

1 **Feedback in the β -catenin destruction complex imparts bistability and cellular**
2 **memory**

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34 **ABSTRACT**

35 Wnt ligands are considered classical morphogens, for which the strength of the cellular
36 response is proportional to the concentration of the ligand. Herein, we show an emergent property
37 of bistability arising from feedback among the Wnt destruction complex proteins that target the
38 key transcriptional co-activator β -catenin for degradation. Using biochemical reconstitution, we
39 identified positive feedback between the scaffold protein Axin and the kinase GSK3. Theoretical
40 modeling of this feedback between Axin and GSK3 predicted that the activity of the destruction
41 complex exhibits bistable behavior. We experimentally confirmed these predictions by
42 demonstrating that cellular cytoplasmic β -catenin concentrations exhibit an "all-or-none" response
43 with sustained memory (hysteresis) of the signaling input. This bistable behavior was transformed
44 into a graded response and memory was lost through inhibition of GSK3. These findings provide
45 a mechanism for establishing decisive, switch-like cellular response and memory upon Wnt
46 pathway stimulation.

47 **One Sentence Summary:**

48 Positive feedback within the β -catenin destruction complex gives rise to bistability and memory in
49 response to Wnt stimulation, imparting signal transduction accuracy and insulation.

50

51 RESULTS

52 Wnt/ β -catenin signaling is involved in organism development, stem cell maintenance and
53 is misregulated in human disease. At the core of this signaling pathway is the β -catenin destruction
54 complex, comprised of the kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 alpha
55 (CK1 α), and the scaffolding proteins Axin and Adenomatous polyposis coli (APC). In the absence
56 of Wnt ligands, phosphorylation of the transcriptional co-activator β -catenin within the destruction
57 complex targets β -catenin for ubiquitin-mediated proteasomal degradation, thereby maintaining
58 low levels of cytoplasmic and nuclear β -catenin. Wnt signaling inhibits phosphorylation of β -
59 catenin to block its turnover; accumulated β -catenin subsequently enters the nucleus to mediate a
60 Wnt-specific transcriptional program required for animal development and tissue homeostasis (1).

61 Although Wnt ligands are considered classical morphogens, Wnt gradients are dispensable
62 for proper patterning during development in some contexts (2-4). To better understand the
63 biochemical function of the β -catenin destruction complex and to assess how critical steps within
64 the complex impact behavior of the Wnt pathway, we performed biochemical reconstitutions of
65 the destruction complex with *Xenopus* egg extracts and purified proteins. Based on these
66 measurements, we developed mathematical simulations of destruction complex dynamics and
67 validated our model by performing single-cell analyses of β -catenin behavior.

68 Previous studies in cultured mammalian cells and *in vitro* reconstitution have shown that
69 the scaffold protein Axin is a direct target of GSK3 (5, 6). Because *Xenopus* egg extracts are

70 readily amenable to biochemical studies and faithfully recapitulate signaling dynamics that control
71 β -catenin turnover (7), we examined the regulation of Axin by GSK3 in extracts (Fig. 1A).
72 Consistent with previous studies (5), inhibition of GSK3 with LiCl induced Axin turnover (Fig.
73 1B,C). Stabilization of Axin required both the GSK3 phosphorylation sites at serine 322 and serine
74 326, and the GSK3 binding site (GBS) on Axin (Fig. 1C) (8).

75 As Axin is the limiting component of the destruction complex, overexpression of Axin
76 promotes β -catenin degradation and inhibits Wnt signaling even in the absence of APC (9, 10).
77 The limiting concentration of Axin provides a simple means for insulating a discrete pool of GSK3
78 that specifically targets β -catenin for phosphorylation (10). In addition, given its role as a scaffold,
79 Axin is ideally positioned to regulate the activity of GSK3, thereby promoting both Axin stability
80 and β -catenin degradation. We initially examined GSK3 activity in *Xenopus* egg extracts using a
81 phospho-specific antibody that recognizes GSK3 β phosphorylation at serine 9 (pS9 GSK3), which
82 limits GSK3 β activity. The addition of recombinant Axin to extracts resulted in a marked reduction
83 in pS9 GSK3 (Fig. 1D). The requirement for a phosphatase in β -catenin degradation has been
84 reported (11). Thus, we tested the effect of the phosphatase inhibitor okadaic acid (OA) on pS9
85 GSK3. We found that OA prevented the Axin-mediated reduction of pS9 GSK3 in *Xenopus*
86 extracts (Fig. 1D), suggesting an OA-sensitive phosphatase requirement at this regulatory step.

87 To identify Axin regions that bind co-factors necessary for pS9 GSK3 dephosphorylation,
88 we performed domain deletion analysis by expressing Axin mutants in HEK 293 cells (Fig. 1E).

89 As expected, full-length Axin promoted loss of the inhibitory phosphorylation of GSK3, and OA
90 blocked this effect, suggesting phosphatase dependence of GSK3 activation. Similarly, the
91 deletion of the GSK3 binding site (GBS) or the phosphatase 2A (PP2A) domains of Axin prevented
92 Axin-mediated inhibition of GSK3 phosphorylation, suggesting these regions are essential for
93 GSK3 activation by Axin. In contrast, Axin lacking its β -catenin binding site (β cat-BS), APC
94 binding site (RGS), or DIX domain still promoted the removal of the inhibitory serine 9
95 phosphorylation on GSK3; thus, these sites are not required for Axin-mediated removal of serine
96 9 phosphorylation on GSK3. Additionally, Axin was recently shown to contain a short linear motif
97 (SLiM) that interacts with the B56 subunit of PP2A (12, 13). We made alanine mutants of this
98 conserved SLiM sequence (Fig. S1) and found that SLiM 4A Axin mutants could not remove pS9
99 on GSK3 (Fig. 1F).

100 To test if PP2A could directly act on pS9 GSK3, we performed *in vitro* reconstitution using
101 purified components of the destruction complex. We found that PP2A exhibited a preference for
102 pS9 GSK3 (Fig. 1G) versus the β -catenin sites phosphorylated by GSK3 (phospho-serine 33, serine
103 37, and threonine 41; Fig. 1H). Based on these findings, we propose the following model: the
104 majority of cytoplasmic GSK3 is in or fluctuating as the pS9 GSK3 state, which normally limits
105 its activity. Upon pS9 GSK3 binding to Axin, pS9 GSK3 is targeted for dephosphorylation by
106 Axin-bound PP2A. Dephosphorylated GSK3 is active and phosphorylates Axin to promote its
107 stabilization. Active, dephosphorylated GSK3 and phosphorylated Axin (bound to APC) comprise

108 a destruction complex state that is "fully activated" to phosphorylate β -catenin, targeting it for
109 ubiquitin-mediated proteasomal degradation.

110 We built a theoretical model based on our biochemical observations to better understand
111 the reaction kinetics within the β -catenin destruction complex (Fig. 2A). GSK3 concentration was
112 kept constant as it is predicted to be degraded at a relatively slow rate (10). Rates of Axin synthesis
113 and degradation were based on our *Xenopus* extract data and previous work (10). We translated
114 our model (Fig. 2A) into a set of ordinary differential equations (ODEs) (TableS1) and solved
115 them numerically and analytically in steady-state conditions (Fig. S2). The reaction rates and rate
116 constants used in the model are listed in Table S2 and Table S3, respectively. As shown in Fig.
117 2A, our model showed a positive feedback loop between Axin and GSK3.

118 The function of the destruction complex is to promote the phosphorylation and subsequent
119 ubiquitin-mediated degradation of β -catenin. When Axin^P and GSK3 are high, β -catenin is low,
120 and the pathway is "off." When Axin^P and GSK3 are low (e.g., via Wnt activation), cytoplasmic
121 and nuclear β -catenin is high, and the pathway is "on." Consistent with previous work, we modeled
122 the Wnt signal to act on the active, destruction complex-bound GSK3 by directly increasing the
123 inhibitory rate (k_1) (14) and calculated the steady-state concentration of β -catenin. To model
124 pathway activation, we started initially with a low value of k_1 , which was followed by a gradual
125 increase. For each k_1 value, the β -catenin concentration was determined and plotted as "naive."
126 Additionally, we solved the equations with decreasing values of k_1 , starting with a high value, and

127 referred to this as "pre-activated." As shown in Fig. 2B, for a range of $k_1 < 0.85$, we found that the
128 Wnt signal strength needed to stabilize β -catenin from the naive state was higher than the signal
129 strength needed to maintain β -catenin once the system had been pre-activated. In contrast, when
130 dephosphorylation of GSK3 by Axin^P was omitted from the model, the effect of k_1 was identical
131 for "naïve" and "pre-activated" states (Fig. 2C).

132 Our modeling suggests the β -catenin destruction complex has two stable states, "off" and
133 "on". This bistability may result from self-perpetuating states such as positive feedback or double-
134 negative feedback (15). A bistable system is characterized by two alternative steady states, an off-
135 state and an on-state, without intermediate states (Fig. 3A) (15). In a population of cells, the
136 inflection point of the switching will be set by subtle variation in the concentrations and rates of
137 pathway components. This is why, in a uniform sheet of cells, one often observes a salt-and-pepper
138 phenotype rather than perfect collective switching. To simulate the heterogeneous response of a
139 population of cells, we ran the simulation for 2,000 cells where we randomly selected a k_1 value
140 for each cell from a normal distribution (Fig. S3). We then plotted the distribution of β -catenin
141 with an increase in the mean value of k_1 (Fig. 3B) that demonstrates the expectation for β -catenin
142 response in a noisy tissue culture system.

143 We then validated whether the positive feedback between Axin and GSK3 observed in
144 *Xenopus* egg extracts can lead to a bistable response in mammalian cells activated by Wnt ligands.
145 Accurate single-cell quantification of soluble β -catenin has been challenging due to the high

146 concentrations of non-signaling β -catenin at adherens junctions. We combined automated imaging
147 with custom cell-identification software (Fig. 3C) to analyze primary, immortalized human colonic
148 epithelial cells (HCECs) (16). By varying concentrations of purified, recombinant Wnt3a, and
149 measuring nuclear β -catenin, we found that at low Wnt3a concentrations, signaling is in the off-
150 state (nuclear β -catenin is absent), whereas, at high Wnt concentrations, signaling is in the on-state
151 (nuclear β -catenin is present) (Fig. 3D). At intermediate Wnt3a doses, we found a mixed
152 population of cells that were either in the off- or the on-state, with a bistable range between 2 and
153 6 nM Wnt3a. The β -catenin response to increasing Wnt3a exhibited a Hill-coefficient of ~ 6 ,
154 suggesting cooperativity among responding factors (Fig. S4).

155 For a system to be genuinely bistable, it must exhibit hysteresis, i.e., the concentration of
156 Wnt needed to induce a given response (after Wnt exposure) is lower than the concentration of
157 Wnt required to mediate the initial response (15). An alternative possibility for the salt-and-pepper
158 β -catenin phenotype we observed is the existence of a monostable transcritical bifurcation, a
159 mechanism often found in phase separation settings, in which a two-state system switches states
160 at a single inflection point (i.e., turning on and turning off occur at the same ligand concentration)
161 (17). To test this, we fully activated cells with high concentrations of Wnt ligand and then
162 measured the concentrations of Wnt needed to maintain the on-state (Fig. 4A). We observed that
163 cells that were previously treated with a high concentration of Wnt maintained nuclear β -catenin

164 throughout the experiment (Fig. 4B,C) and that this hysteretic behavior persisted several hours
165 after the initial Wnt3a treatment (Fig. S5, S6A).

166 We next tested whether the bistable response observed in HCEC cells in response to Wnt3a
167 treatment was due to positive feedback between Axin and GSK3 revealed in our biochemical
168 experiments (Fig. 1). Our modeling suggested bistability was lost by removing the function of
169 Axin^P in the dephosphorylation of GSK3 (Fig. 5A,B). Wnt ligand-mediated activation of the Wnt
170 pathway occurs via a mechanism involving inhibition of GSK3-mediated β -catenin
171 phosphorylation (14, 18, 19). We predict, however, that direct inhibition of GSK3 with a small
172 molecule inhibitor that targets its ATP catalytic pocket would break the biochemical GSK3/Axin
173 feedback loop by being insensitive to Axin^P-dependent dephosphorylation of pS9 GSK3 (Fig. 5C).
174 We treated HCEC cells with the GSK3 inhibitor CHIR99021 (GSK3i) (20) and found that, in
175 contrast with the Wnt3a treatment regimen, activation of the pathway with GSK3i treatment failed
176 to promote the bistable behavior of nuclear β -catenin (Fig. 5D). Unlike Wnt3a, the effects of
177 GSK3i on β -catenin nuclear accumulation were readily reversible, and the nuclear β -catenin signal
178 was lost rapidly (without any observable evidence of hysteresis) after the removal of GSK3i (Fig.
179 S6B). Hence, for all GSK3i experiments, we used time points for which we observed a steady-
180 state response after GSK3i treatment, i.e., a minimum of 6 hours treatment (Fig. S7A), but we
181 could not wash out the inhibitor because β -catenin levels rapidly reset to baseline (Fig. S7B).
182 Finally, we observed that GSK3i caused cells to respond in a monostable, graded manner (Fig. 5D,

183 S8). These experimental findings further support the conclusion from our biochemical
184 reconstitution and mathematical model: bistability and hysteresis in Wnt signaling are driven by
185 positive feedback between Axin and GSK3.

186 **DISCUSSION**

187 These experiments demonstrate that a biochemical feedback loop between GSK3 and Axin
188 maintains the β -catenin destruction complex in a stable off- or on-state. This switch-like behavior
189 requires the mutual regulation of GSK3 and Axin via antagonistic behaviors of an additional kinase
190 and phosphatase. We also provided evidence that the PP2A phosphatase acts on GSK3 to remove
191 the inhibitory phosphorylation on serine 9. Modeling of these biochemical events predicted that
192 cells would respond in a binary manner to Wnt pathway stimulation, which was supported by our
193 experiments in human colonic epithelial cells. Additionally, these cells displayed memory to Wnt
194 stimulation, and β -catenin remained in the nucleus even after Wnt ligands had been removed.
195 These results suggest the β -catenin destruction complex displays robustness by existing in two
196 self-sustaining attractor states of active and inactive, which provides a mechanism for suppressing
197 potentially deleterious fluctuations in concentrations and activities of pathway components.

198 Bistability has emerged as a foundational principle in signal transduction (21), yet its
199 existence has been elusive in the Wnt pathway. Beyond suppressing noise within the Wnt pathway,
200 positive feedback in the β -catenin destruction complex provides a mechanism to insulate a pool of
201 GSK3 required in the complex from the total cellular GSK3, thereby preventing crosstalk with

202 other GSK3-regulating pathways such as PI3K/AKT and MAPK (22, 23). Furthermore, the
203 existence of both bistable and graded responses could explain why long-range Wnt morphogen
204 activity is dispensable in certain in vivo contexts but essential in others(24). The phenomena
205 described herein shed light on a foundational structure of the Wnt/ β -catenin pathway that instills
206 robustness and, when perturbed, could lead to vulnerabilities in the accurate processing of Wnt
207 signals.

208

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220

221 **References:**

- 222 1. R. Nusse, H. Clevers, Wnt/beta-Catenin Signaling, Disease, and Emerging Therapeutic
223 Modalities. *Cell* **169**, 985-999 (2017).
- 224 2. C. Alexandre, A. Baena-Lopez, J. P. Vincent, Patterning and growth control by membrane-
225 tethered Wingless. *Nature* **505**, 180-185 (2014).
- 226 3. L. A. Baena-Lopez, X. Franch-Marro, J. P. Vincent, Wingless promotes proliferative
227 growth in a gradient-independent manner. *Science signaling* **2**, ra60 (2009).

- 228 4. G. Morata, G. Struhl, Developmental biology: Tethered wings. *Nature* **505**, 162-163
229 (2014).
- 230 5. H. Yamamoto *et al.*, Phosphorylation of axin, a Wnt signal negative regulator, by glycogen
231 synthase kinase-3beta regulates its stability. *The Journal of biological chemistry* **274**,
232 10681-10684 (1999).
- 233 6. K. Willert, S. Shibamoto, R. Nusse, Wnt-induced dephosphorylation of axin releases beta-
234 catenin from the axin complex. *Genes Dev* **13**, 1768-1773 (1999).
- 235 7. A. Salic, E. Lee, L. Mayer, M. W. Kirschner, Control of beta-catenin stability:
236 reconstitution of the cytoplasmic steps of the wnt pathway in *Xenopus* egg extracts. *Mol*
237 *Cell* **5**, 523-532 (2000).
- 238 8. L. Ji *et al.*, The SIAH E3 ubiquitin ligases promote Wnt/beta-catenin signaling through
239 mediating Wnt-induced Axin degradation. *Genes Dev* **31**, 904-915 (2017).
- 240 9. T. Nakamura *et al.*, Axin, an inhibitor of the Wnt signalling pathway, interacts with beta-
241 catenin, GSK-3beta and APC and reduces the beta-catenin level. *Genes Cells* **3**, 395-403
242 (1998).
- 243 10. E. Lee, A. Salic, R. Kruger, R. Heinrich, M. W. Kirschner, The roles of APC and Axin
244 derived from experimental and theoretical analysis of the Wnt pathway. *PLoS biology* **1**,
245 E10 (2003).
- 246 11. J. M. Seeling *et al.*, Regulation of beta-catenin signaling by the B56 subunit of protein
247 phosphatase 2A. *Science* **283**, 2089-2091 (1999).
- 248 12. E. P. T. Hertz *et al.*, A Conserved Motif Provides Binding Specificity to the PP2A-B56
249 Phosphatase. *Mol Cell* **63**, 686-695 (2016).
- 250 13. T. Kruse *et al.*, Mechanisms of site-specific dephosphorylation and kinase opposition
251 imposed by PP2A regulatory subunits. *EMBO J* **39**, e103695 (2020).
- 252 14. C. S. Cselenyi *et al.*, LRP6 transduces a canonical Wnt signal independently of Axin
253 degradation by inhibiting GSK3's phosphorylation of beta-catenin. *Proc Natl Acad Sci U*
254 *S A* **105**, 8032-8037 (2008).
- 255 15. J. E. Ferrell, Jr., Self-perpetuating states in signal transduction: positive feedback, double-
256 negative feedback and bistability. *Curr Opin Cell Biol* **14**, 140-148 (2002).
- 257 16. A. I. Roig *et al.*, Immortalized epithelial cells derived from human colon biopsies express
258 stem cell markers and differentiate in vitro. *Gastroenterology* **138**, 1012-1021 e1011-1015
259 (2010).
- 260 17. J. G. Albeck, J. M. Burke, S. L. Spencer, D. A. Lauffenburger, P. K. Sorger, Modeling a
261 snap-action, variable-delay switch controlling extrinsic cell death. *PLoS biology* **6**, 2831-
262 2852 (2008).
- 263 18. A. R. Hernandez, A. M. Klein, M. W. Kirschner, Kinetic responses of beta-catenin specify
264 the sites of Wnt control. *Science* **338**, 1337-1340 (2012).
- 265 19. G. Wu, H. Huang, J. Garcia Abreu, X. He, Inhibition of GSK3 phosphorylation of beta-
266 catenin via phosphorylated PPPSPXS motifs of Wnt coreceptor LRP6. *PLoS One* **4**, e4926
267 (2009).
- 268 20. F. H. Tran, J. J. Zheng, Modulating the wnt signaling pathway with small molecules.
269 *Protein Sci* **26**, 650-661 (2017).
- 270 21. A. Goldbeter, Dissipative structures in biological systems: bistability, oscillations, spatial
271 patterns and waves. *Philos Trans A Math Phys Eng Sci* **376**, (2018).

- 272 22. V. W. Ding, R. H. Chen, F. McCormick, Differential regulation of glycogen synthase
273 kinase 3beta by insulin and Wnt signaling. *The Journal of biological chemistry* **275**, 32475-
274 32481 (2000).
- 275 23. S. S. Ng *et al.*, Phosphatidylinositol 3-kinase signaling does not activate the wnt cascade.
276 *The Journal of biological chemistry* **284**, 35308-35313 (2009).
- 277 24. R. A. Stewart, A. B. Ramakrishnan, K. M. Cadigan, Diffusion and function of Wnt ligands.
278 *PLoS genetics* **15**, e1008154 (2019).
- 279 25. C. A. Thorne *et al.*, A biochemical screen for identification of small-molecule regulators
280 of the Wnt pathway using *Xenopus* egg extracts. *J Biomol Screen* **16**, 995-1006 (2011).
- 281 26. C. R. Cabel *et al.*, Single-Cell Analyses Confirm the Critical Role of LRP6 for Wnt
282 Signaling in APC-Deficient Cells. *Dev Cell* **49**, 827-828 (2019).
- 283

284 **FIGURE LEGENDS**

285 **Fig. 1. GSK3 and Axin mutually activate in *Xenopus* extracts and mammalian cells.**

286 (A) Experimental scheme. Cytoplasmic fraction of *Xenopus* egg extracts. Extracts are collected,
287 spiked with radiolabeled (rad) [³⁵S] β-catenin or [³⁵S] Axin, and aliquots are removed at the
288 indicated time points for analysis by SDS-PAGE and autoradiography. (B) Turnover of
289 radiolabeled [³⁵S] β-catenin or [³⁵S]Axin in *Xenopus* extracts. LiCl (GSK3i) and NaCl (Control)
290 (50 mM each) were added to extracts as indicated. (C) As in (B), turnover of Axin, AxinSA
291 (serine 322 and 326 mutated to alanine), and AxinΔGBS (GSK3 binding site) in *Xenopus*
292 extracts. (D) Axin promotes dephosphorylation of pS9 GSK3 in *Xenopus* extract, which is
293 blocked by OA (200 nM). MBP-Axin (10 nM) was added to egg extract in the presence or
294 absence of OA (10 nM), and pS9 GSK3 and GSK3 were detected by immunoblotting. (E) The
295 β-catenin binding site, APC binding site, and DIX domain are dispensable for Axin-mediated
296 dephosphorylation of pS9 GSK3. Myc-tagged Axin truncation mutants were transfected into
297 HEK293 cells, as indicated, and immunoblotting was performed. For OA treatment, cells were
298 incubated with 10 nM OA for 2 hrs prior to lysis. (F) Expression of FLAG-tagged wild-type
299 Axin (Axin SLiM WT) and FLAG-tagged Axin with mutations in the conserved B56 binding site
300 that prevent the interaction of B56 with Axin (Axin SLiM 4A) in HEK293. (G) Reconstitution
301 of pS9 GSK3 dephosphorylation by PP2A in the presence of Axin. Recombinant Axin (1 μM)
302 and GSK3 (10 μM) were incubated with ATP for 30 min to allow for the autophosphorylation of

303 GSK3. PP2A (1 μ M) and OA (10 nM) were added for an additional 30 min, and samples were
304 immunoblotted for pS9 GSK3. **(H)** PP2A preferentially dephosphorylates pS9 GSK3 versus β -
305 catenin in the presence of Axin. The reaction was performed as in (F) but with the addition of β -
306 catenin (10 μ M).

307

308 **Fig. 2. Mathematical modeling of the core β -catenin destruction complex components give**

309 **rise to bistable Wnt activity. (A)** Wiring diagram of β -catenin destruction complex feedback.

310 Phosphorylated forms are denoted with "P." The model consists of a positive feedback loop

311 between GSK3 and Axin^P. **(B)** We assume that an input Wnt signal changes the rate constant (k_1)

312 in the phosphorylation flux of GSK3 (I_8). The model shows bistable response in β -catenin. **(C)**

313 Bistability is lost when GSK3 is dephosphorylated by a phosphatase activity that is independent

314 of Axin^P. Consequently, a graded β -catenin response is observed.

315

316 **Fig. 3. Human colonic epithelial cells respond to Wnt in a bistable manner.**

317 **(A)** Depiction of the difference between a graded versus a bistable response in an epithelial

318 monolayer. **(B,D)** Density plots of nuclear β -catenin against Wnt concentrations in HCECs

319 under simulated and experimental conditions show a bistable range of 3-6 nM Wnt3a. Steps of

320 automated image processing to quantify nuclear β -catenin immunofluorescence signal. Inserts

321 show representative images of HCECs treated with Wnt at 6 hrs steady state. **(C)** Steps of
322 automated image processing to quantify nuclear β -catenin immunofluorescence signal.

323

324 **Fig. 4. Cells exhibit memory of Wnt stimulation.**

325 **(A)** Scheme of the experimental approach to pre-stimulate colonic cells. **(B,C)** Model
326 prediction of hysteresis and experimental results from Wnt3a dose-response analyses. Wnt3a
327 dose-response density plots of nuclear β -catenin for HCECs treated with Wnt3a for the first time
328 (naive) or previously pulsed with a high dose of Wnt3a (pre-stimulated). Results from simulated
329 and experimental conditions are shown.

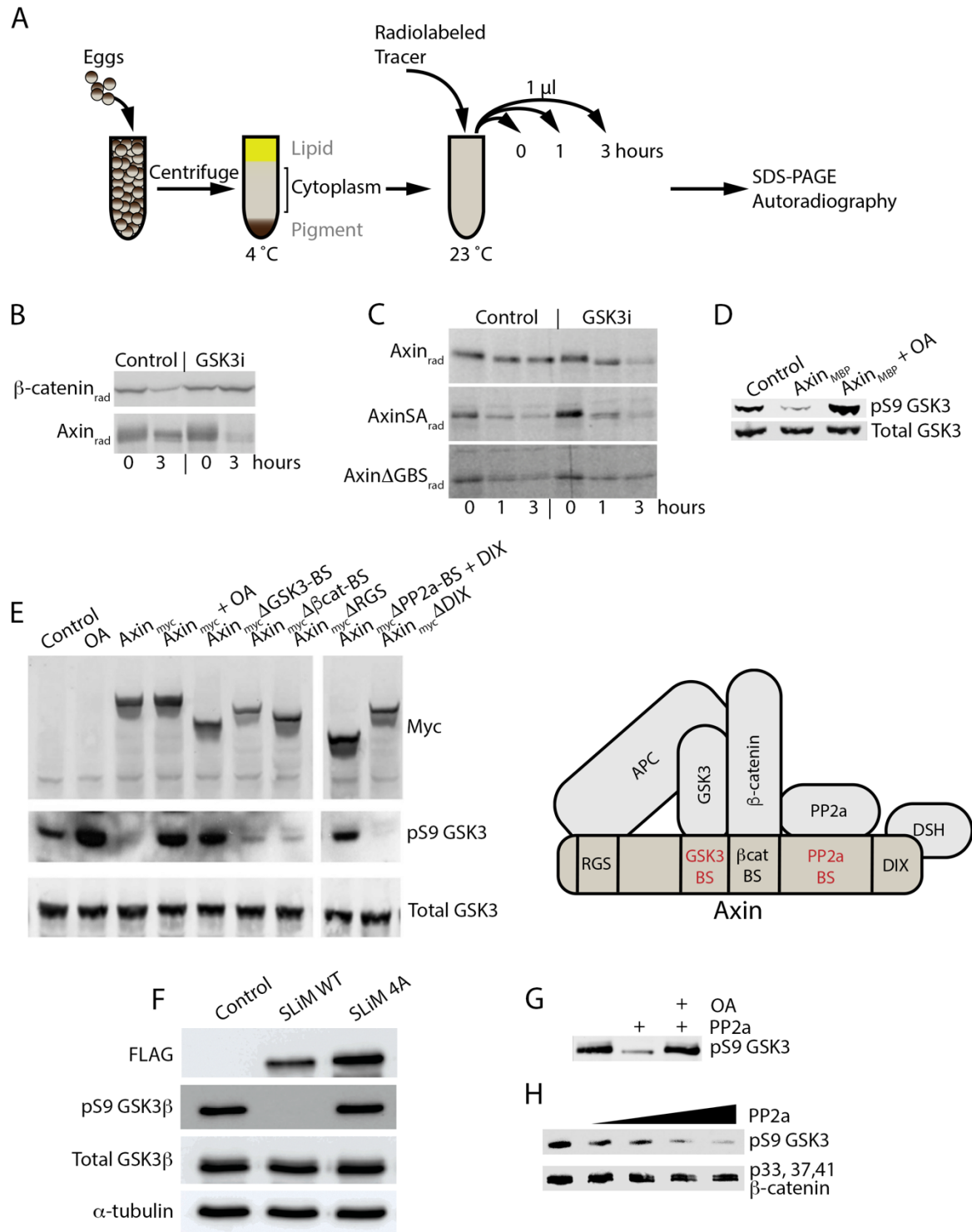
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331 **Fig. 5. Disrupting positive feedback removes bistability.**

332 **(A)** Wiring diagram of β -catenin destruction complex feedback. GSK3i disrupts the positive
333 feedback loop by removing the dependency of Axin^p concentration on dephosphorylation of
334 GSK3 (denoted by red X in the diagram) **(B)** CHIR00921 is epistatic to the Axin/PP2a regulation
335 due to direct interaction with the ATP catalytic site on GSK3. **(C)** Model prediction of graded
336 response and experimental results from CHIR009921 dose-response analyses. **(D)** GSK3
337 inhibition with CHIR99021 treatment results in a graded, monostable response in HCECs.

338

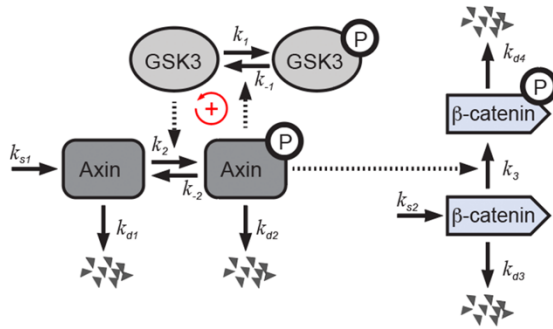
Figure 1



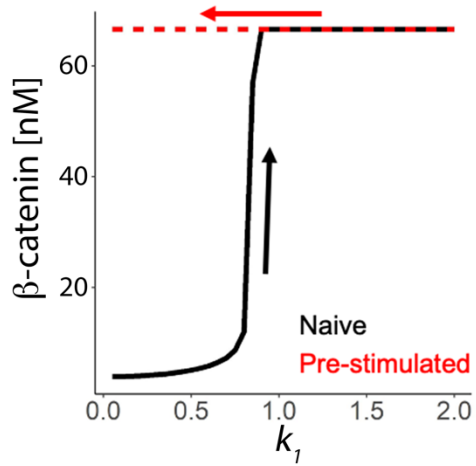
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Figure 2

A



B



C

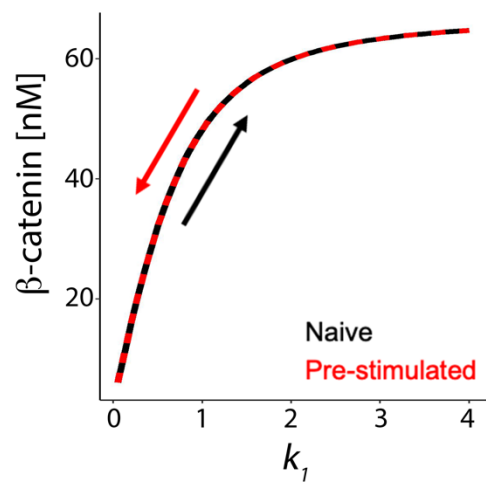


Figure 3

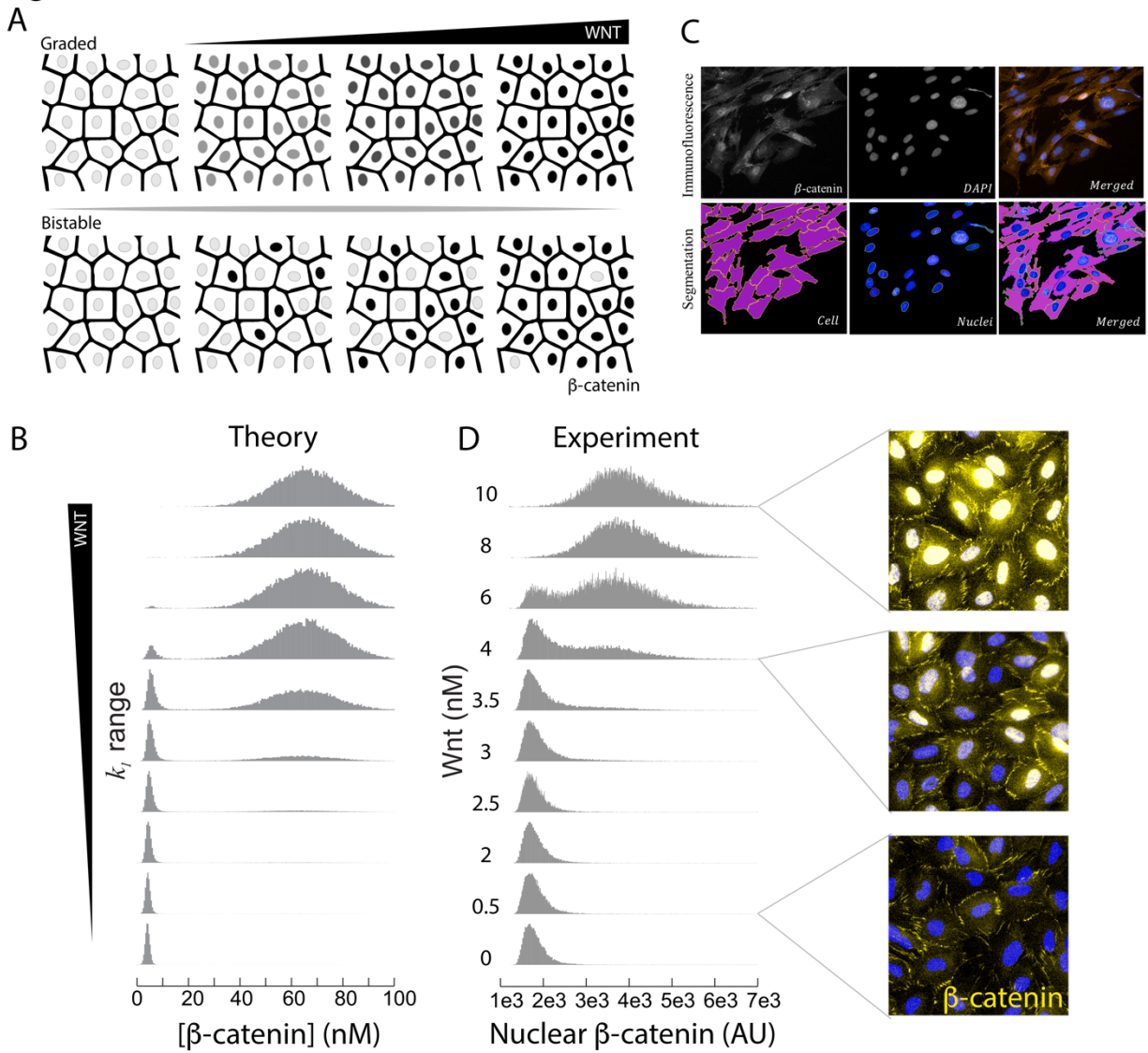


Figure 4

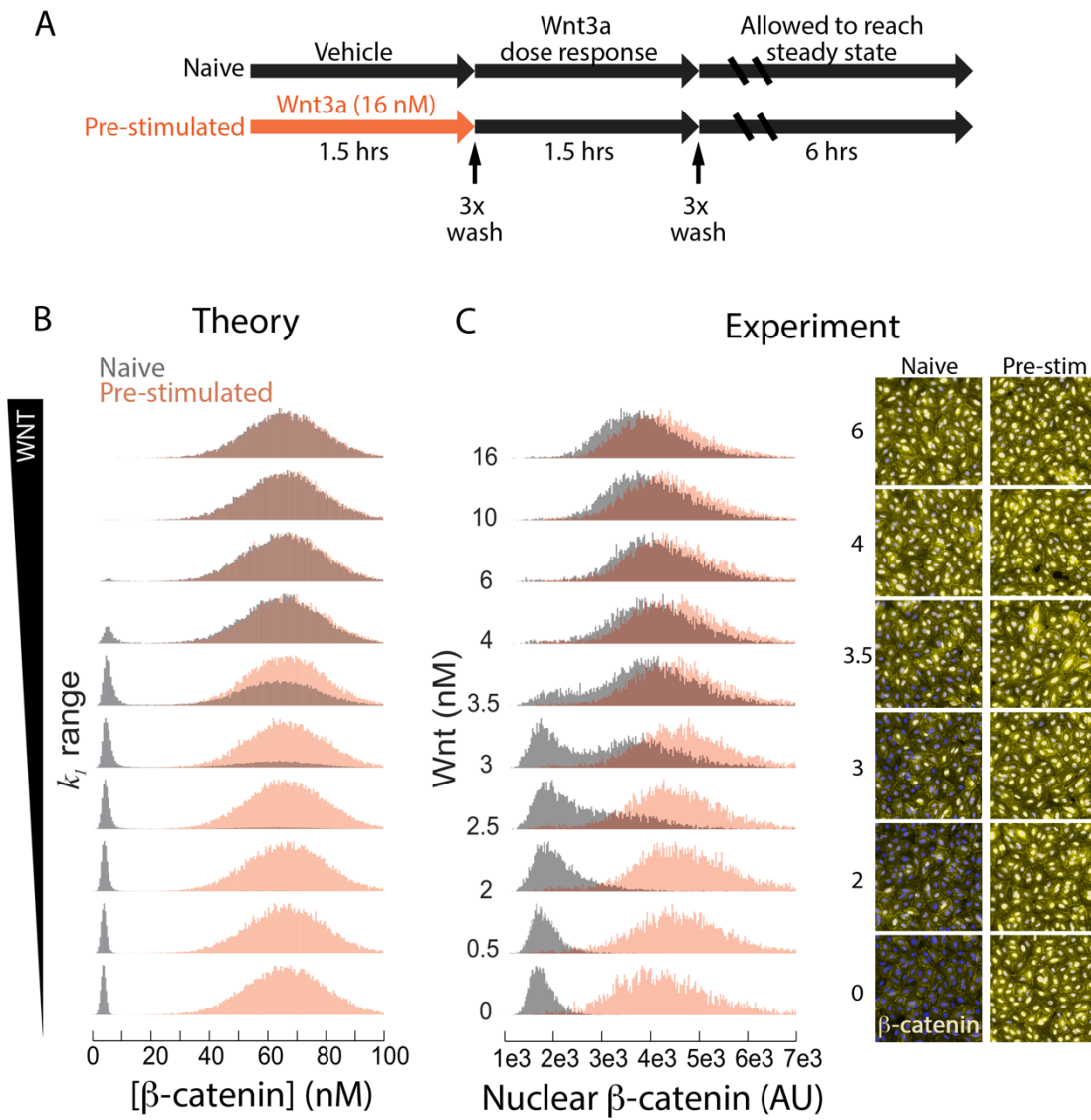
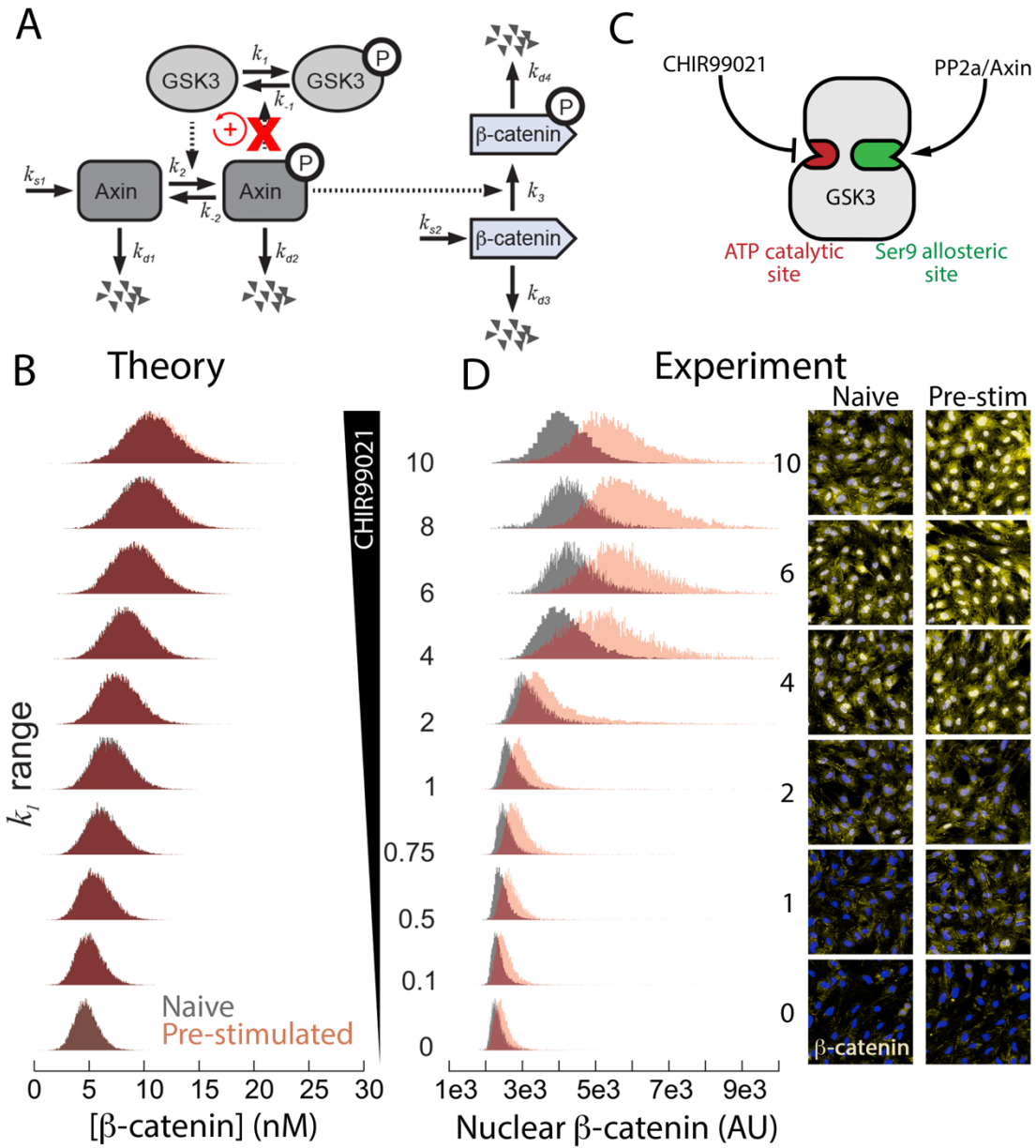


Figure 5



344

Supplementary Materials for

345 **Feedback in the β -catenin destruction complex imparts bistability and cellular** 346 **memory**

347

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351 **Materials and Methods**

352 Plasmids and radiolabeled proteins

353 Radiolabeled β -catenin and Axin were generated in rabbit reticulocyte lysates (Promega)
354 according to the manufacturer's instructions. Degradation assays were performed based on
355 previously published methods (7, 25).

356

357 *Xenopus* extract studies

358 *Xenopus* embryos were *in vitro* fertilized, dejellied and extracts prepared as previously described
359 (7).

360

361 Immunoblots

362 Cells were lysed in non-denaturing buffer (50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA,
363 1% w/v Triton X-100) and the soluble fraction was used for immunoblotting. For Axin
364 immunoblots, Axin was immunoprecipitated with mouse anti-Axin antibody (Zymed) and
365 immunoblotted with anti-Axin 1 goat antibody (R & D). Total GSK3 and GSK3 pS21 (Cell

366 Signaling) were detected from lysates denatured in lysis buffer containing 1% SDS, protease and
367 phosphatase inhibitors.

368

369 Kinase assays

370 *In vitro* kinase assays were performed as previously described (14).

371

372 Cell Culture

373 HEK293 were purchased from ATCC and cultured based in ATCC protocols. Human colonic
374 epithelial cells (HCECs) were cultured in 5% CO₂ in DMEM supplemented with 10% FBS, 1x
375 penicillin-streptomycin and 1x glutamax.

376

377 Bistability experiments

378 HCECs were plated at 20,000 cells/well in fluorescent 96-well plates (Greiner Bio-One;
379 Cat#655090) on day 0. Cells were incubated and allowed to reach 100% confluence. On day 2,
380 cells were treated with increasing concentrations of recombinant human Wnt3A (R&D; Cat#5036-
381 WN-500, with carrier) for 1.5 h. Cells were then washed with PBS thrice and complete media
382 (DMEM high glucose containing 10% FBS, 1x glutamax and 1x penicillin-streptomycin) was
383 added. Cells were incubated for 3 h and fixed with 4% paraformaldehyde-sucrose solution.

384

385 Hysteresis: HCECs were plated at 20,000 cells/well in fluorescent 96-well plates (Greiner Bio-
386 One; Cat#655090) on day 0 and allowed to reach 100% confluence on day 2. HCECs were treated
387 with Wnt3a long enough to stimulate the pathway (1.5 h), but short enough to avoid negative
388 feedback from the destruction complex (≤ 6 h) (Fig. S6) by Axin2, a transcriptional target of the

389 Wnt pathway. Cells were treated either without (Naive) or with 16 nM of Wnt3A (Pre-stimulated)
390 for 1.5 h. Cells were washed with PBS three times and subsequently treated with increasing
391 concentrations of Wnt3A for 1.5 h. Cells were washed with PBS three times, replaced with
392 complete media for 3 h and fixed.

393

394 Stimulation through direct GSK3 inhibition (Fig 5): HCECs were plated as described above. In
395 Fig. S7, cells were treated with CHIR99021 at the indicated concentrations and durations. We
396 chose the concentration of 10 μ M for 6h because with these conditions the β -catenin response
397 reached steady state. Cells were treated with increasing concentrations of CHIR99021 (Selleck
398 Chemicals; Cat#S1263) for 6 hrs and then fixed. For the hysteresis experiment using CHIR99021:
399 HCECs were plated as above. On day 2, cells were treated with either DMSO (Naive) or 10 μ M
400 of CHIR99021 (Pre-stimulated) for 6 hrs. Cells were washed with PBS three times, treated
401 CHIR99021 dose curve for 6 hrs and fixed.

402

403 Immunofluorescence

404 Fixed cells were permeabilized with 0.2% Triton X-100, blocked with 2.5% BSA, and stained with
405 β -catenin antibody at 1:300 (BD Biosciences; Cat#610154) diluted in 2.5% BSA. After washing
406 with PBST (0.1% Tween-20), cells were incubated in secondary antibody conjugated to 1:1000
407 Alexa fluor (Invitrogen; Cat#A1103) diluted in 2.5% BSA for 2 hrs in the dark. Cells were washed
408 in PBST and DAPI was used to stain nuclei. Cells were imaged on Perkin Elmer Operetta System
409 using a 20x air objective.

410

411 Nuclear image segmentation

412 To isolate single cell data, nuclear segmentation was performed using the Perkin Elmer Harmony
413 software as previously described (26). β -catenin nuclear intensity was normalized to nuclear area
414 of each cell (nuclear β -catenin). Data analysis codes were custom-built using R.

415

416 Mathematical modeling

417 To better understand sufficiency requirements for the emergence of bistability in our system, we
418 developed a minimal mathematical model of the Wnt pathway dynamics. Our equations describe
419 the dynamics of concentrations of Axin, GSK3, and β -catenin in both their phosphorylated and
420 unphosphorylated states. Intermolecular interactions accounted for in the model follow directly
421 from Fig. 2A. Specifically, we assume that Axin is phosphorylated at a rate that increases with the
422 concentration of unphosphorylated GSK3, whereas GSK3 is dephosphorylated at a rate that
423 increases with the concentration of phosphorylated Axin. We further assume that β -catenin is
424 phosphorylated at a rate proportional to the concentration of phosphorylated Axin, which leads to
425 its rapid depletion (since the rate of phospho- β -catenin degradation is assumed to be much higher
426 than that of its unphosphorylated form). Additionally, Axin and β -catenin are produced at constant
427 rates. Finally, Axin and β -catenin undergo degradation in both their phosphorylated and
428 unphosphorylated forms.

429

430 Dynamical equations that define our model follow straightforwardly from the above assumptions
431 and are given in Table S1. To analyze the model, we first note β -catenin concentration dynamics
432 do not feedback on the dynamics of the other molecules. In this way, the behavior of β -catenin is
433 a readout of the state of the pathway and need not be considered when examining the nature of its
434 possible dynamical states. Additionally, the total amount of GSK3 is constant (see Eq 4) since its

435 degradation is negligible on the timescale of our experiment (I_0). Thus, three dynamical equations
436 (specifying the dynamics of Axin, phospho-Axin, and unphosphorylated GSK3) suffice to specify
437 the behavior of model uniquely (see Table S1, Eq 1-3).

438

439 The key feature of the model is a positive feedback loop between GSK3 dephosphorylation and
440 Axin phosphorylation. Specifically, the rate of GSK3 dephosphorylation increases with the
441 concentration of phosphorylated-Axin (V_{-1} term), whereas the rate of Axin phosphorylation
442 increases with the concentration of (unphosphorylated) Axin (V_2 term). The activity of the pathway
443 regulates the levels of β -catenin through phosphorylation of β -catenin (V_3 term) which is thereby
444 targeted for proteasomal degradation (V_{d4} term). The other terms account for Axin-independent
445 phosphorylation of GSK3 (V_1 term), GSK3-independent Axin dephosphorylation (V_{-2} term), de
446 novo translation of Axin (V_{s1} term) and β -catenin (V_{s2} term), and spontaneous “background”
447 degradation of Axin in its phosphorylated and unphosphorylated states (V_{d2} and V_{d1} terms
448 respectively) as well as that of β -catenin in its unphosphorylated state (V_{d3} term).

449

450 In the simplest initial version of the model, it was assumed that no cooperativity was present in
451 any reaction step such that the terms V_2 and V_{-2} were linear in $[GSK3]$ and $[Axin^p]$ respectively.
452 Setting all time-derivatives to zero, these equations are readily solved for the steady state
453 concentrations (which we did using symbolic algebra software Maple, Maplesoft) to obtain two
454 steady state solutions. Setting all time-derivatives to zero, these equations are readily solved for
455 the steady state concentrations (which we did using symbolic algebra software Maple, Maplesoft)
456 to obtain two steady state solutions, one of which is stable and the other unstable. This result
457 indicates that the simplest version of the equations cannot exhibit bistability since bistability

458 requires the coexistence of three steady state solutions (two stable and one unstable). That the
459 simple version of the model does not exhibit bistability can be established rigorously by
460 applying chemical reaction network theory (CRNT) [The Chemical Reaction Network Toolbox,
461 Windows Version | Zenodo].

462

463 Hence, to explain bistability, we modified the model to account for the possible presence of
464 cooperativity in both Axin phosphorylation by GSK3 as well as in its dephosphorylation. We note
465 these are assumptions; however, our analysis shows that cooperativity in (at least some of the)
466 intermolecular interactions is required to generate bistable dynamics. The equations are still
467 possible to solve analytically, though the solutions are quite lengthy and thus not presented here.
468 Accounting for possible presence of cooperativity does lead to three solution branches, as indicated
469 in the bifurcation diagram in Fig. S2. To explore the stability of the different branches, we
470 performed numerical simulations where our dynamical equations were solved using an explicit
471 Euler forward scheme. We found that in an open set of parameter values, two stable branches can
472 co-exist with an unstable branch thus implying that our minimal model can account for bistability
473 of the pathway (Fig. S2).

474

475 In our minimal model, a steady state with zero concentrations of both phospho-Axin and
476 unphosphorylated GSK3 is present for all values of model parameters. Arguably, this feature is
477 non-generic since it is not present when phospho-GSK3 can be dephosphorylated spontaneously
478 (in the absence of phospho-Axin). However, our model is a good approximation of this later
479 situation if the rate of spontaneous GSK3 dephosphorylation is negligible. It may be shown that a
480 small rate of spontaneous GSK3 dephosphorylation will change the appearance of the bifurcation
481 diagram in Fig. S2 to produce a single root locus where two saddle-node bifurcations are connected

482 by an unstable branch. For high rates of spontaneous GSK3 dephosphorylation, bistability is lost
483 and only a single stable branch will remain. These predictions are in principle testable, provided
484 one can control the rate of GSK3 dephosphorylation for example by a phosphatase, presumably
485 PP2a. However, this work is beyond the scope of the present study.

486

487 To further examine the dynamics of the pathway and to more closely compare theoretical
488 predictions to experimental results, we examined the response of the pathway to quasi-static
489 variation of a control parameter. Specifically, we varied the rate of spontaneous GSK3
490 phosphorylation (k_1) and examined the resulting steady-state concentration of β -catenin. This was
491 done using two procedures that we term “Naive” and “Pre-stimulated”. In the former case, the
492 value of k_1 was initially set to a low value and was then increased quasi-statically. In the latter
493 case, k_1 was gradually decreased, starting with a large initial value. Fig. 2 shows the plots of the
494 steady-state β -catenin levels as a function of k_1 corresponding to the two simulated protocols. It is
495 seen that the two curves do not coincide, indicating the presence of hysteresis. Specifically, in the
496 Pre-stimulated case when k_1 was large initially, the initial levels of β -catenin were correspondingly
497 high and remained high for a substantial range of (decreasing) k_1 values. In the Naive case, when
498 k_1 was increased from a value initially set to be low, steady-state β -catenin levels remained much
499 lower than seen in the Pre-stimulated case (for the same values of k_1). This hysteretic behavior is
500 a key hallmark of bistability and is readily anticipated from the phase portrait given in Fig. S2.

501

502 We next asked if our minimal computational model can interpret our observations on the
503 distribution of Wnt pathway activation levels in cell populations (Fig. 3D, 4C, 5D). Clearly, the
504 precise level of nuclear β -catenin varies from cell to cell. To capture this variation, we considered

505 an ensemble of cells, with each cell having a different random value of spontaneous GSK3
506 phosphorylation k_1 . Expectedly, this results in a stochastic distribution of steady-state β -catenin
507 levels. Next, we examined changes in the simulated distribution of pathway activation as the mean
508 value of k_1 was varied according to the two protocols described above and as shown in Fig. 2. We
509 found that the distribution of β -catenin levels becomes bimodal within a finite intermediate range
510 of the mean k_1 values. This is another key hallmark of bistability and is in complete agreement
511 with the experimental data (see Fig. 4B and 4C). At the same time, average β -catenin levels follow
512 the same qualitative trend as was seen in Fig. S9, where the deterministic dynamics without
513 stochastic variation in k_1 were examined. Finally, we asked if the dynamics of the pathway may
514 be rendered monostable by means of perturbations that interfere with the positive feedback
515 between Axin and GSK3. To this end, we made the rate of Axin phosphorylation independent of
516 GSK3 concentration. In this case, the dynamics became monostable such that no hysteresis was
517 seen when k_1 was first increased and then decreased quasi-statically, see Fig. S9E.

518

519 To account for the effect of Wnt stimulation on the pathway, we considered four alternative
520 scenarios. Specifically, in our model, Wnt can stabilize β -catenin by 1) increasing k_1 , 2)
521 decreasing k_{-1} , 3) increasing k_{-2} or 4) decreasing k_2 . Expectedly, in all these cases, the system
522 exhibits hysteresis (Fig. S9), since, generically, the presence of hysteresis does not depend on the
523 particular choice of the control parameter (as long as varying the control parameter allows to move
524 from a bistable to a monostable regime).

525

526 **Table S1: Set of ordinary differential equations for the system in Fig. 2A.**

527
$$\frac{d[Axin]}{dt} = -V_2 + V_{-2} - V_{a1} + V_{s1} \quad (1)$$

528 $\frac{d[Axin^p]}{dt} = +V_2 - V_{-2} - V_{d2} \quad (2)$

529 $\frac{d[GSK3\beta]}{dt} = -V_1 + V_{-1} \quad (3)$

530 $[GSK3\beta] + [GSK3\beta^p] = GSK3\beta_{tot} = constant \quad (4)$

531 $\frac{d[\betacatenin^p]}{dt} = V_3 - V_{d4} \quad (5)$

532 $\frac{d[\betacatenin]}{dt} = V_{s2} - V_{d3} - V_3 \quad (6)$

533

534 **Table S2: Reaction rates for the model.**

535 $V_1 = k_1 [GSK3\beta]$

536 $V_{-1} = k_{-1} ([GSK3\beta_{Tot}] - [GSK3\beta])[Axin^p]$

537 $V_2 = k_2 [GSK3\beta]^2 [Axin]$

538 $V_{-2} = k_{-2} [Axin^p]^2$

539 $V_{d1} = k_{d1} [Axin]$

540 $V_{d2} = k_{d2} [Axin^p]$

541 $V_{s1} = k_{s1}$

542 $V_{d4} = k_{d4} [\betacatenin^p]$

543 $V_3 = k_3 [\betacatenin] [Axin^p]$

544 $V_{s2} = k_{s2}$

545 $V_{d3} = k_{d3} [\betacatenin]$

546

547 **Table S3: Rate constants used in the model.**

Variable	Value	Definition	Ref
----------	-------	------------	-----

k_{s1}	$8.2 \times 10^{-5} \text{ nM min}^{-1}$	Axin synthesis rate	(10)
k_{d1}	0.167 min^{-1}	Axin degradation rate constant	(10)
k_{d2}	0.004 min^{-1}	Axin ^P degradation rate constant	Assumption
k_1	Fig S9A & Fig 2B : $0.05-2 \text{ [min}^{-1}]$ Fig S9B: $1 \text{ [min}^{-1}]$ Fig. S9C: $1 \text{ [min}^{-1}]$ Fig. S9D: $1 \text{ [min}^{-1}]$ Fig. S9E & Fig 2C: $0.05-4 \text{ [min}^{-1}]$	phosphorylation rate of GSK3	
k_{-1}	Fig S9A & Fig 2B : $1 \text{ [nM}^{-1} \text{ min}^{-1}]$ Fig S9B: $0.05-4 \text{ [nM}^{-1} \text{ min}^{-1}]$ Fig. S9C: $1 \text{ [nM}^{-1} \text{ min}^{-1}]$ Fig. S9D: $2 \text{ [nM}^{-1} \text{ min}^{-1}]$ Fig. S9E & Fig 2C: $0.001 \text{ [min}^{-1}]$	Dephosphorylation rate of GSK3 ^P	
k_2	Fig S9A & Fig 2B : $2 \text{ [nM}^{-2} \text{ min}^{-1}]$ Fig S9B: $2 \text{ [nM}^{-2} \text{ min}^{-1}]$ Fig. S9C: $0.05-10 \text{ [nM}^{-2} \text{ min}^{-1}]$ Fig. S9D: $2 \text{ [nM}^{-2} \text{ min}^{-1}]$ Fig. S9E & Fig 2C: $2 \text{ [nM}^{-2} \text{ min}^{-1}]$	phosphorylation rate of Axin	
k_{-2}	Fig S9A & Fig 2B : $2 \text{ [nM}^{-1} \text{ min}^{-1}]$ Fig S9B: $2 \text{ [nM}^{-1} \text{ min}^{-1}]$ Fig. S9C: $2 \text{ [nM}^{-1} \text{ min}^{-1}]$ Fig. S9D: $0.05-10 \text{ [nM}^{-1} \text{ min}^{-1}]$ Fig. S9E & Fig 2C: $2 \text{ [nM}^{-1} \text{ min}^{-1}]$	Dephosphorylation rate of Axin ^P	

k_3	$5\text{nM}^{-1} \text{min}^{-1}$	Phosphorylation rate of β -catenin	Assumption
k_{s2}	0.42 nM min^{-1}	Synthesis rate of β -catenin	(10)
k_{d3}	$6.3 \times 10^{-3} \text{ min}^{-1}$	Degradation rate of β catenin	(10)
k_{d4}	0.42 min^{-1}	Degradation rate of β -catenin ^P	(10)
$GSK3_{Tot}$	50nM	Total concentration of GSK3	(10)

548

549 **Table S4: Two initial conditions used to solve the ODEs.**

Experiment[nM]	Axin	Axin ^P	GSK3	β catenin	β catenin ^P
Naive	4×10^{-4}	5×10^{-3}	50	1	30
Pre-stimulated	6.1×10^{-4}	1×10^{-6}	2	30	1

550

551 **Fig S1. (A)** Cartoon of Axin SLiM site mutant effect on GSK3 activation. **(B)** Alignment of the
 552 B56 binding site sequence in four species reveals strong evolutionary conservation.

553

554 **Fig S2.** Solving the ODEs analytically leads to three fixed points. We explored the stability of the
 555 different branches using numerical simulations where our dynamical equations were solved. Two
 556 of the fixed points were stable and can co-exist while the third fixed point was unstable.

557

558 **Fig S3.** To account for heterogeneity of the cells, we ran the simulation for 2000 cells and in each

559 run we randomly choose a k_1 value from a normal distribution instead of a single value. **(A)** Range
560 of k_1 used for the model in which there is a positive feedback loop and bistability response is
561 observed as shown in Fig. 3B and Fig. 4B. **(B)** Range of k_1 used for the model where the positive
562 feedback loop is broken, and we see a graded response as shown in Fig. 5D.

563

564 **Fig S4.** Plot of mean β -catenin response to increasing and decreasing Wnt3a stimulation from Fig.
565 3D, ($EC_{50} = 3.78$ nM; $n_H = 6.35$).

566

567 **Fig S5.** Plot of mean β -catenin response to increasing and decreasing Wnt3a stimulation from Fig.
568 4D.

569

570 **Fig S6.** **(A)** HCECs were treated with recombinant Wnt3a (16 nM) for one hours, washed three
571 times and regular growth media minus Wnt3a was added back. Nuclear β -catenin is plotted.
572 Nuclear β -catenin remains high at 6 h after removal but afterward comes back down. **(B)** HCECs
573 were treated with different concentrations of the CHIR99021 for various durations. Cells were
574 allowed to reach steady-state using nuclear localization of β -catenin as a readout. The effect of
575 CHIR99021 on nuclear β -catenin localization in HCECs is reversible 3h after removal of the
576 compound. CHIR99021 was used at 10 μ M concentration for six hours.

577

578 **Fig S7.** **(A)** HCECs were treated with different concentrations of GSK3i CHIR99021 for various
579 durations. Nuclear localization of β -catenin is plotted. β -catenin steady state was achieved at 6
580 hours under sustained CHIR99021 stimulation. **(B)** β -catenin hysteresis experiment was performed
581 with identical treatment times as was described in Fig. 4A, except CHIR99021 was used instead

582 of Wnt3a. Unlike the effect observed in cells treated with Wnt3a, cells treated with CHIR99021
583 cannot maintain memory of the stimulation and return to basal β -catenin concentrations at the 6 hr
584 timepoint.

585

586 **Fig S8.** Plot of mean β -catenin response to increasing and decreasing CHIR99021 stimulation from
587 Fig. 5D.

588

589 **Fig S9.** We assumed four different scenarios where in each scenario one of k_1 , k_2 , k_{-2} , and k_{-1}
590 rate constants changes and the others remain constant with increasing Wnt. **(A)** Wnt increases k_1
591 with $k_{-2}=2$, $k_2=2$, and $k_{-1}=1$. **(B)** Wnt decreases k_{-1} with $k_{-2}=2$, $k_2=2$, and $k_1=1$. **(C)** Wnt
592 decreases k_2 with $k_{-2}=2$, $k_1=1$, $k_{-1}=1$. **(D)** Finally Wnt increases k_{-2} with $k_2=2$, $k_1=1$, $k_{-1}=2$. As
593 shown in these four panels for a range of these parameters we see bistability in the concentrations
594 of the molecules. **(E)** We simulated adding GSK3i as disrupting the positive feedback loop and
595 leading to a graded response in β -catenin concentration rather than a bistable response.

596

597

598

Fig S1

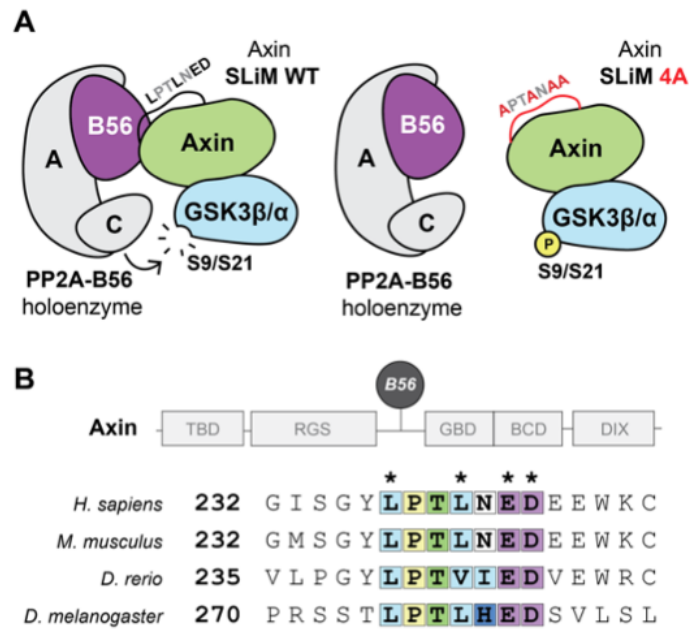


Fig S2

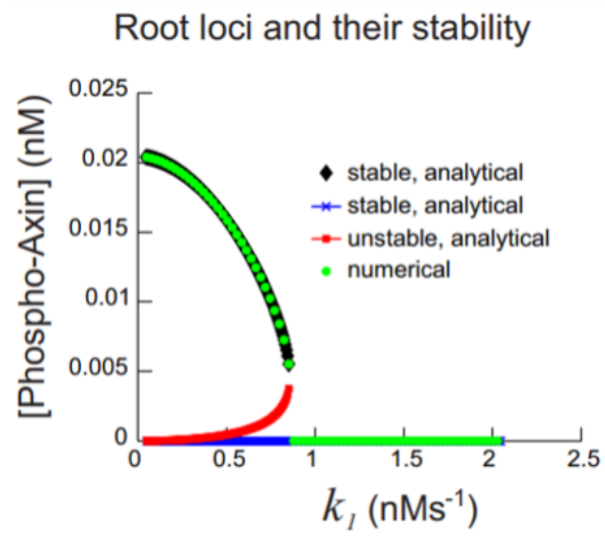


Fig S3

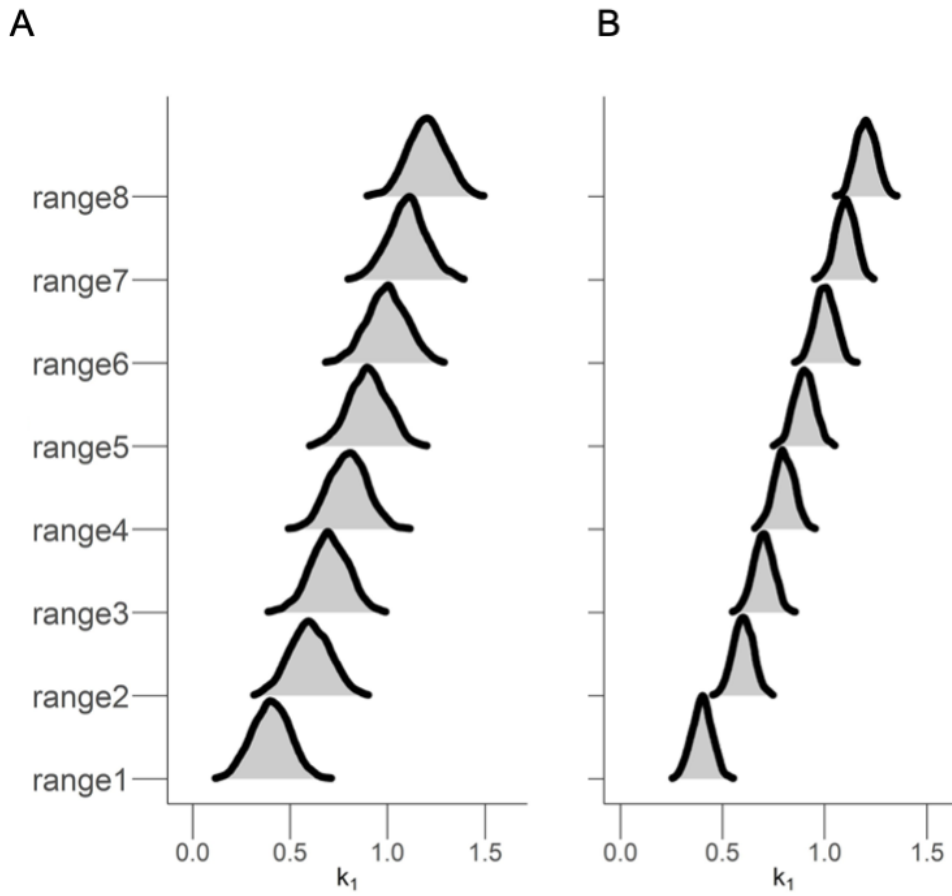


Fig S4

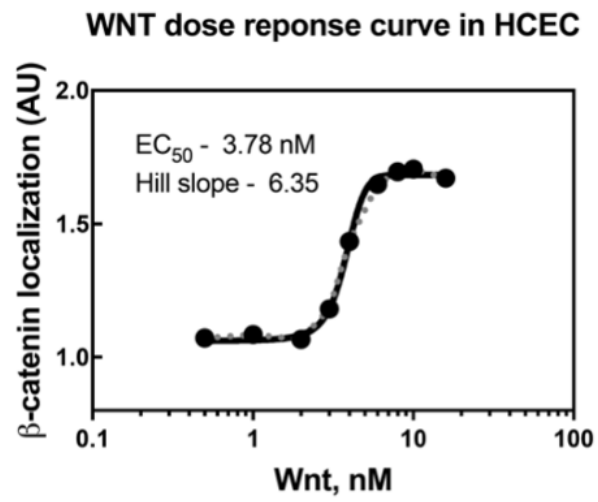


Fig S5

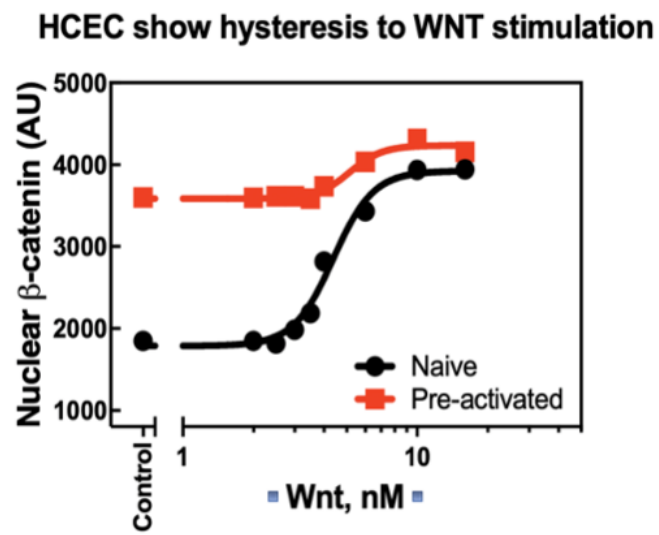


Fig S6

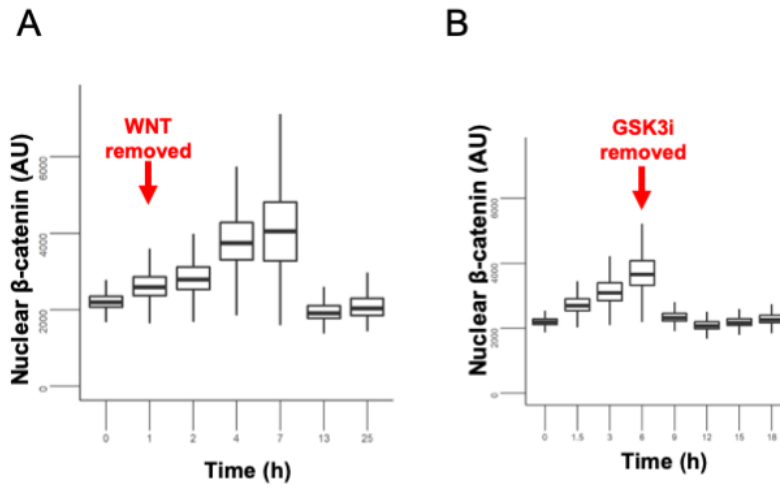
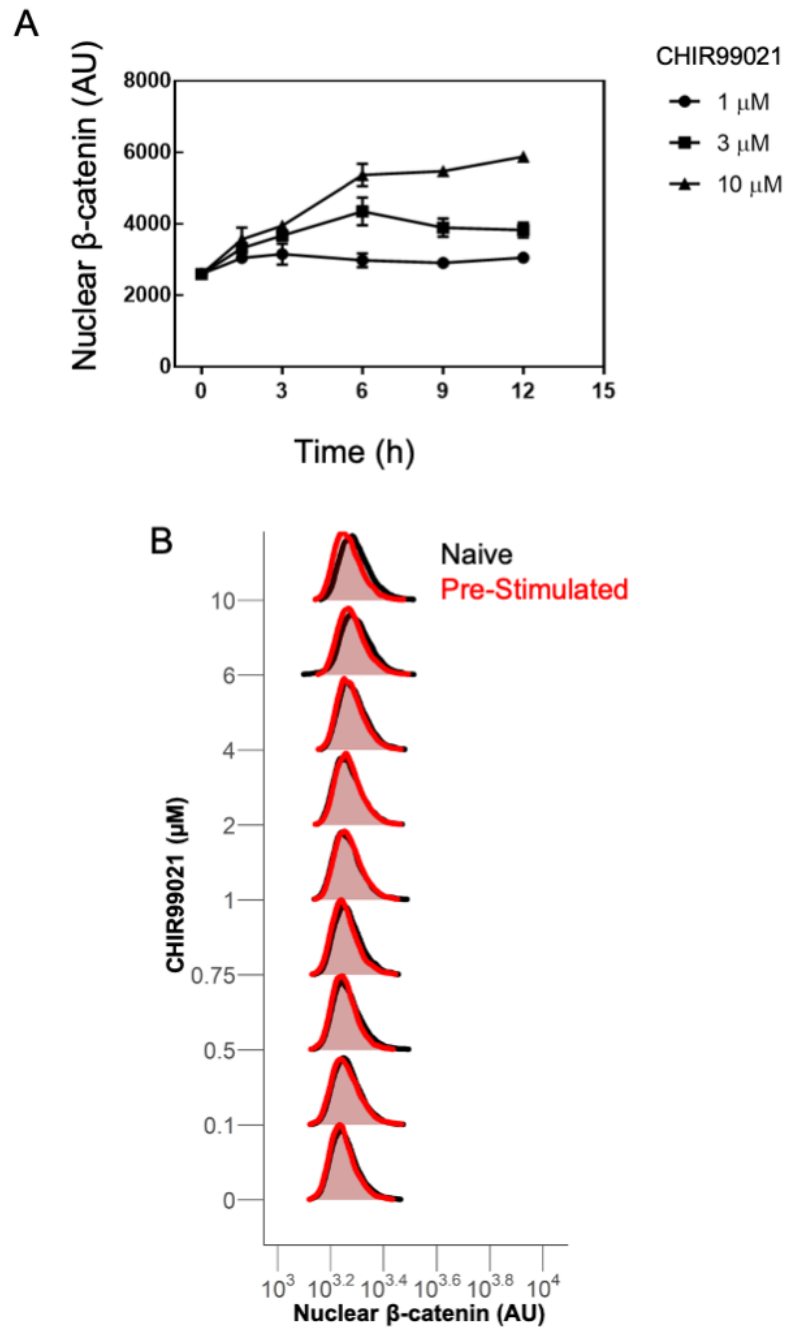


Fig S7



605

Fig S8

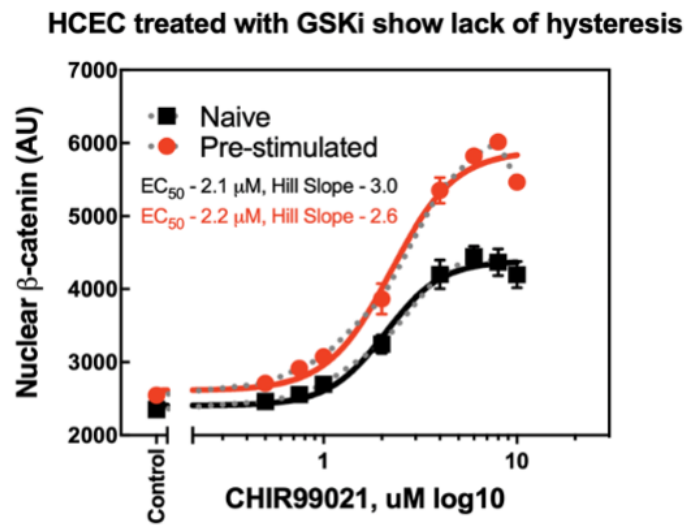
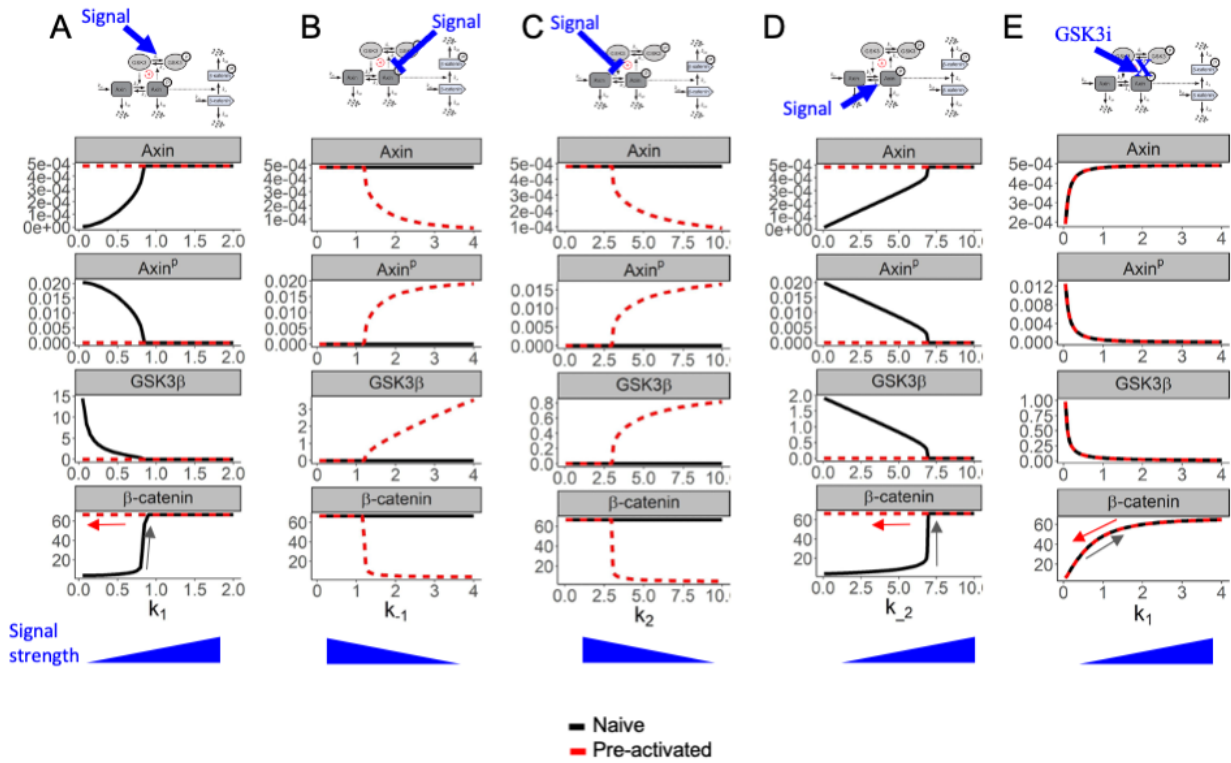


Fig S9



607

608