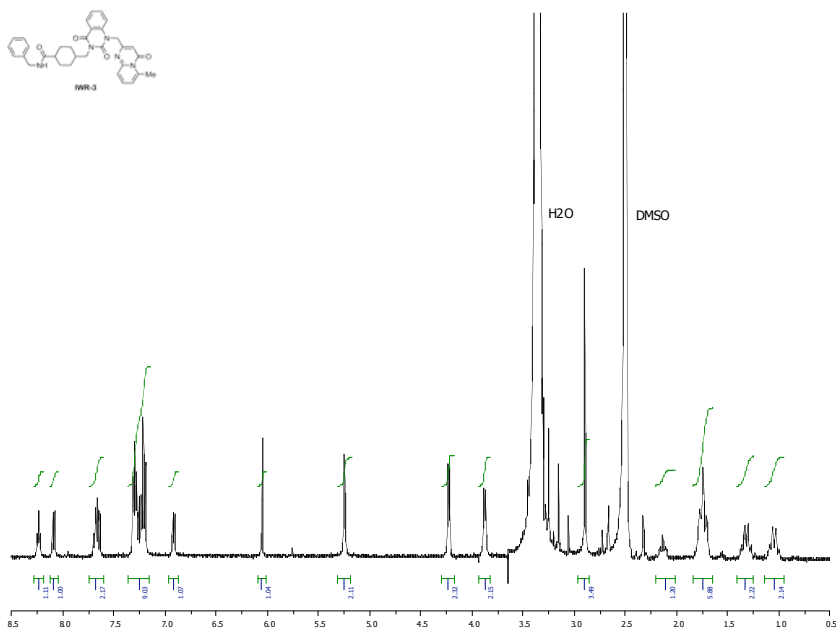
IWP-1: ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 12.42 (br, 1H, NH), 8.98 (t, $J = 5.9$ Hz, 1H, NH), 8.73 (d, $J = 7.8$ Hz, 1H), 8.38 (d, $J = 7.8$ Hz, 1H), 7.99 (dd, $J = 7.8, 7.6$ Hz, 1H), 7.92 (dd, $J = 7.8, 7.6$ Hz, 1H), 7.65 (d, $J = 8.2$ Hz, 2H), 7.63 (d, $J = 8.5$ Hz, 1H), 7.54 (d, $J = 2.3$ Hz, 1H), 7.08 (d, $J = 8.2$ Hz, 2H), 7.02 (dd, $J = 8.5, 2.3$ Hz, 1H), 4.28 (d, $J = 5.9$ Hz, 2H), 3.83 (s, 3H), 3.80 (s, 3H); ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ 179.7, 168.3, 163.7, 158.7, 158.4, 156.2, 155.7, 137.9, 134.3, 133.9, 132.8, 132.3, 128.0, 127.5, 127.3, 126.8, 126.7, 121.2, 114.9, 113.7, 104.7, 55.6, 55.5, 42.4; MS(ES^+) calcd for $\text{C}_{26}\text{H}_{22}\text{N}_5\text{O}_5\text{S}$ ($\text{M}+\text{H}$) $^+$ 516.1, found 516.1.

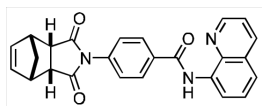




IWR-3: ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 8.24 (t, $J = 6.0$ Hz, 1H, NH), 8.09 (dd, $J = 7.8$, 0.4 Hz, 1H), 7.70–7.64 (m, 2H), 7.32–7.19 (m, 9H), 6.92 (d, $J = 6.9$ Hz, 1H), 6.05 (s, 1H), 5.25 (s, 2H), 4.23 (d, $J = 6.0$ Hz, 2H), 3.88 (d, $J = 6.9$ Hz, 2H), 2.90 (s, 3H), 2.16–2.11 (m, 1H), 1.77–1.71 (m, 5H), 1.37–1.28 (m, 2H), 1.09–1.00 (m, 2H); ^{13}C NMR (75 MHz, CDCl_3) δ 175.1, 161.5, 161.2, 160.6, 153.4, 151.0, 143.6, 139.9, 136.9, 135.4, 128.3, 128.2, 127.1, 126.7, 124.5, 123.0, 118.9, 115.1, 114.8, 101.9, 47.2, 46.9, 44.0, 41.8, 35.7,

29.7, 28.9, 24.1; $\text{MS}(\text{ES}^+)$ calcd for $\text{C}_{33}\text{H}_{34}\text{N}_5\text{O}_4$ ($\text{M}+\text{H}$) $^+$ 564.3, found 564.1.





IWR-1: ^1H NMR (400 MHz, CDCl_3) δ 10.72 (s, 1H, NH), 8.89 (dd, $J = 7.3, 1.1$ Hz, 1H), 8.81 (dd, $J = 4.1, 1.2$ Hz, 1H), 8.17 (dd, $J = 8.2, 1.1$ Hz, 1H), 8.13 (d, $J = 8.4$ Hz, 2H), 7.56 (dd, $J = 8.2, 7.3$ Hz, 1H), 7.53 (dd, $J = 8.2, 1.2$ Hz, 1H), 7.46 (dd, $J = 8.2, 4.1$ Hz, 1H), 7.37 (d, $J = 8.4$ Hz, 2H), 6.28 (s, 2H), 3.53 (s, 2H), 3.47 (s, 2H), 1.80 (d, $J = 8.8$, 1H), 1.62 (d, $J = 8.8$, 1H); ^{13}C

NMR (100 MHz, CDCl_3) δ 176.5, 164.6, 148.4, 138.8, 136.5, 135.2, 134.9, 134.8, 134.5, 128.2, 128.0, 127.5, 126.9, 122.0, 121.8, 116.6, 52.4, 46.0, 45.7; MS(ES^+) calcd for $\text{C}_{25}\text{H}_{20}\text{N}_3\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 410.2, found 410.1.

