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## Quantitative live-cell imaging and computational modelling yield novel insight into endogenous WNT/CTNNB1 signaling dynamics — [Source link](#)

S.M.A. de Man, Gooitzen Zwanenburg, T. van der Wal, Mark A. Hink ...+1 more authors

**Institutions:** University of Amsterdam

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2 into endogenous WNT/CTNNB1 signaling dynamics

3 Author information

4 S.M.A. de Man<sup>1</sup>, G. Zwanenburg<sup>2,^</sup>, T. van der Wal<sup>1</sup>, M.A. Hink<sup>3,4,\*,^</sup>, R. van Amerongen<sup>1,\*,^,#</sup>

5 <sup>1</sup> Developmental, Stem Cell and Cancer Biology, Swammerdam Institute for Life Sciences, University  
6 of Amsterdam, Amsterdam, The Netherlands

7 <sup>2</sup> Biosystems Data Analysis, Swammerdam Institute for Life Sciences, University of Amsterdam,  
8 Amsterdam, The Netherlands

9 <sup>3</sup> Molecular Cytology, Swammerdam Institute for Life Sciences, University of Amsterdam,  
10 Amsterdam, the Netherlands

11 <sup>4</sup> van Leeuwenhoek Centre for Advanced Microscopy, Swammerdam Institute for Life Sciences,  
12 University of Amsterdam, Amsterdam, the Netherlands

13 \* These authors contributed equally

14 ^ corresponding authors: [r.vanamerongen@uva.nl](mailto:r.vanamerongen@uva.nl) (RvA, lead contact), [m.a.hink@uva.nl](mailto:m.a.hink@uva.nl) (MAH),  
15 [g.zwanenburg@uva.nl](mailto:g.zwanenburg@uva.nl) (GZ)

16 # Twitter: @wntlab

## 17 Abstract

18 WNT/CTNNB1 signaling regulates tissue development and homeostasis in all multicellular  
19 animals. Multiple aspects of the underlying molecular mechanism remain poorly understood  
20 and critical information on endogenous WNT/CTNNB1 signaling dynamics is missing. Here we  
21 combine CRISPR/Cas9-mediated genome editing and quantitative live-cell microscopy to  
22 measure diffusion characteristics of fluorescently tagged, endogenous CTNNB1 in human cells  
23 with high spatiotemporal resolution under both physiological and oncogenic conditions.  
24 State-of-the-art functional imaging reveals that a substantial fraction of CTNNB1 resides in  
25 slow-diffusing complexes in the cytoplasm, irrespective of the activation status of the  
26 pathway. The identity of this cytoplasmic CTNNB1 complex changes according to the  
27 phosphorylation status of CTNNB1 as it undergoes a major reduction in size when  
28 WNT/CTNNB1 is (hyper)activated. We also measure the concentration of complexed and free  
29 CTNNB1 in both the cytoplasm and the nucleus before and after WNT stimulation, and use  
30 these parameters to build a minimal computational model of WNT/CTNNB1 signaling. Using  
31 this integrated experimental and computational approach, our work reveals that WNT  
32 pathway activation regulates the dynamic distribution of CTNNB1 across different functional  
33 pools by modulating three regulatory nodes: the cytoplasmic destruction complex,  
34 nucleocytoplasmic shuttling and nuclear retention.

## 35 Introduction

36 WNT signaling is one of the most ancient pattern-forming cell signaling cascades. It drives  
37 many biological processes from the onset of embryogenesis until adulthood in all multicellular  
38 animals (reviewed in van Amerongen and Nusse, 2009; Holstein, 2012; Loh et al., 2016). WNT  
39 signaling remains important throughout the lifespan of the organism and controls stem cell  
40 maintenance in many mammalian tissues, including the breast, intestine and skin (van  
41 Amerongen et al., 2012; Barker et al., 2007; Lim et al., 2013). Disruption of the pathway causes  
42 disease, with hyperactivation being a frequent event in human colorectal and other cancers  
43 (reviewed in Nusse & Clevers, 2017; Wiese, Nusse, & van Amerongen, 2018).

44 The key regulatory event in WNT/CTNNB1 signaling (traditionally known as ‘canonical WNT  
45 signaling’) is the accumulation and nuclear translocation of the transcriptional co-activator  
46 beta-catenin (CTNNB1) (Figure 1A). In the absence of WNT signaling, rapid turnover by the  
47 so-called destruction complex maintains low levels of CTNNB1. This cytoplasmic complex  
48 consists of the scaffold proteins APC and AXIN, which bind CTNNB1, and the serine/threonine  
49 kinases CSNK1 and GSK3, which subsequently phosphorylate residues S45, T41, S37 and S33  
50 (Amit et al., 2002; Liu et al., 2002). This primes CTNNB1 for ubiquitination by E3 Ubiquitin  
51 Protein Ligase BTRC and subsequent proteasomal degradation (Aberle et al., 1997; Latres et  
52 al., 1999). In the current working model for WNT/CTNNB1 signaling, binding of WNT ligands  
53 to the FZD/LRP receptor complex sequesters and inhibits the destruction complex at the  
54 membrane in a process that involves DVL (Bilic et al., 2007; Schwarz-Romond et al., 2007).  
55 This allows newly synthesized CTNNB1 to accumulate. Upon stimulation, CTNNB1 also  
56 translocates to the nucleus, where CTNNB1 binds to TCF/LEF transcription factors to regulate  
57 target gene transcription as part of a larger transcriptional complex (Behrens et al., 1996;  
58 Fiedler et al., 2015; Molenaar et al., 1996; van Tienen et al., 2017).

59 The working model for WNT/CTNNB1 signaling described above is the result of almost 40  
60 years of research. The use of traditional genetic and biochemical approaches has allowed  
61 identification of the core players, as well as dissection of the main signaling events. However,  
62 multiple aspects of WNT/CTNNB1 signaling remain poorly understood. For instance the exact  
63 molecular composition of the destruction complex as well as the mechanism for its inhibition  
64 remain unclear (reviewed in Tortelote et al., 2017), and how WNT/CTNNB1 signaling regulates  
65 the subcellular distribution of CTNNB1 requires further scrutiny.

66 Most biochemical techniques lead to loss of spatial information and averaging of cell-to-cell  
67 heterogeneity, since proteins are extracted from their cellular context. Additionally, temporal  
68 information is usually limited to intervals of several minutes or hours. Live-cell microscopy  
69 offers better spatiotemporal resolution. Currently, however, many of these studies are  
70 conducted by overexpressing the protein(s) of interest. This can severely affect activation,  
71 localization and complex formation (Gibson et al., 2013; Mahen et al., 2014). Although  
72 stabilization of CTNNB1 by WNT signaling has been extensively studied, very few studies have  
73 focused on the spatiotemporal dynamics of this process – especially at the endogenous level  
74 (Chhabra et al., 2019; Massey et al., 2019; Rim et al., 2020).

75 Here we use CRISPR/Cas9 mediated genome editing in haploid cells to generate clonal cell  
76 lines that express fluorescently tagged CTNNB1. Using confocal imaging and automated cell  
77 segmentation we quantify the dynamic subcellular increase of endogenous CTNNB1 upon  
78 WNT stimulation. Moreover, using Fluorescence Correlation Spectroscopy (FCS) and Number  
79 and Brightness (N&B) analysis we measure the mobility and concentration of CTNNB1,  
80 providing detailed information on CTNNB1 containing complexes in the cytoplasm and  
81 nucleus. Next, we use these biophysical parameters to build a computational model of

82 WNT/CTNNB1 signaling that predicts the levels and subcellular distribution of CTNNB1 across  
83 its cytoplasmic and nuclear pools. Using this integrated experimental and computational  
84 approach, we find that WNT regulates the dynamic distribution of CTNNB1 across different  
85 functional pools by modulating three regulatory nodes: cytoplasmic destruction,  
86 nucleocytoplasmic shuttling and nuclear retention. Finally, we strengthen the link between  
87 our data and the model via specific experimental perturbations, which shows that the  
88 regulatory nodes responsible for nuclear retention and nuclear shuttling of CTNNB1 are  
89 equally important under physiological and oncogenic conditions.

## 90 Results

### 91 Generation and functional validation of clonal HAP1<sup>SGFP2-CTNNB1</sup> cell lines

92 To visualize and quantify the spatiotemporal dynamics of WNT/CTNNB1 signaling at the  
93 endogenous level, we fluorescently tagged CTNNB1 in mammalian cells using CRISPR/Cas9  
94 mediated homology directed repair (Ran et al., 2013) (Figure 1). To preserve the existing  
95 (epi)genetic control mechanisms of *CTNNB1* expression, only the coding sequence for SGFP2,  
96 a monomeric, bright and photostable green fluorescent protein (Kremers et al., 2007), was  
97 seamlessly inserted at the starting ATG of the *CTNNB1* coding sequence in HAP1 cells, a WNT-  
98 responsive near haploid cell line (Figure 1B, Figure 1 supplement 2A)(Andersson et al., 1987;  
99 Carette et al., 2011; Kotecki et al., 1999; Lebensohn et al., 2016). The choice for this haploid  
100 cell line ensured homozygous tagging of CTNNB1 (Figure 1C), thus overcoming the limitations  
101 of polyploid cell lines where genome editing often results in a combination of correctly and  
102 incorrectly edited alleles (Canaj et al., 2019).

103 We isolated clonal cell lines with the desired modification by FACS sorting (Figure 1D-F) with  
104 a gating strategy that specifically selected for haploid cells (Figure 1 supplement 1) as HAP1

105 cells can become diploid or polyploid over time (Essletzbichler et al., 2014; Yaguchi et al.,  
106 2018). Genome editing of wild-type HAP1 (HAP1<sup>WT</sup>) cells resulted in a small population with  
107 low SGFP2 fluorescence (0.2%) (Figure 1D-E). The intensity, but not the number of cells in this  
108 population increased upon treatment with CHIR99021, a potent and selective GSK3 inhibitor  
109 (Bain et al., 2007), providing a strong indication that these fluorescent events corresponded  
110 to HAP1 cells in which the *SGFP2* sequence was successfully knocked into the endogenous  
111 *CTNNB1* locus (HAP1<sup>SGFP2-CTNNB1</sup>) (Figure 1F). While scarless tagging of endogenous genes in  
112 HAP1 cells was relatively cumbersome (only 0.2% gated events), PCR based screening and  
113 sanger sequencing revealed that the desired repair occurred with almost 90% efficiency  
114 within this population (Figure 1 supplement 2).

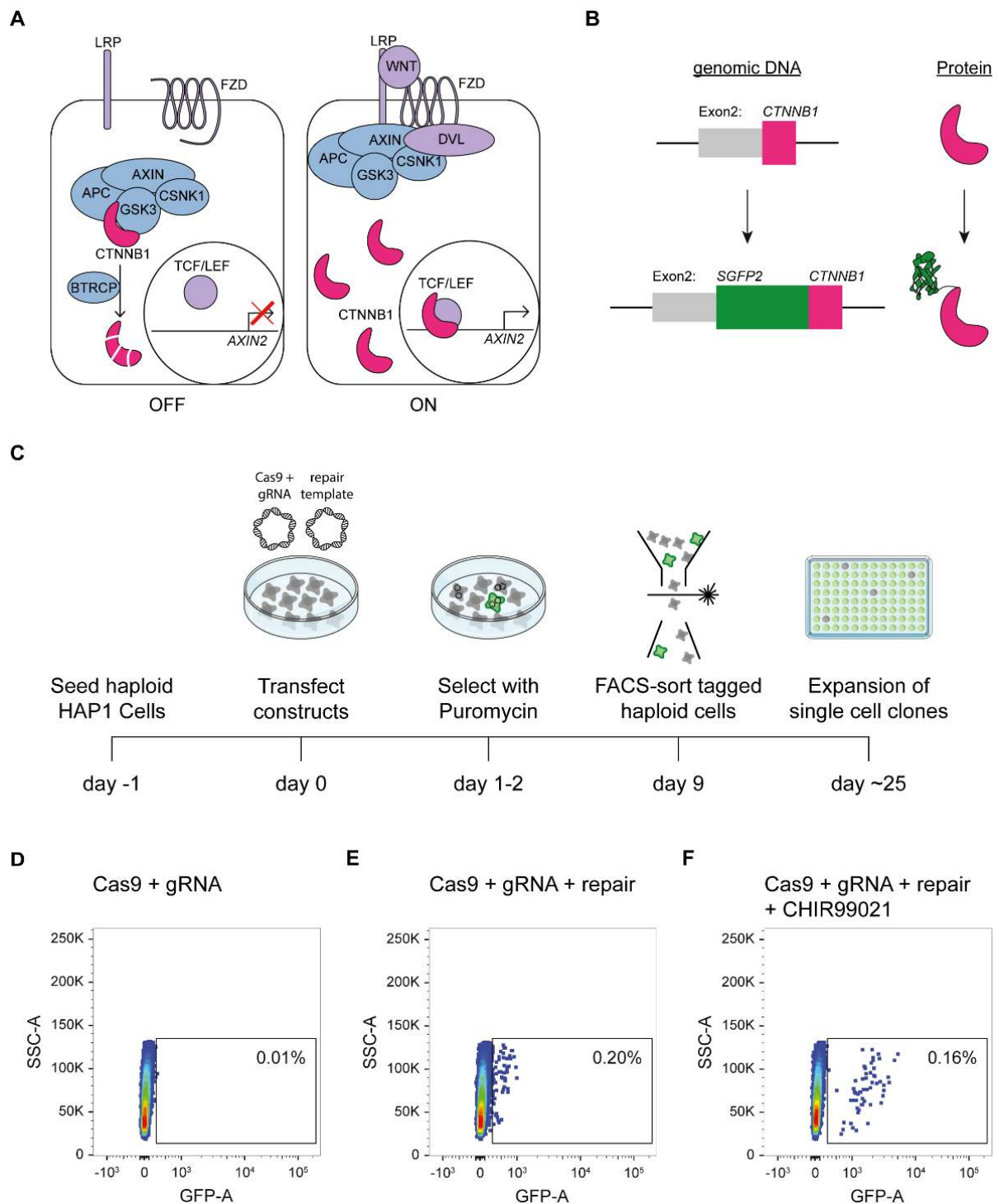
115 **Figure 1:** Generation of HAP1<sup>SGFP2-CTNNB1</sup> cell lines. A) Cartoon depicting the current model of the WNT/CTNNB1 pathway. In the absence of  
116 WNT ligands (left, "OFF"), free cytoplasmic CTNNB1 is captured by the destruction complex consisting of AXIN, APC, CSNK1 and GSK3, which  
117 leads to its phosphorylation, BTRC mediated ubiquitination and subsequent proteasomal degradation, resulting in low levels of CTNNB1 in  
118 the cytoplasm and nucleus. Binding of the WNT protein (right, "ON") to the FZD and LRP receptors inhibits the destruction complex through  
119 DVL. CTNNB1 accumulates in the cytoplasm and subsequently translocates to the nucleus, where it promotes the transcription of target  
120 genes, such as AXIN2, as a co-activator of TCF/LEF transcription factors. B) Cartoon depicting exon 2 of the *CTNNB1* locus, which contains  
121 the start codon, and the CTNNB1 protein before (top) and after (bottom) introduction of the SGFP2 by CRISPR/Cas9 mediated homology  
122 directed repair. C) Schematic of the experimental workflow and timeline for generating HAP1<sup>SGFP2-CTNNB1</sup> clones. Cas9, gRNA and repair  
123 templates are transfected as plasmids. The repair template contains the coding sequence of SGFP2 surrounded by 800 bp homology arms  
124 on either side and lacks the gRNA recognition site (see supplement 2 of this figure). A short puromycin selection step is included from 24-48  
125 hours after transfection to enrich for transfected cells. Haploid, GFP-positive cells are sorted and single cell clones are expanded for further  
126 analysis. D-F) FACS plots illustrating control (D) and SGFP2-CTNNB1 tagged cells (E-F). D) Cells transfected with Cas9 and gRNA in the absence  
127 of a repair template were used to set the gate for SGFP2-positive events. E) A small population of cells expressing low levels of SGFP2 can  
128 be detected when cells are transfected with Cas9, gRNA and repair template. F) Treatment for 24 hours of cells similar to those depicted in  
129 (D) with 8 $\mu$ M CHIR99021 does not change the amount of cells that are SGFP2 positive, but increases the SFP2 signal, most likely reflecting  
130 an increase in SGFP2-tagged beta catenin levels on a per cell basis and supporting the notion that the gated events indeed represent  
131 successfully tagged cells.

132 The following figure supplements are available for Figure 2:

133 **Figure supplement 1:** FACS Gating strategy for haploid HAP1 cells.

134 **Figure supplement 2 :** *SGFP2-CTNNB1* locus.

135



136

137 To verify that the SGFP2 tag did not interfere with CTNNB1 function, three clonal  
 138 HAP1<sup>SGFP2-CTNNB1</sup> cell lines were further characterized using established experimental readouts  
 139 for WNT/CTNNB1 signaling (Figure 2 and Figure 2 supplement 1). Western blot analysis  
 140 confirmed that the HAP1<sup>SGFP2-CTNNB1</sup> clones did not contain any untagged CTNNB1 but only  
 141 expressed the SGFP2-CTNNB1 fusion protein (Figure 2A). Moreover, the total levels of SGFP2-

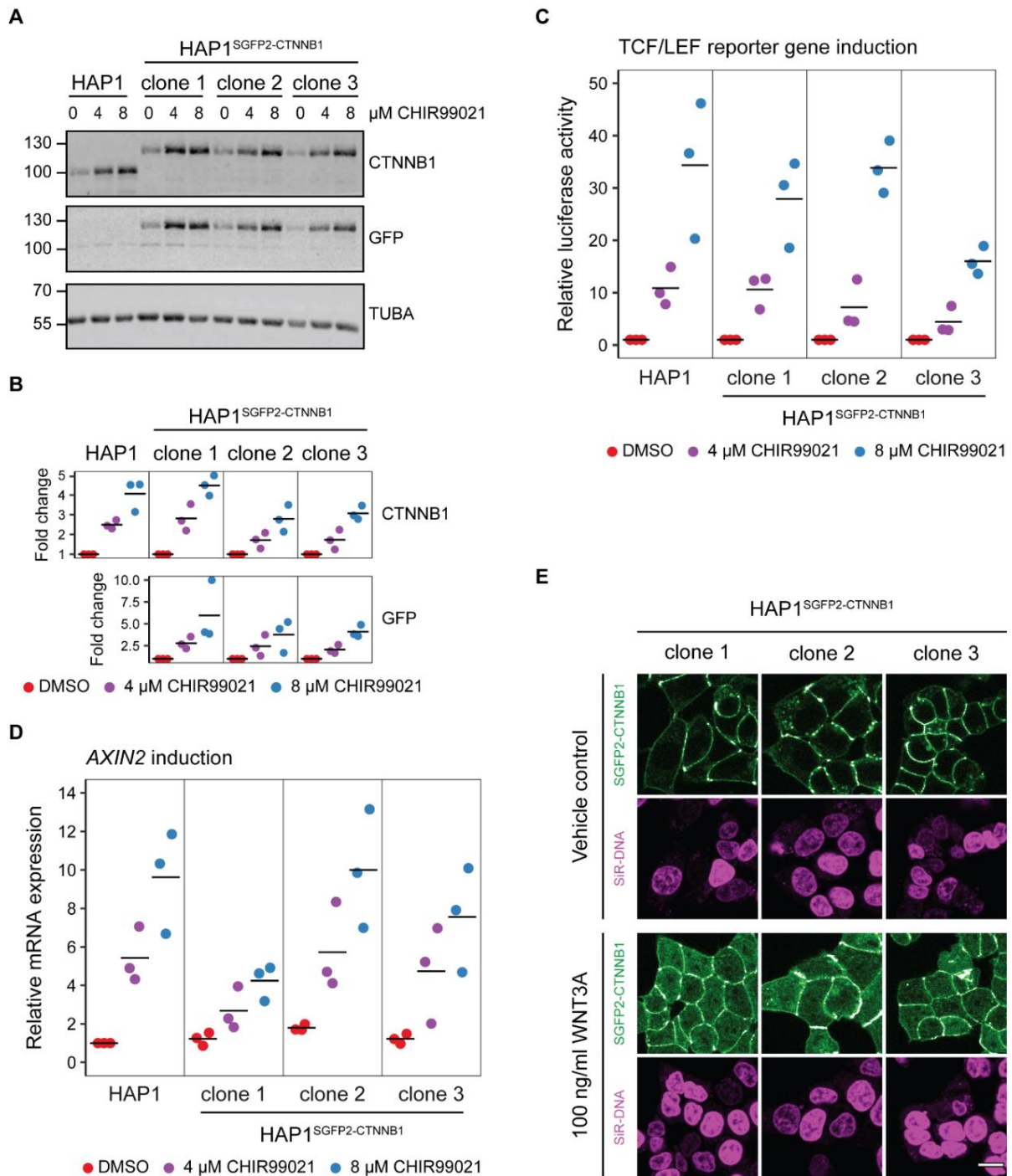


142 CTNNB1 in tagged cell lines increased to the same extent as wild-type CTNNB1 in untagged  
143 cells in response to CHIR99021 treatment (Figure 2A-B). Similarly, untagged and tagged  
144 CTNNB1 induced target gene expression in equal measure, as measured by a TCF/LEF  
145 responsive luciferase reporter (Hu et al., 2007) (Figure 2C), and increased transcription of the  
146 universal WNT/CTNNB1 target *AXIN2* (Lustig et al., 2002) (Figure 2D). Finally, while  
147 unstimulated cells mainly showed SGFP2-CTNNB1 localization at the adherens junction,  
148 treatment with purified WNT3A protein (Figure 2E) and CHIR99021 (Figure 2 supplement 1E)  
149 increased SGFP2-CTNNB1 levels in the cytoplasm and nucleus consistent with its signaling  
150 function.

151 Taken together, WNT-responsive changes in CTNNB1 levels, localization and activity are  
152 preserved after CRISPR/Cas9 mediated homozygous tagging of CTNNB1. Although there is  
153 some variation between the three clones with respect to CTNNB1 stabilization and target  
154 gene activation, this is likely due to the sub-cloning of these cell lines rather than the targeting  
155 per se.

156 **Figure2:** Functional validation of three independent HAP1<sup>SGFP2-CTNNB1</sup> clones. A) Western blot, showing CTNNB1 (HAP1<sup>WT</sup>) and SGFP2-CTNNB1  
157 (HAP1<sup>SGFP2-CTNNB1</sup> clone 1, 2 and 3) accumulation in response to CHIR99021 treatment. All panels are from one blot that was cut at the 70 kDa  
158 mark and was stained with secondary antibodies with different fluorophores for detection. Top: HAP1<sup>WT</sup> cells express CTNNB1 at the  
159 expected wild-type size. Each of the three clonal HAP1<sup>SGFP2-CTNNB1</sup> cell lines only express the larger, SGFP2-tagged form of CTNNB1, that runs  
160 at the expected height (~27 kDa above the wild-type CTNNB1). Middle: Only the tagged clones express the SGFP2-CTNNB1 fusion protein,  
161 as detected with an anti-GFP antibody at the same height. Bottom: alpha-Tubulin (TUBA) loading control. A representative image of n=3  
162 independent experiments is shown. B) Quantification of Western blots from n=3 independent experiments, including the one in (A),  
163 confirming that the accumulation of CTNNB1 in response to WNT/CTNNB1 pathway activation is comparable between HAP1<sup>WT</sup> and HAP1<sup>SGFP2-CTNNB1</sup>  
164 cells. Horizontal bar indicates the mean. C) Graph depicting the results from a MegaTopflash dual luciferase reporter assay, showing  
165 comparable levels of TCF/LEF reporter gene activation for HAP1<sup>WT</sup> and HAP1<sup>SGFP2-CTNNB1</sup> cells in response to CHIR99021 treatment. Data points  
166 from n=3 independent experiments are shown. Horizontal bar indicates the mean. Values are depicted relative to the DMSO control, which  
167 was set to 1 for each individual cell line. D) Graph depicting *AXIN2* mRNA induction in response to CHIR99021 treatment, demonstrating  
168 that induced expression of an endogenous target gene is comparable between HAP1<sup>WT</sup> and HAP1<sup>SGFP2-CTNNB1</sup> cells. Data points represent n=3  
169 independent experiments. Horizontal bar represents the mean. *HPRT* was used as a reference gene. Values are depicted relative to the  
170 HAP1<sup>WT</sup> DMSO control, which was set to 1. E) Representative confocal microscopy images of the three HAP1<sup>SGFP2-CTNNB1</sup> clones after 4-hour  
171 vehicle control or 100ng/ml WNT3A treatment from n=1 biological experiment, revealing intracellular accumulation of SGFP2-CTNNB1  
172 (green). Nuclei were counterstained with SiR-DNA dye (magenta). Scale bar is 10µm.

173 The following figure supplements are available for Figure 2:  
174 **Figure supplement 1:** Verification of the WNT/CTNNB1 responsiveness of HAP1 cells.



175

176 Live imaging of endogenous SGFP2-CTNNB1 during WNT pathway activation

177 To better understand the temporal dynamics of endogenous CTNNB1 stabilization, we  
 178 performed live-cell imaging over 12 hours in HAP1<sup>SGFP2-CTNNB1</sup> cells (Figure 3, Supplementary  
 179 Movie 1-2) with different levels of WNT stimulation. Unstimulated cells showed a stable  
 180 CTNNB1 signal at the cell membrane throughout the imaging time course (Figure 3A,

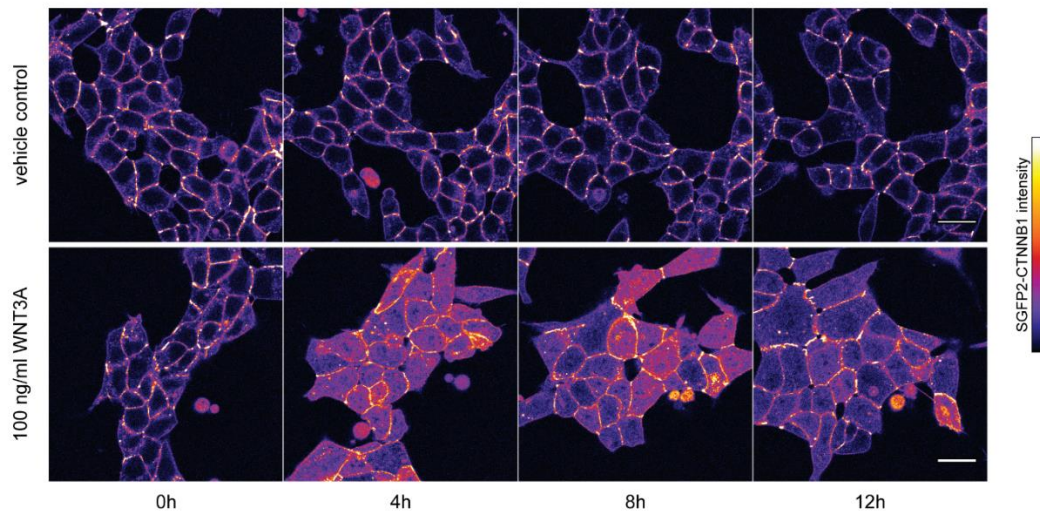
181 Supplementary Movie 1). The membrane localization of CTNNB1 is consistent with its  
182 structural role in adherens junctions (Valenta et al., 2012; Yap et al., 1997), which we will not  
183 consider further in the current study. Stimulation with different concentrations of purified  
184 WNT3A resulted in a heterogeneous response pattern, with some cells in the population  
185 showing a far more prominent increase in CTNNB1 levels in the cytoplasm and nucleus than  
186 others (Figure 3A, Figure 3 supplement 2A-B, Supplementary Movie 2).

187 To quantify these dynamic changes, we developed a custom-built automated segmentation  
188 pipeline in CellProfiler™ (Figure 3D). Quantification showed that the dynamics of CTNNB1  
189 accumulation were independent of the dose of WNT3A (Figure 3B-C, Supplementary Movies  
190 4-5). Treatment with 100 ng/ml WNT3A increased SGFP2-CTNNB1 fluorescence 1.74-fold  
191 (mean, 95% CI 1.73-1.76) in the cytoplasm and 3.00-fold (mean, 95% CI 2.97-3.03) in the  
192 nucleus, with similar results in the other two HAP1<sup>SGFP2-CTNNB1</sup> clones (Figure 3 supplement 1).

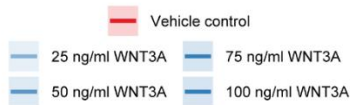
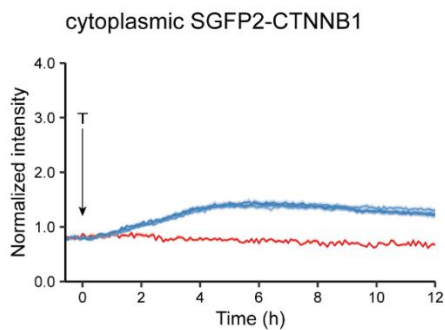
193 Our quantification further shows that nuclear accumulation of CTNNB1 is favored over  
194 cytoplasmic increase (compare the fold-changes in Figure 3B-C). Moreover, the first  
195 statistically significant increases in fluorescence intensity in the cytoplasm could be detected  
196 after ~45 minutes of treatment (Supplementary Movie 4, Figure 3 supplement 2C), whereas  
197 in the nucleus an increase was first statistically significant after ~30 minutes (Supplementary  
198 Movie 5, Figure 3 supplement 2D). To examine the relation between the cytoplasmic and  
199 nuclear CTNNB1 pools more closely, we calculated the ratio between nuclear and cytoplasmic  
200 intensities of SGFP2-CTNNB1 (Figure 3D, Supplementary Movie 6). In untreated cells, the  
201 nuclear/cytoplasmic ratio was 0.652 (mean [3-5 hours], 95% CI 0.649-0.657), showing that  
202 SGFP2-CTNNB1 was preferentially localized to the cytoplasm (Figure 3D, Figure 3 supplement  
203 3). For the first 3 hours after WNT3A, nuclear CTNNB1 levels rose considerably faster than

204 cytoplasmic CTNNB1 levels until the nuclear/cytoplasmic ratio showed a slight nuclear  
205 enrichment of 1.08 (mean [3-5 hours] 95% CI 1.07-1.10) for 100 ng/ml WNT3A. This indicates  
206 that not only the turn-over, but also the subcellular localization of CTNNB1 is actively  
207 regulated both before and after WNT pathway activation.

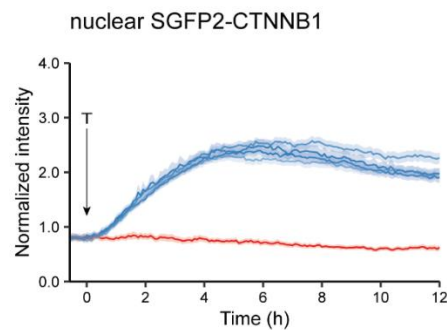
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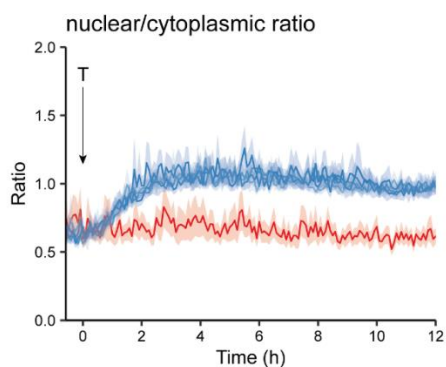
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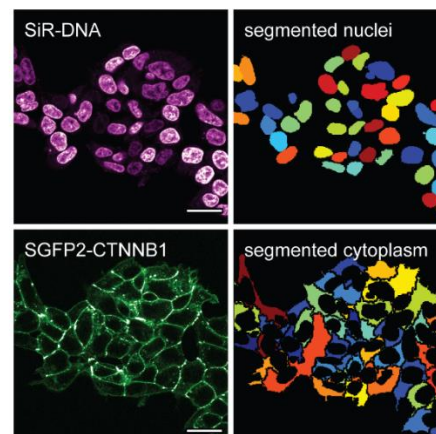
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D



E



208

209 **Figure 3:** Live imaging of HAP1SGFP2-CTNNB1. A) Representative stills from confocal time-lapse experiments corresponding to  
210 Supplementary Movies 1-2, showing an increase of SGFP2-CTNNB1 after treatment with 100 ng/ml WNT3A (bottom) relative to a vehicle  
211 control (BSA) treated sample (top). Scale bar = 20  $\mu$ m. B-D) Quantification of time-lapse microscopy series, using the segmentation pipeline  
212 shown in (E). Arrow indicates the moment of starting the different treatments (T, see legend in B for details). B-C) Graph depicting the  
213 normalized intensity of SGFP2-CTNNB1 in the cytoplasm (B) or nucleus (C) over time. Solid lines represent the mean normalized fluorescence  
214 intensity and shading indicates the 95% confidence interval. n=155-393 cells for each condition and time point, pooled data from n=3  
215 independent biological experiments. D) Graph depicting the nuclear/cytoplasmic ratio of SGFP2-CTNNB1 over time, calculated from raw  
216 intensity values underlying (B) and (C). E) Segmentation of nuclei (top) and cytoplasm (bottom) based in the SiR-DNA signal and SGFP2-  
217 CTNNB1 signal. Scale bar = 20 $\mu$ m.

218 The following figure supplements are available for Figure 3:

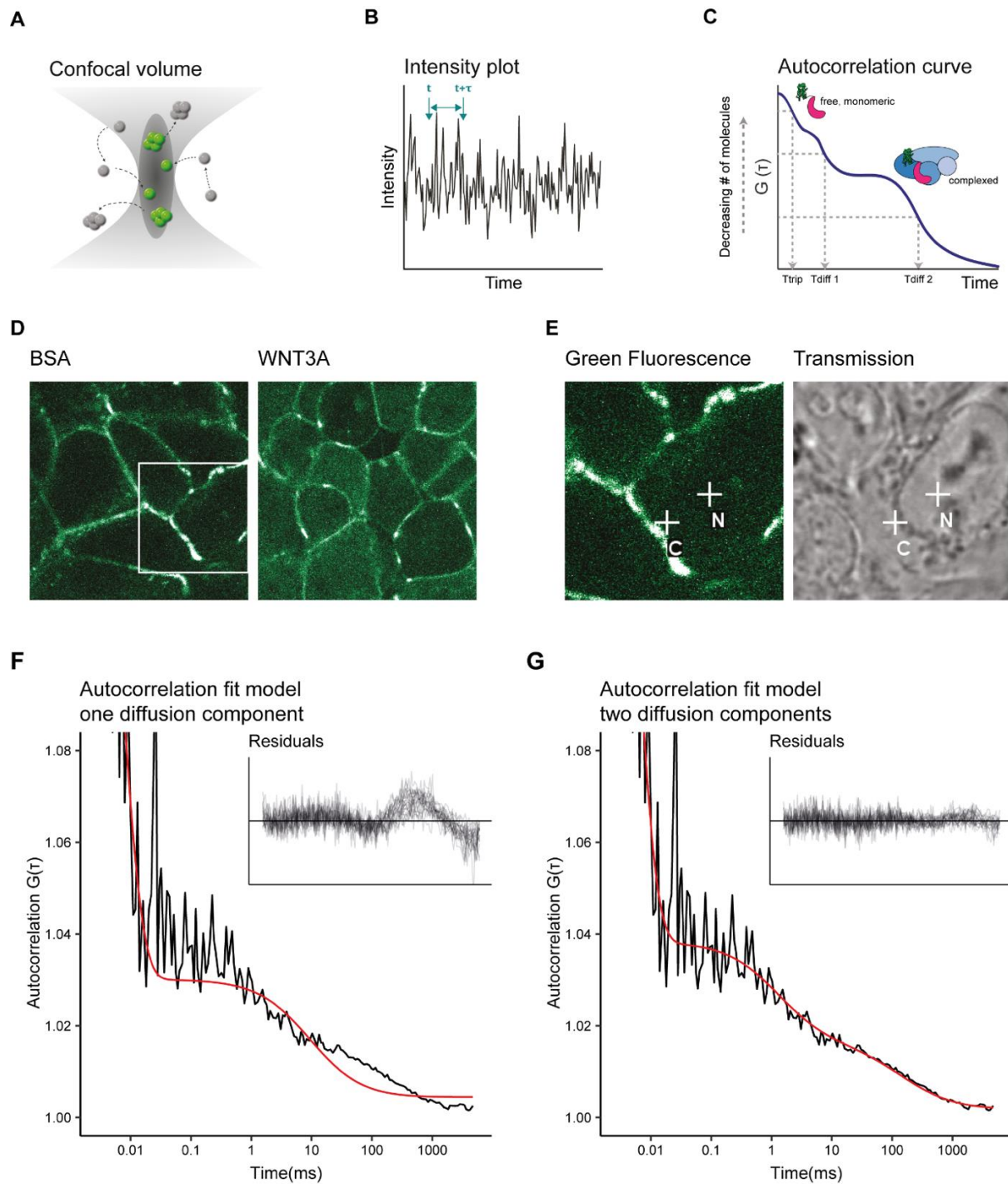
219 **Figure supplement 1:** Verification of imaging results with WNT3A three independent HAP1<sup>SGFP2-CTNNB1</sup> clones.

220 **Figure supplement 2:** Difference analysis of SGFP2-CTNNB1 fluorescence.

221 **Figure supplement 3:** Unnormalized nuclear and cytoplasmic intensity measurements

## 222 Establishing a fitting model for SGFP2-CTNNB1 diffusion

223 Having measured the relative changes in the cytoplasmic and nuclear levels of CTNNB1 in  
224 response to WNT3A stimulation, we next sought to exploit our experimental system to  
225 quantify additional molecular properties of CTNNB1 in each of these subcellular  
226 compartments using Fluorescence Correlation Spectroscopy (FCS). FCS is a powerful method  
227 to measure the mobility and absolute levels of fluorescent particles in a nanomolar range,  
228 compatible with typical levels of signaling proteins in a cell (reviewed in Hink, 2014). It has for  
229 instance been used to gain insight into the assembly of DVL3 supramolecular complexes  
230 (Yokoyama et al., 2012), the endogenous concentrations and mobility of nuclear complexes  
231 (Holzmann et al., 2019; Lam et al., 2012), and most recently, to quantify ligand-receptor  
232 binding reactions in the WNT pathway (Eckert et al., 2020). In point FCS, the fluorescence  
233 intensity is measured in a single point (Figure 4A,D-E). Diffusion of labeled particles, in this  
234 case SGFP2-CTNNB1, causes fluctuation of the fluorescence signal over time (Figure 4B). By  
235 correlating the fluorescence intensity signal to itself over increasing time-intervals, an  
236 autocorrelation curve is generated (Figure 4C). To extract relevant biophysical parameters,  
237 such as mobility (a measure for size) and the absolute numbers of the fluorescent particles  
238 (corresponding to their concentration), this autocorrelation curve is fitted with an  
239 appropriate model.



240

241 **Figure 4:** Two diffusion-component fit-model for SGFP2-CTNNB1 FCS measurements. A) Schematic representation of the point FCS  
 242 technique, depicting the confocal volume with fluorescent particles diffusing in and out. Particles in FCS are defined by their coherent  
 243 movement; therefore, a particle can be made up of monomers or multimers in isolation or complexed to unlabeled molecules. B) Schematic  
 244 representation of intensity fluctuations over time as measured in the confocal volume. Fluctuations are the result of both photo-physics  
 245 (e.g. blinking of the fluorophore) and diffusion. C) Graphical representation of the two diffusion-component fitting model used for our  
 246 autocorrelation curves.  $T_{trip}$  describes the blinking of the SGFP2 fluorophore and the after-pulsing artefact.  $T_{diff1}$  and  $T_{diff2}$  describe the  
 247 monomeric and complexed form of SGFP2-CTNNB1, respectively. Details of all fitting parameters are described in Materials and Methods.  
 248 D) Representative confocal images of HAP1<sup>SGFP2-CTNNB1</sup> cells treated for 4 hours with BSA (left) or 100 ng/ml WNT3A (right). E) Zoom in of the  
 249 white rectangle in (D), with representative locations of FCS measurement points for cytoplasm (C) and nucleus (N) indicated with white  
 250 crosses in the SGFP2-CTNNB1 channel and transmission channel. F-G) Fitting of a representative autocorrelation curve with one unfixed  
 251 diffusion-component (F) or a two diffusion-component model (G), where the first diffusion component was fixed to the speed of free  
 252 monomeric SGFP2-CTNNB1 ( $14.9\mu\text{m}^2/\text{s}$ ) and the second diffusion component was unfixed. The black line represents the autocorrelation  
 253 curve generated from the FCS measurement, the red line represents the fitted model. The residuals after fitting of 25 individual curves are  
 254 shown in the upper right corner of the graphs.

255 We first attempted to fit the autocorrelation curves obtained with point FCS measurements  
256 of HAP1<sup>SGFP2-CTNNB1</sup> cells with a one-component model (i.e. containing one single diffusion  
257 speed for SGFP2-CTNNB1). This model was unable to fit most of our data (Figure 4F). The  
258 current literature suggests that while a large portion of CTNNB1 is present as a monomer  
259 (Gottardi and Gumbiner, 2004; Maher et al., 2010), CTNNB1 is also present in multiprotein  
260 complexes in the cytoplasm and in the nucleus (reviewed in Gammons and Bienz, 2018).  
261 Therefore, we next used a model with two diffusion components, in which the first diffusion  
262 component was fixed to the diffusion speed of monomeric, unbound SGFP2-CTNNB1 (14.9  
263  $\mu\text{m}^2/\text{s}$ ) and the second diffusion component was limited to slower speeds compatible with  
264 point-FCS imaging (see materials and methods for details). This model provided good fits for  
265 our autocorrelation curves obtained in both cytoplasmic and nuclear point FCS measurements  
266 (Figure 4G), consistent with the presence of free monomeric CTNNB1 and larger CTNNB1  
267 containing complexes in both the nucleus and cytoplasm.

#### 268 Quantification of absolute SGFP2-CTNNB1 concentrations

269 Using this fitting model, we determined, for the first time, the absolute concentrations of  
270 endogenous CTNNB1 in living cells in presence and absence of a physiological WNT stimulus  
271 (Figure 5A). In the absence of WNT3A, we determined the total concentration of SGFP2-  
272 CTNNB1 to be 180 nM (median, 95%CI 127-218) in the cytoplasm and 122 nM (median, 95%CI  
273 91-158) in the nucleus. This is consistent with the nuclear exclusion we observed with  
274 confocal imaging (Figure 3).

275 In the presence of WNT3A, we measured a 1.2-fold increase in the total SGFP2-CTNNB1  
276 concentration to 221 nM (median, 95%CI 144-250 nM) in the cytoplasm. This increase was  
277 smaller than expected from fluorescence intensity measurements (Figure 3B), for which we

278 currently have no explanation (Figure 5 supplement 1). In the nucleus the concentration  
 279 increased 2.0-fold to 240 nM (median, 95%CI 217-325) upon pathway activation. Nuclear  
 280 concentrations of SGFP2-CTNNB1 therefore exceed cytoplasmic concentrations after WNT3A  
 281 treatment, consistent with the nuclear accumulation observed with live imaging (Figure 3).  
 282 These concentrations are in a similar range as those previously determined by quantitative  
 283 mass spectrometry in different mammalian cell lines (Kitazawa et al., 2017; Tan et al., 2012).  
 284 Of note, the exact concentrations can vary between cell types and may be dependent on the  
 285 intricacies and assumptions that underlie each individual technique.

286 **Table 1:** Total number of SGFP2-CTNNB1 molecules and calculated concentrations obtained from FCS measurements in n=3 independent  
 287 experiments. The concentration is calculated from the number of molecules and the calibrated confocal volume (see material and methods).  
 288 The number of molecules is consistent with those measured with N&B analysis (Figure 5 supplement 1A, Supplementary file 1)

|             |           |    | Number of molecules |         | Concentration (nM) |         |
|-------------|-----------|----|---------------------|---------|--------------------|---------|
| compartment | treatment | n  | median              | 95% CI  | median             | 95% CI  |
| Cytoplasm   | BSA       | 21 | 80                  | 70-116  | 180                | 127-218 |
|             | WNT3A     | 21 | 95                  | 85-122  | 221                | 144-250 |
| Nucleus     | BSA       | 21 | 63                  | 53-72   | 122                | 91-158  |
|             | WNT3A     | 18 | 135                 | 127-150 | 240                | 217-325 |

289  
 290 Our two-component fitting model also allowed us to discriminate between pools of SGFP2-  
 291 CTNNB1 with different mobility, i.e. fast diffusing monomeric CTNNB1 (Figure 5B) and slow  
 292 diffusing complexed CTNNB1 (Figure 5C). In the nucleus, the concentration of fast moving  
 293 CTNNB1 increased 2.0-fold from 87 nM (median, 95%CI 78-119) to 170 nM (median, 95%CI  
 294 147-214), while slow moving CTNNB1 concentration increased 3.9-fold from 22 nM (median,  
 295 95%CI 4-40) to 86 nM (median, 95%CI 67-114). This is also reflected by the increase in the  
 296 bound fraction of SGFP2-CTNNB1 the nucleus (Figure 5D). The preferential increase of the  
 297 slow-moving fraction is consistent with the notion that upon WNT stimulation CTNNB1 will  
 298 become associated with the chromatin in a TCF dependent transcriptional complex (or “WNT  
 299 enhanceosome”).



300 **Table 2:** Number and concentration of SGFP2-CTNNB1 molecules with the fast or slow diffusion coefficient obtained from FCS measurements  
 301 in n=3 independent experiments. The concentration is calculated from the number of molecules and the calibrated confocal volume (see  
 302 material and methods).

|             |           |    | Fast SGFP2-CTNNB1   |        |                    |         | Slow SGFP2-CTNNB1   |        |                    |        |
|-------------|-----------|----|---------------------|--------|--------------------|---------|---------------------|--------|--------------------|--------|
|             |           |    | Number of molecules |        | Concentration (nM) |         | Number of molecules |        | Concentration (nM) |        |
| compartment | treatment | n  | median              | 95% CI | median             | 95% CI  | median              | 95% CI | median             | 95% CI |
| Cytoplasm   | BSA       | 21 | 51                  | 40-63  | 91                 | 66-139  | 29                  | 20-37  | 57                 | 38-76  |
|             | WNT3A     | 21 | 60                  | 47-80  | 145                | 76-168  | 35                  | 30-41  | 68.                | 57-76  |
| Nucleus     | BSA       | 21 | 48                  | 41-66  | 87                 | 78-119  | 13                  | 2-22   | 22                 | 4-40   |
|             | WNT3A     | 18 | 96                  | 81-101 | 170                | 147-214 | 47                  | 37-49  | 86                 | 64-104 |

303

304 Of note, in the cytoplasm, the concentration of both fast and slow SGFP2-CTNNB1 increased  
 305 upon WNT3A treatment (Figure 5B-C), with the fraction of bound SGFP2-CTNNB1 remaining  
 306 equal between stimulated (median 0.38, 95%CI 0.29-0.46) and unstimulated cells (median  
 307 0.34, 95%CI 0.31-0.4) (Figure 5D). The fact that a large portion of CTNNB1 remains in a  
 308 complex after WNT stimulation, challenges the view that mainly monomeric CTNNB1  
 309 accumulates, as commonly depicted in the textbook model (Figure 1A).

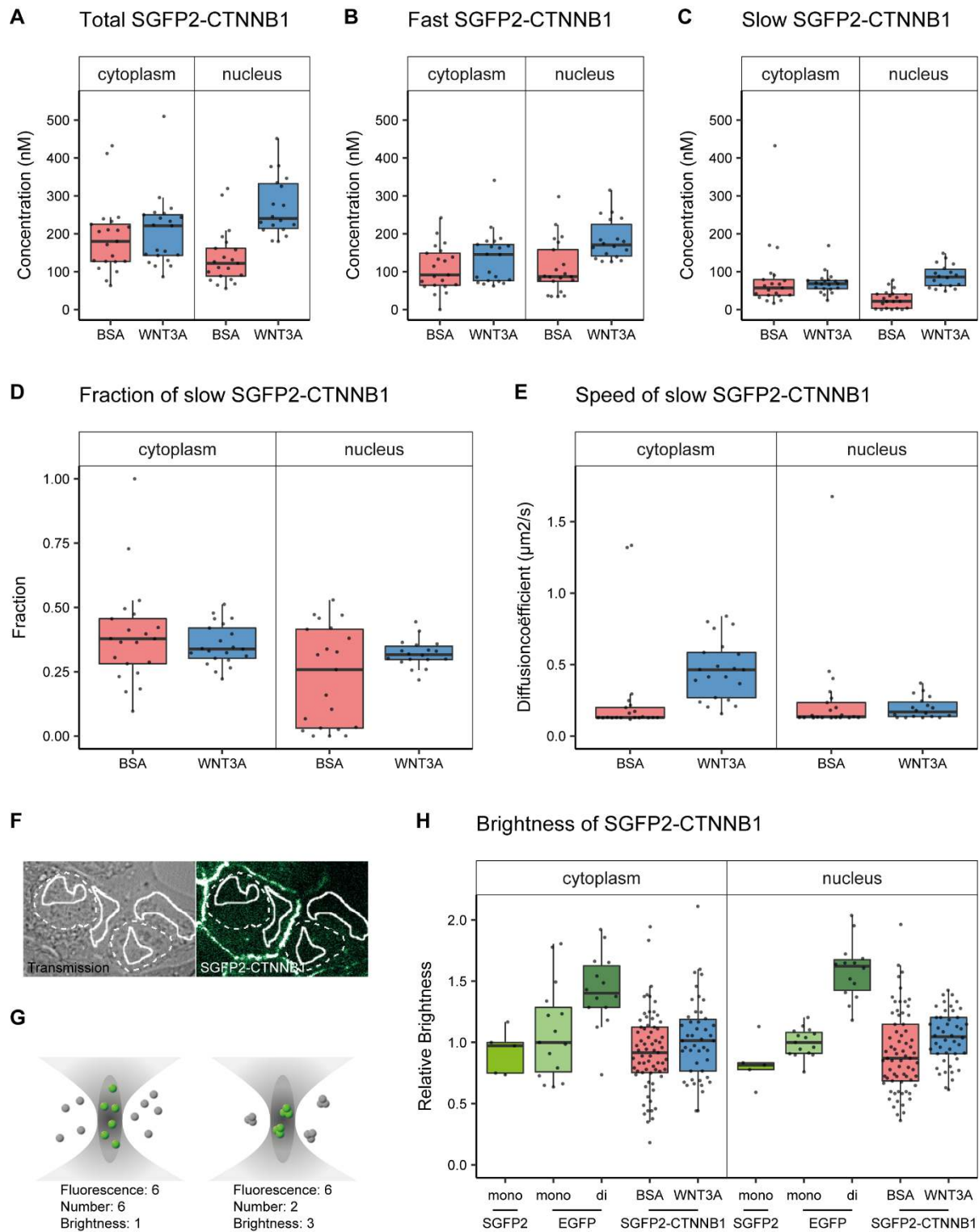
310 **Figure 5:** Abundance and mobility of SGFP2-CTNNB1 molecules in living cells after 4 hours WNT3A treatment or control. Details on sample  
 311 size and statistics can be found in supplementary file 1. A) Graph depicting the total concentration of SGFP2-CTNNB1 particles (monomeric  
 312 plus complexed) as measured with FCS. B) Graph depicting the concentration of SGFP2-CTNNB1 particles with the fast diffusion component  
 313 (i.e. free monomeric). C) Graph depicting the concentration of SGFP2-CTNNB1 containing particles with the slow diffusion component (i.e.  
 314 complex associated). D-E) Graphs depicting the fraction (D) and speed (E) of the second diffusion component (i.e. SGFP2-CTNNB1 containing  
 315 complex) measured by FCS. F) Example of typical regions of interest in two cells used in N&B analysis. Solid line represents the analysis ROI,  
 316 dashed line, marks the outline of the nuclear envelope. G) Schematic representation of a confocal volume with different brightness species.  
 317 On the left are 6 monomers with a brightness of 1, on the right 2 trimers with a brightness of 3, both result in a fluorescence of 6. N&B  
 318 analysis is able to extract the number and the brightness of such samples, for more detail see supplement 1 of this figure. H) Graph depicting  
 319 the molecular brightness of SGFP2-CTNNB1 in the cytoplasm and nucleus relative to controls as measured with N&B in the same subcellular  
 320 compartments. EGFP monomer was used for normalization and EGFP dimer as a control for N&B measurements.

321 The following supplements are available for Figure 6:

322 **Figure supplement 1:** Quantification of SGFP2-CTNNB1 particles, fluorescence and fluorescence lifetime.

323 **Figure supplement 2:** Number and Brightness analysis

324 **Supplementary File 1:** Lists all summary statistics (mean, median, 95% confidence intervals, differences, p-values) of the FCS and N&B  
 325 parameters show in Figure 6 and Figure 6 supplement 1.



327 Quantification of SGFP2-CTNNB1 mobility

328 While we cannot determine the exact composition of the SGFP2-CTNNB1 complex, we do  
329 obtain biophysical parameters that are linked to its size. For instance, the diffusion coefficient  
330 of the nuclear SGFP2-CTNNB1 complex was  $0.17 \mu\text{m}^2\text{s}^{-1}$ , (median, 95%CI 0.14-0.22) in cells  
331 treated with purified WNT3A (Figure 5E). This is comparable to the diffusion coefficients  
332 measured for other chromatin bound transcriptional activators (Lam et al., 2012), which  
333 further supports that this pool represents the WNT enhanceosome.

334 In the cytoplasm, we determined the second diffusion coefficient of SGFP2-CTNNB1 to be  
335  $0.13 \mu\text{m}^2\text{s}^{-1}$  (median, 95% CI 0.13-0.17) in the absence of WNT3A stimulation (Figure 5E). This  
336 is indicative of very large complexes containing SGFP2-CTNNB1 that move with diffusion  
337 kinetics comparable to those previously observed for the 26S proteasome (Pack et al., 2014).  
338 After WNT3A treatment, the speed of the cytoplasmic complex increased 3.5-fold to  $0.46$   
339  $\mu\text{m}^2\text{s}^{-1}$  (95% CI of the median 0.37-0.57). Because a 3.5-fold change in speed would result in  
340  $3.5^3$ -change in size for a spherical particle (assuming Einstein-Stokes, see equation 7 in the  
341 material and methods section for details), this indicates that the size of the cytoplasmic  
342 CTNNB1 complex drastically changes when the WNT pathway is activated. Thus, although the  
343 fraction of CTNNB1 that resides in a complex remains the same, the identity of the  
344 cytoplasmic complex is quite different in unstimulated and WNT3A stimulated cells.

345 Determining the multimerization status of SGFP2-CTNNB1

346 Recent work suggests that the CTNNB1 destruction complex (also known as the  
347 “degradosome”) is a large and multivalent complex, mainly as the result of AXIN and APC  
348 multimerization (reviewed in Schaefer and Peifer, 2019). The second diffusion coefficient,  
349 determined by our FCS measurements (Figure 5E), is consistent with this model. Such a large,  
350 multivalent destruction complex would be expected to have multiple CTNNB1 binding sites.  
351 To measure the multimerization status (i.e. the number of bound SGFP2-CTNNB1 molecules)  
352 within this cytoplasmic complex, we performed Number and Brightness (N&B) analysis  
353 (Figure 5F-G, Figure 5 supplement 2). N&B is a fluorescence fluctuation spectroscopy  
354 technique similar to point FCS, but it makes use of image stacks acquired over time rather  
355 than individual point measurements (Digman et al., 2008). By quantifying the variance in  
356 fluorescence intensity of this stack, not only the number of particles but also their brightness  
357 can be determined. The number of particles we determined using N&B, were highly similar to  
358 those obtained with FCS (compare Figure 5 supplement 1A with Table 1).

359 Because brightness is an inherent property of a fluorophore, a change in brightness is a  
360 measure of the number of fluorophores per particle. In our case, the brightness is indicative  
361 of the number of SGFP2-CTNNB1 molecules per complex. As N&B does not incorporate  
362 diffusion kinetics, we cannot differentiate between monomeric (which would have a  
363 brightness of one) and complexed CTNNB1 (which would have a brightness exceeding one if  
364 multiple CTNNB1 molecules reside in a single complex). Therefore, the measured brightness  
365 of SGFP2-CTNNB1 in our N&B analysis is an average of both fractions. We observe that the  
366 total pool of SGFP2-CTNNB1 in both the cytoplasm and nucleus has a brightness similar to  
367 EGFP and SGFP2 monomers (Figure 5H, Table 3). Because we found a large fraction of SGFP2-

368 CTNNB1 to reside in a complex using point FCS (Figure 5C-D), this suggests that few, if any, of  
369 these complexes contain multiple SGFP2-CTNNB1 molecules. If the cytoplasmic SGFP2-  
370 CTNNB1 containing complex indeed represents a large, multivalent destruction complex, this  
371 would imply that under physiological conditions, quite unexpectedly, most CTNNB1 binding  
372 sites are unoccupied in both the absence and presence of WNT3A.

373 **Table 3:** Brightness of SGFP2-compared relative to EGFP-monomer and -dimer controls in n=2 independent experiments. P-values were  
374 calculated using PlotsOfDifferences that uses a randomization test (Goedhart, 2019). Note that only the EGFP-dimer is significantly different  
375 to the EGFP-monomer control, while SGFP2-CTNNB1 is not.

| Fluorophore  | Compartment | Treatment      | n  | median | 95CI median | P-value to matched control (EGFP monomer in the nucleus or cytoplasm) |
|--------------|-------------|----------------|----|--------|-------------|---|
| EGFP-monomer | Cytoplasm   | NA             | 15 | 1      | 0.79 - 1.34 | 1.000   |
| EGFP-dimer   | Cytoplasm   | NA             | 14 | 1.4    | 1.29 - 1.60 | 0.011*  |
| SGFP2-CTNNB1 | Cytoplasm   | BSA            | 69 | 0.92   | 0.83 - 1.00 | 0.738   |
| SGFP2-CTNNB1 | Cytoplasm   | 100ng/ml WNT3A | 46 | 1.01   | 0.93 - 1.11 | 0.919   |
| EGFP-monomer | Nucleus     | NA             | 15 | 1      | 0.91 - 1.07 | 1.000   |
| EGFP-dimer   | Nucleus     | NA             | 14 | 1.62   | 1.44 - 1.69 | <0.001*   |
| SGFP2-CTNNB1 | Nucleus     | BSA            | 69 | 0.87   | 0.78 - 0.96 | 0.192   |
| SGFP2-CTNNB1 | Nucleus     | 100ng/ml WNT3A | 46 | 1.05   | 0.95 - 1.15 | 0.578   |

376

### 377 A minimal computational model of WNT/CTNNB1 signaling

378 Quantitative measurements and physical parameters of WNT pathway components and their  
379 interactions remain limited (Kitazawa et al., 2017; Lee et al., 2003; Tan et al., 2012), especially  
380 in living cells. As we obtained measurements of different functional pools of CTNNB1, we next  
381 sought to integrate these biophysical parameters in a minimal computational model of WNT  
382 signaling to identify the critical nodes of regulation of subcellular SGFP2-CTNNB1 distribution  
383 (Figure 6A, Table 4-5, Materials and Methods). This minimal model is based on a previous  
384 model of Kirschner and colleagues (Lee et al., 2003), and incorporates the new data obtained  
385 in our study, supplemented with parameters from the literature (Lee et al., 2003; Tan et al.,  
386 2012).

387 Our model diverges from the model presented by Lee et al. on two major points. First, the  
388 model is simplified by replacing the details of the destruction complex formation cycle and

389 the individual actions of APC and AXIN with a single, fully formed destruction complex. We  
390 chose this option because our study does not provide new quantitative data on the formation  
391 and dynamics of the destruction complex, but does provide absolute concentrations of  
392 CTNNB1 that is in a bound state in the cytoplasm. Second, we explicitly include shuttling of  
393 CTNNB1 between the cytoplasm and nucleus in both directions (Schmitz et al., 2013; Tan et  
394 al., 2014).

395 Thus, our model (Figure 6A) describes the binding of cytoplasmic CTNNB1 ('CB') to the  
396 destruction complex ('DC') leading to its phosphorylation and degradation (described by  $k_3$ ),  
397 which releases the DC. Transport of CTNNB1 from the cytoplasm to the nucleus, allows  
398 nuclear CTNNB1 ('NB') to bind to TCF forming a transcriptional complex (NB-TCF). When WNT  
399 is present in the system, we describe the inactivation of the destruction complex ('DC\*') by  
400 DVL. The model is available as interactive app at  
401 [https://wntlab.shinyapps.io/WNT\\_minimal\\_model/](https://wntlab.shinyapps.io/WNT_minimal_model/) and allows users to explore the effects of  
402 modulating different equilibria and constants in an intuitive way.

403 Our model faithfully recapitulates the dynamic changes that we observe with functional  
404 imaging (compare Figure 6B-F to Figure 3 and 5). Moreover, it reveals two critical regulatory  
405 nodes in addition to the requisite inactivation of the destruction complex (described by  
406  $k_5/k_4$ ). The first additional node of regulation is nuclear import and export (or 'shuttling',  
407 described by  $k_6/k_7$ ). Upon WNT stimulation, the ratio of  $k_6/k_7$  needs to increase in order for  
408 the model to match the free CTNNB1 concentrations we measured by FCS (Table 5, Figure  
409 5B). Thus, the balance shifts from nuclear export before WNT, to nuclear import after WNT.  
410 The second additional node of regulation is the association of CTNNB1 with the TCF  
411 transcriptional complex (or 'retention'), described by  $k_9/k_8$ . Upon WNT stimulation, the ratio

412 of  $k_9/k_8$  needs to decrease by almost a factor of 10 in order for the model to reproduce the  
 413 concentrations of free and bound CTNNB1 in the nucleus as measured by FCS (Table 5, Figure  
 414 6F, Figure 5B-C). Thus, association of CTNNB1 to the TCF transcriptional complex is favored  
 415 after WNT stimulation.

416 In summary, our model suggests that WNT/CTNNB1 signaling is regulated at three distinct  
 417 levels of the signal transduction pathway: destruction complex inactivation,  
 418 nucleocytoplasmic shuttling and nuclear retention.

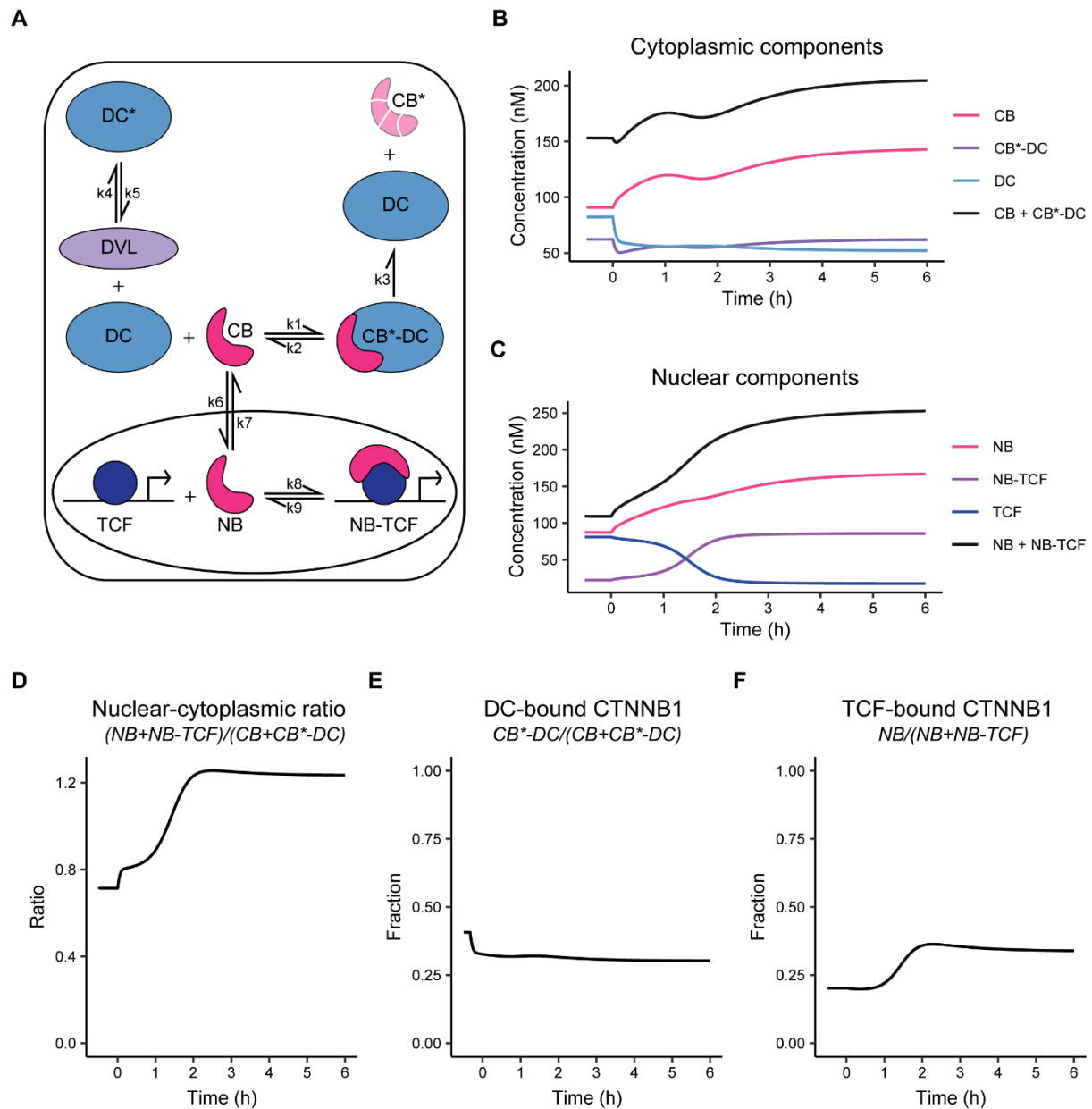
419 **Table 4:** Variables Minimal Model of WNT signaling.

| Model name       | Variable         | Compound                        | Values obtained from                   | WNT OFF (nM) | WNT ON (nM) |
|------------------|------------------|---------------------------------|--|--------------|-------------|
| CB               | $x_1$            | Free cytoplasmic CTNNB1         | FCS data this report                   | 91           | 145         |
| DC               | $x_2$            | Free destruction complex        | Model equations                        | 82.4         | 52          |
| CB*-DC           | $x_3$            | DC-bound phosphorylated CTNNB1  | FCS data this report*                  | 62.5*        | 62.5*       |
| DC*              | $x_4$            | Inactivated destruction complex | Model equations                        | 0            | 30.5        |
| NB               | $x_5$            | Free nuclear CTNNB1             | FCS data this report                   | 87           | 170         |
| TCF              | $x_6$            | Free TCF                        | Model equations                        | 81           | 17          |
| NB-TCF           | $x_7$            | TCF-bound nuclear CTNNB1        | FCS data this report                   | 22.2         | 86          |
| TCF <sup>0</sup> | TCF <sup>0</sup> | Total TCF                       | $x_7$ and Tan et al., 2012 - Figure 11 | 103          | 103         |

420 \*Under the assumption that  $k_3$  does not change, the levels of CB\*-DC remain equal. Since there was no significant difference between the  
 421 concentration of slow SGFP2-CTNNB1 (Table 2) the average of both medians was used

422 **Table 5:** Equilibrium conditions for the Minimal Model of WNT signaling. All rates are multiplied with factor  $R=20$ , so that the equilibrium is  
 423 reached at 4.5h according to Figure 4 C, D.

| Rate constant     |                     | Biological process   | Values based on                               | WNT OFF | WNT ON |
|-------------------|---------------------|--|---|---------|--------|
| $b$               | $\text{nMmin}^{-1}$ | CTNNB1 synthesis   | $v_{12}$ from Lee                             | 0.423   | 0.423  |
| $\frac{k_2}{k_1}$ | nM                  | Binding to and phosphorylation by the destruction complex of cytoplasmic CTNNB1    | $K_8$ from Lee                                | 120     | 120    |
| $k_3$             | $\text{min}^{-1}$   | Dissociation and degradation of phosphorylated CTNNB1 from the destruction complex | Deduced from $b$ and $x_3$                    | 0.0068  | 0.0068 |
| $\frac{k_5}{k_4}$ | nM                  | Inactivation of the destruction complex by activated DVL                           | Fitted to $x_1$ and $x_7$                     | N.A.    | 1.7    |
| $\frac{k_6}{k_7}$ |                     | Ratio between nuclear import and export of CTNNB1                                  | Deduced from $x_1$ and $x_5$                  | 0.96    | 1.17   |
| $\frac{k_9}{k_8}$ | nM                  | Dissociation of nuclear CTNNB1 from TCF  | Deduced from $x_5$ , TCF <sup>0</sup> , $x_7$ | 320     | 33.6   |



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**Figure 6:** Computational model of WNT/CTNNB1 based on FCS concentrations for free and complexed CTNNB1 (Table 1-2). A) Schematic overview of the model. DC=destruction complex, DC\*= DVL-inactivated DC, CB=cytoplasmic CTNNB1, CB\*=phosphorylated CB, NB=nuclear CTNNB1, TCF=TCF/LEF transcription factors, DVL=WNT-activated DVL. The model assumes that there is no activated DVL in the absence of WNT, therefore  $k_5/k_4$  do not play any role in the WNT 'OFF' equilibrium. Note that CB\* is degraded and therefore plays no role in the model. B) Graph depicting the modelled concentrations of cytoplasmic components over time. The black line indicates total concentration of cytoplasmic CTNNB1, corresponding to Figure 4C. C) Graph depicting the modelled concentrations of nuclear components over time. The black line indicates total concentration of nuclear CTNNB1, corresponding to Figure 4D. D) Graph depicting the ratio of total nuclear and cytoplasmic CTNNB1 over time, corresponding to the measurements in 4E. E) Graph depicting the DC-bound CTNNB1 fraction ratio over time. F) Graph depicting the TCF-bound CTNNB1 fraction ratio over time.



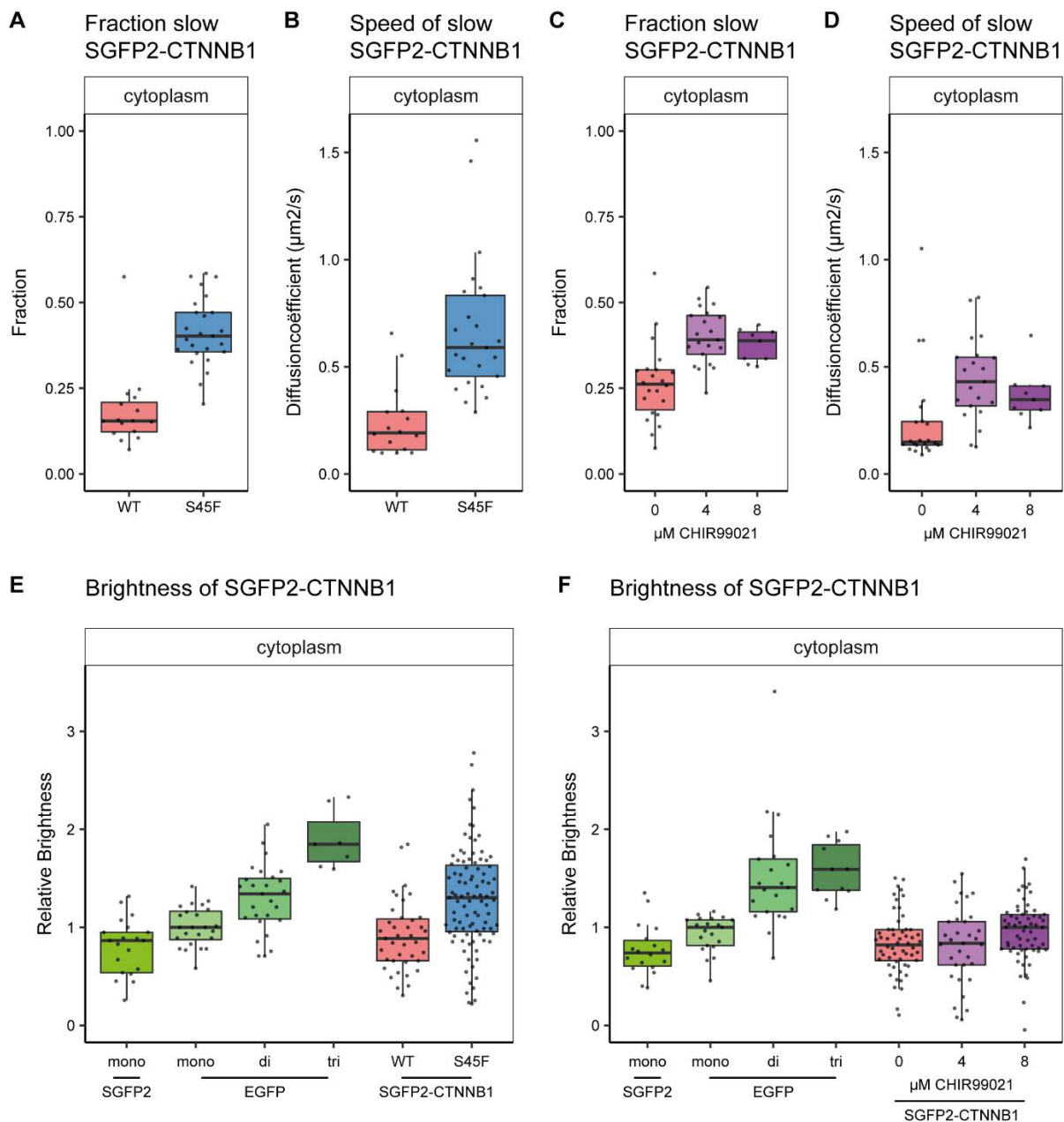
434 Perturbing the system to mimic oncogenic WNT signaling

435 WNT signaling is often disrupted in cancer (reviewed in Polakis, 2000; Zhan, Rindtorff, &  
436 Boutros, 2017), in many cases due to inactivating mutations of negative regulators or  
437 activating mutations in CTNNB1 itself (Bugter et al., 2020). One of the earliest identified  
438 mutations in CTNNB1 was a substitution of serine-45 for a phenylalanine (S45F) (Morin et al.,  
439 1997). This mutation removes the CSNK1 priming phosphorylation site on CTNNB1 that is  
440 needed for sequential phosphorylation by GSK3, and thus blocks its proteasomal degradation  
441 (Amit et al., 2002; Liu et al., 2002).

442 We generated the S45F mutation in one of our HAP1<sup>SGFP2-CTNNB1</sup> cell lines through a second  
443 step of CRISPR/Cas9 mediated genome editing (Fig 7 supplement 1A-D). As expected, the  
444 mutation resulted in higher CTNNB1 levels (Figure 7 supplement 1E-F) and constitutive  
445 downstream activation of the pathway (Figure 7 supplement 1G-H). Next, we used this cell  
446 line for two purposes. First, we used FCS and N&B to compare the complex-state of wild-type  
447 and mutant CTNNB1 in the cytoplasm (Figure 7). Second, we reproduced the same  
448 perturbation in silico to strengthen the link between our experimental data and the  
449 computational model (Figure 8).

450 Similar to the situation detected under physiological conditions (Figure 5D), we find a large  
451 fraction of SGFP2-CTNNB1<sup>S45F</sup> to reside in a cytoplasmic complex (Figure 7A). As observed for  
452 physiological stimulation with WNT3A (Figure 5E), the speed of this complex is increased in  
453 SGFP2-CTNNB1<sup>S45F</sup> compared to unstimulated HAP1<sup>SGFP2-CTNNB1</sup> cells (Figure 7B). We find  
454 similar behavior when we block the GSK3 mediated phosphorylation of wild-type CTNNB1  
455 using CHIR99021 (Figure 7C-D). The reduction in cytoplasmic complex size therefore must  
456 occur downstream of CTNNB1 phosphorylation. Intriguingly, our N&B analyses suggest that

457 these smaller S45F mutant cytoplasmic complexes have a higher occupancy of CTNNB1  
 458 (Figure 7E) than the those in WNT3A (Figure 5H) or CHIR99021 (Figure 7F) stimulated wildtype  
 459 cells. The S45F mutant (median 1.304, 95% CI 1.139-1.418,  $p=0.002$ ) was significantly brighter  
 460 than the SGFP2 monomer control (median 0.866, 95% CI 0.573 - 0.949), where the WT tagged  
 461 HAP1 cells again did not diverge from the monomer (0.886, 95% CI 0.722-1,  $p=0.845$ )  
 462 (Supplementary file 1).



463

464 **Figure7** Cytoplasmic complex characteristics in absence of SGFP2-CTNNB1 N-terminal phosphorylation. The S45F mutant was introduced  
465 using CRISPR (see Figure 7 supplement 1) and CHIR treated and control cells were measured after 24 hours. Details on sample size and  
466 statistics can be found in supplementary file 1. A) Graph depicting the fraction of particles with the second diffusion component (i.e. SGFP2-  
467 CTNNB1 containing complex) measured by FCS for S45F mutant B) Graph depicting the speed of the second diffusion component (i.e. SGFP2-  
468 CTNNB1 containing complex) measured by FCS for S45F mutant. C).Graph depicting the fraction of particles with the second diffusion  
469 component (i.e. SGFP2-CTNNB1 containing complex) measured by FCS after 24h treatment with CHIR99021 C) Graphs depicting the speed  
470 of the second diffusion component (i.e. SGFP2-CTNNB1 containing complex) measured by FCS after 24h treatment with CHIR99021 .E-F)  
471 Graphs depicting the molecular brightness of SGFP2-CTNNB1 in the cytoplasm and nucleus relative to controls as measured with N&B in the  
472 same subcellular compartments for S45F mutant CTNNB1 (E) or after 24 hours of CHIR99021 treatment (F). EGFP monomer was used for  
473 normalization and EGFP dimer and trimer as controls for N&B measurements.  
474 The following supplements are available for Figure 7:  
475 **Figure 7 supplement 1:** Generation and characterization of a S45F mutant cell line (HAP1<sup>SGFP2-CTNNB1(S45F)</sup>)

476 The S45F mutant shows a substantial increase in SGFP2-CTNNB1 levels in the cytoplasm and  
477 nucleus (Figure 8A). As this constitutive mutation does not provide any kinetic information,  
478 we also measured the dynamic response of SGFP2-CTNNB1 to CHIR99021-mediated GSK3  
479 inhibition (Figure 8 supplement 1, Supplementary movie 3). We see similar initial kinetics as  
480 for WNT3A stimulation. However, in contrast to what is observed for WNT3A treatment, no  
481 plateau was reached at the highest concentration of CHIR99021 (8  $\mu$ M). The fact that  
482 intracellular SGFP2-CTNNB1 levels in the 8  $\mu$ M CHIR99021 condition continued to accumulate,  
483 suggests that negative feedback, for example through AXIN2 (Lustig et al., 2002) or through  
484 internalization of receptor complexes (Agajanian et al., 2019), is overridden under these  
485 circumstances. Of note, the quantification also confirms that there is cell to cell heterogeneity  
486 in the response, regardless of whether WNT/CTNNB1 signaling is activated at the level of the  
487 receptor (WNT3A treatment) or at the level of the destruction complex (CHIR99021  
488 treatment), as can be seen from the spread of intensities measured from individual cells  
489 (Figure 3 Supplement 2A-B).

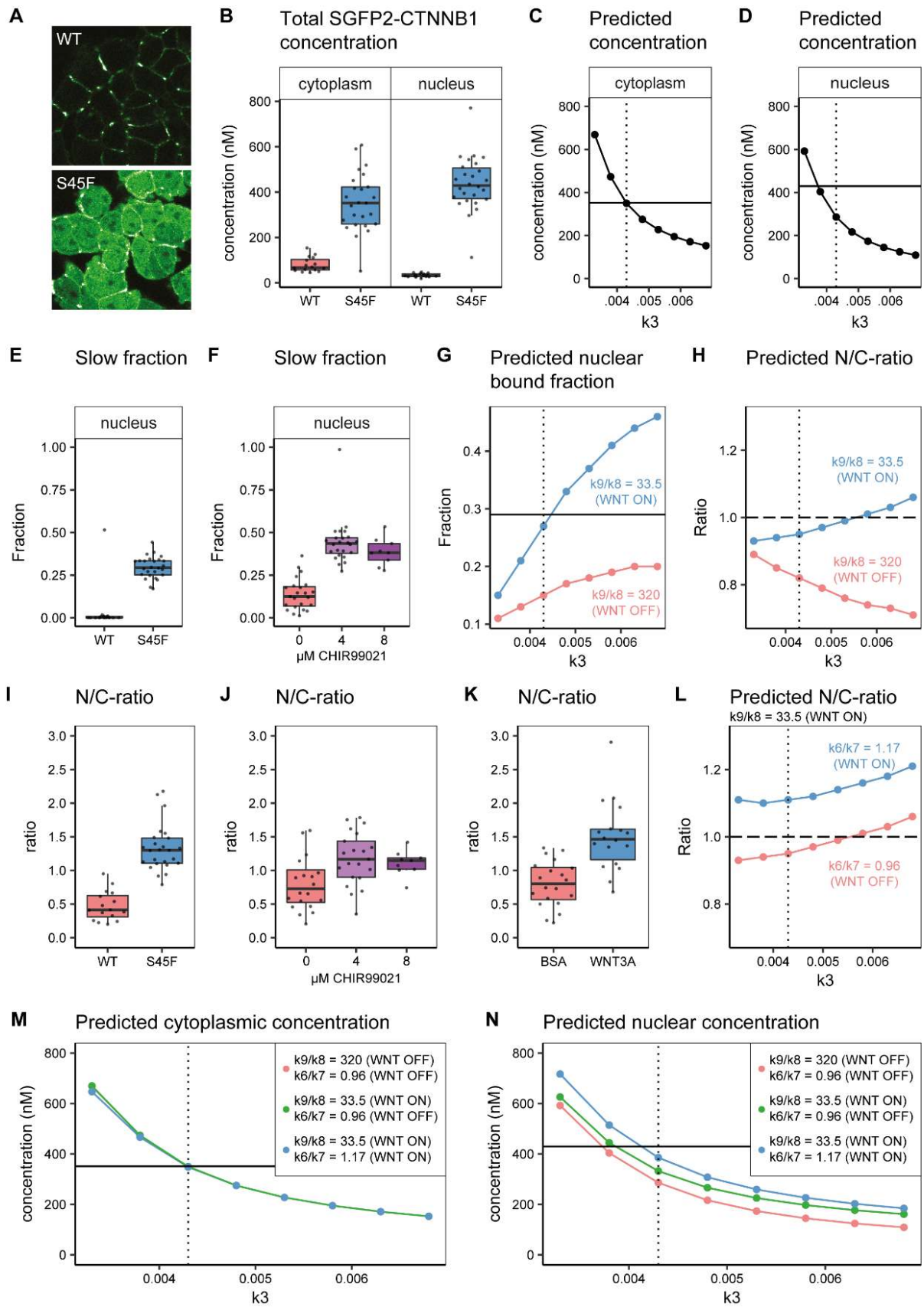
490 Finally, we compared our biological measurements from these perturbation experiments to  
491 our computational model predictions. Both the S45F mutation and CHIR99021 treatment  
492 disrupt one of the critical nodes in the model, namely the degradation of phosphorylated  
493 CTNNB1 (corresponding to k3, Figure 6A). With FCS and N&B we quantified the accumulation  
494 of CTNNB1 levels of mutant SGFP2-CTNNB1<sup>S45F</sup> (Figure 8B, Figure 8 supplement 2A) and wild-

495 type SGFP2-CTNNB1 upon CHIR99021 treatment (Figure 8 supplement 2B-C). Both exceeded  
496 the levels observed with physiological WNT3A stimulation (Figure 3A-C, Figure 5A). In our  
497 computational model we simulated reduced degradation by lowering the value of  $k_3$ . A  
498 reduction in  $k_3$  from its initial value ( $k_3=0.0068$ , Table 5) to  $k_3=0.0043$ , accurately predicted  
499 the higher cytoplasmic concentration measured for the S45F mutant (Figure 8C), but a further  
500 reduction of  $k_3=0.0038$  was needed to match the measured nuclear concentration (Figure  
501 8D). However, reducing  $k_3$  alone was not sufficient to reproduce either the fraction of  
502 CTNNB1 that is bound in the nucleus (Figure 8E-G) or the overall nuclear enrichment of  
503 CTNNB1 (Figure 8H). The latter requires a predicted nuclear/cytoplasmic (N/C) ratio greater  
504 than one, as observed in both physiological and constitutively active WNT/CTNNB1 signaling  
505 (Figure 8I-K).

506 The increase in the bound fraction of SGFP2-CTNNB1 in the nucleus was comparable between  
507 our mutant cell line (Figure 8E), CHIR99021 (Figure 8F) and WNT3A (Figure 5D) treatment. To  
508 match this experimental observation, we adjusted the  $k_9/k_8$  ratio as was also required for  
509 physiological WNT3A signaling (Table 5, Figure 8G). This shows the importance of this  
510 regulatory node not only in physiological, but also in oncogenic signaling. At the same time,  
511 the adjustment of  $k_9/k_8$  on top of  $k_3$  still does not predict the observed nuclear enrichment  
512 of CTNNB1 (Figure 8H). After changing the nuclear shuttling ratio  $k_6/k_7$  to the ratio we fitted  
513 for the WNT ON situation (Table 5), the model now also reproduces the nuclear enrichment  
514 of CTNNB1 (Figure 8L). In figures 8M-N, we show that the changes in parameters  $k_6/k_7$  and  
515  $k_9/k_8$  have little effect on the CTNNB1 concentration in the cytoplasm, but do substantially  
516 affect the nuclear concentrations of CTNNB1. This suggest that processes downstream of  
517 CTNNB1 degradation play a significant and active role in the CTNNB1 dynamics of the cell.

518 Taken together, our computational model can describe both physiological and oncogenic  
519 signaling. Moreover, it underlines the importance of CTNNB1 regulation downstream of  
520 destruction complex activity and confirms a critical role for nuclear import and nuclear  
521 retention.

522 **Figure 8:** In silico and experimental perturbation of WNT signaling. Details on experimental sample size and statistics can be found in  
523 supplementary file 1. A) Representative confocal images of HAP1-SGFP2WT (WT, top) and HAP1-SGFP2S45F (S45F, bottom) cells acquired  
524 with the same image settings. The S45F mutation leads to the accumulation and nuclear enrichment of CTNNB1 in the cell. B) Graph depicting  
525 the total concentration of SGFP2-CTNNB1 particles (monomeric plus complexed) as measured with FCS. C-D) Inhibition of CTNNB1  
526 degradation is modelled as a reduction in the value of  $k_3$ . C) Graph depicting the predicted total cytoplasmic CTNNB1 concentration as a  
527 function of  $k_3$ . A reduction in  $k_3$  from 0.0068 (Table 5, WNT ON and WNT OFF conditions) to  $\sim 0.0043$  (dotted line) corresponds to the  
528 cytoplasmic concentration observed (solid line). D) Graph depicting the predicted total nuclear CTNNB1 concentration as a function of  $k_3$ .  
529 The solid horizontal line indicates the concentration measured for the S45F mutant by FCS. Note that the value of  $k_3$  that matches the  
530 observed cytoplasm concentration (dotted line) does not match the experimentally determined concentration in the nucleus (solid line). E-  
531 F) Graphs depicting the fraction of particles with the second diffusion component (i.e. SGFP2-CTNNB1 containing complex) measured by FCS  
532 for wild-type and S45F mutant (E) and after 24-hour CHIR99021 treatment (F). The increase in the bound fraction in the oncogenic mutant  
533 or after GSK3 inhibition we find, is comparable to what we observed in WNT3A stimulated cells (Figure 5D). G) Graph showing the predicted  
534 nuclear bound fraction of CTNNB1 as a function of  $k_3$  with the TCF/CTNNB1 binding affinity of the model (Table 4) for WNT OFF ( $k_9/k_8=320$ ,  
535 pink line) and for WNT ON ( $k_9/k_8=33.5$ , blue line). Note that for WNT ON, the value for the nuclear bound fraction approximates the  
536 experimentally determined slow fraction for the S45F mutant (solid line, panel E) at the value for  $k_3$  that matches the cytoplasmic  
537 concentration of CTNNB1 (dotted line). H) Graph showing the predicted nuclear/cytoplasmic (N/C)-ratio as a function of  $k_3$  with TCF/CTNNB1  
538 binding affinity of the model (Table 4) for WNT OFF ( $k_9/k_8=320$ , pink line) and WNT ON ( $k_9/k_8=33.5$ , blue line). Note that, although for WNT  
539 ON the value of the N/C-ratio increases with  $k_3$ , there is still nuclear exclusion (N/C-ratio lower than 1, dashed line) at the value of  $k_3$  that  
540 matches the cytoplasmic CTNNB1-concentration (dotted line). I-K) The N/C-ratio as measured by FCS for wild-type and S45F mutant (I), after  
541 24-hour CHIR99021 treatment (J) and after 4 hour WNT3A treatment (K). Note that all perturbations lead to nuclear accumulation (N/C-  
542 value exceeding 1). L) Graph showing the predicted N/C-ratio as a function of  $k_3$  with the WNT ON value for  $k_9/k_8$  with the nuclear shuttling  
543 ratio of the model ( $k_6/k_7$  Table 4), corresponding to WNT OFF ( $k_6/k_7=0.96$ , pink line) and WNT ON ( $k_6/k_7=1.17$ , blue line), respectively. Note  
544 that the WNT ON value of  $k_6/k_7$  increases the N/C-ratio to nuclear accumulation at the value for  $k_3$  that matches the cytoplasmic  
545 concentration (dotted line). M) Graph depicting the predicted total cytoplasmic CTNNB1 concentration as a function of  $k_3$  with WNT ON and  
546 WNT OFF values for  $k_9/k_8$  and  $k_6/k_7$ . Note that modulation of  $k_9/k_8$  and  $k_6/k_7$  has no effect on the predicted cytoplasmic concentration of  
547 CTNNB1. The horizontal solid line is the experimentally determined cytoplasmic CTNNB1 concentration (cf. panel B); the vertical dotted line  
548 is at the value of  $k_3$  that best reproduces this experimental finding in the model. N) Graph depicting the predicted total nuclear CTNNB1  
549 concentration as a function of  $k_3$  for WNT ON and WNT OFF values for  $k_9/k_8$  and  $k_6/k_7$ . Note that if both  $k_9/k_8$  and  $k_6/k_7$  are changed from  
550 their WNT OFF values the predicted nuclear concentration of CTNNB1 better matches the experimentally determined concentration  
551 (horizontal solid line) at the value for  $k_3$  that matches the cytoplasm concentration (vertical dotted line).



## 553 Discussion

554 WNT signaling is critical for tissue development and homeostasis. Although most core players  
555 and many of their molecular interactions have been uncovered, dynamic spatiotemporal  
556 information with sufficient subcellular resolution remains limited. As both genome editing  
557 approaches and quantitative live-cell microscopy have advanced further, the goal of studying  
558 WNT/CTNNB1 signaling at endogenous expression levels in living cells now is within reach.  
559 Maintaining endogenous expression levels is important, as overexpression may lead to  
560 altered stoichiometry of signaling components, as well as changes in subcellular localization  
561 (Gibson et al., 2013; Mahen et al., 2014). Indeed, it has been shown that exogenously  
562 expressed CTNNB1 is less signaling competent, probably due to its post-translational  
563 modification status (Hendriksen et al., 2008). Here we generated functional HAP1<sup>SGFP2-CTNNB1</sup>  
564 knock-in cell lines to study the dynamic behavior and subcellular complex state of  
565 endogenous CTNNB1 in individual living human cells in both a physiological and oncogenic  
566 context.

567 Using live-cell microscopy and automated cell segmentation, we observe that endogenous  
568 CTNNB1 only increases 1.7-fold in the cytoplasm and 3.0-fold in the nucleus after WNT3A  
569 treatment, which is consistent with the literature (Jacobsen et al., 2016; Kafri et al., 2016;  
570 Massey et al., 2019).

571 Next, we used state-of-the-art, quantitative microscopy to measure the absolute  
572 concentration of CTNNB1 within different subcellular compartments and in different  
573 complex-states in living cells. The findings from these experiments challenge the dogma that  
574 mainly monomeric CTNNB1 accumulates upon WNT pathway stimulation. Moreover, our  
575 integrative approach of quantitative imaging and computational modelling revealed three

576 critical nodes of CTNNB1 regulation, namely CTNNB1 degradation, nuclear shuttling and  
577 nuclear retention, which together describe the CTNNB1 turnover, subcellular localization and  
578 complex status under both physiological and oncogenic conditions.

#### 579 Cytoplasmic regulation of CTNNB1

580 Using FCS, we determined that in unstimulated HAP1 cells a substantial fraction of SGFP2-  
581 CTNNB1 is associated with a very large, slow-diffusing cytoplasmic complex (Figure 4-5). The  
582 main known cytoplasmic complex containing CTNNB1 is the destruction complex. The  
583 combined weight of the individual destruction complex components (AXIN, APC, CSNK1 and  
584 GSK3) would be expected to result in a much higher mobility than that displayed by the  
585 cytoplasmic CTNNB1-containing complex we observed. However, evidence is growing that  
586 the destruction complex forms large phase separated aggregates (also termed biomolecular  
587 condensates) (reviewed in Schaefer and Peifer, 2019). Oligomerization of AXIN and APC  
588 underlies the formation of these aggregates, and this in turn appears to be required for  
589 efficient degradation of CTNNB1 (Fiedler et al., 2011; Kunttas-Tatli et al., 2014; Pronobis et  
590 al., 2017; Spink et al., 2000). There is some evidence that these aggregates form at (near)  
591 endogenous levels (Fagotto et al., 1999; Faux et al., 2008; Mendoza-Topaz et al., 2011;  
592 Pronobis et al., 2015; Schaefer et al., 2018; Thorvaldsen et al., 2015), but it is still an open  
593 question what the exact composition and size of the destruction complex is in a physiological  
594 context. It should be noted that our imaging does not visualize such aggregates (Figure 3A).  
595 In addition, our N&B data indicate that few, if any, complexes exist that contain multiple  
596 SGFP2-CTNNB1 molecules in the absence or presence of WNT3A stimulation – something that  
597 would be expected in biomolecular condensates formed by the oligomerization of CTNNB1  
598 binding partners. Only following the introduction of an S45F mutation, which results in



599 constitutive inhibition of CTNNB1 phosphorylation and degradation, we observe a brightness  
600 increase that would be compatible with the accumulation of multiple SGFP2-CTNNB1<sup>S45F</sup>  
601 molecules in a single cytoplasmic complex. This indicates that while the destruction complex  
602 might be multivalent in both a physiological and an oncogenic context, CTNNB1 occupancy of  
603 the complex is low under physiological conditions, but increased in oncogenic signaling. This  
604 has major impacts on how we conceptualize the workings of the CTNNB1 destruction  
605 machinery – especially in the context of cancer, since mutations in CTNNB1 (affecting  
606 occupancy) may have very different biochemical consequences than mutations in APC  
607 (affecting multimerization and valency of the destruction complex itself).

608 The mechanism on destruction complex deactivation remains controversial (Tortelote et al.,  
609 2017; Verkaar et al., 2012). The current literature suggests that the destruction complex is  
610 sequestered to the FZD-LRP receptor complex upon WNT pathway stimulation. Several  
611 models exist for how the membrane sequestration inhibits CTNNB1 degradation, including  
612 LRP mediated GSK3 inhibition (Stamos et al., 2014), sequestration of GSK3 in multi vesicular  
613 bodies (Taelman et al., 2010), (partial) dissociation of the destruction complex (Liu et al.,  
614 2005; Tran and Polakis, 2012), and saturation of CTNNB1 within an intact destruction complex  
615 (Li et al., 2012). Our data clearly show that a substantial fraction of CTNNB1 in the cytoplasm  
616 remains bound upon pathway stimulation (Figure 5D). This is not predicted by any of the  
617 above mentioned models and challenges the long-held view that mainly monomeric CTNNB1  
618 accumulates.

619 Additionally, we show that the cytoplasmic CTNNB1 complex in WNT3A or CHIR99021 treated  
620 cells as well as in S45F mutant cells has an increased mobility compared to control cells (Figure  
621 5E). Therefore, while the diffusion coefficient is still very low (indicating a very large complex),

622 this implies it is a vastly different complex than that observed in the absence of WNT  
623 stimulation. The fact that cells in which GSK3 phosphorylation is inhibited through S45F  
624 mutation or CHIR99021 treatment show similar behavior, suggests that the size of the  
625 cytoplasmic complex is directly linked to the phosphorylation status of CTNNB1. The  
626 destruction complex has been shown to associate with (parts of) the ubiquitin and  
627 proteasome machinery (Li et al., 2012; Lui et al., 2011; Schaefer et al., 2020). One interesting  
628 possibility, therefore, is that phosphorylated CTNNB1 is required for coupling the destruction  
629 complex to the ubiquitination and proteasome machinery. In fact, although not explicitly  
630 mentioned in the main text, supplementary table 1 of Li et al., 2012 shows that in HEK293  
631 cells, which harbor no mutation in the core components of the WNT pathway, CTNNB1 was  
632 found to interact with subunits of the proteasome, whereas in the S45F-CTNNB1 mutant cell  
633 line Ls174T these interactions were not detected. In conclusion, although we do not directly  
634 determine its identity, our measured biophysical parameters of the cytoplasmic CTNNB1  
635 complex are consistent with it representing a large, multivalent destruction complex that is  
636 coupled to the proteasome as long as CTNNB1 is being phosphorylated.

### 637 Nuclear regulation of CTNNB1

638 The key function of CTNNB1 downstream of WNT is to regulate transcription of TCF/LEF target  
639 genes (Doumpas et al., 2019; Schuijers et al., 2014). Proteomic analyses have shown that the  
640 WNT enhanceosome consists of CTNNB1, TCF/LEF, PYGO and BCL9 and several other large  
641 proteins (Fiedler et al., 2015; van Tienen et al., 2017). Using FCS, we showed that CTNNB1  
642 resides in a nuclear complex with a diffusion coefficient that is compatible with such a DNA-  
643 bound transcriptional complex (Figure 5E) (Lam et al., 2012).

644 Although CTNNB1 is known to associate with TCF/LEF factors in response to WNT/CTNNB1  
645 signaling to drive transcription (Franz et al., 2017; Schuijers et al., 2014), we also detect low  
646 levels of nuclear CTNNB1 complex in the absence of a WNT stimulus (Figure 5C). The diffusion  
647 coefficient of the nuclear CTNNB1 complex does not change upon the addition of WNT3A  
648 (Figure 5E), suggesting that some CTNNB1 is already associated with the DNA even in the  
649 absence of a WNT stimulus. At this point, we cannot exclude the contribution of TCF/LEF  
650 independent DNA binding (Armstrong et al., 2012; Essers et al., 2005; Kormish et al., 2010),  
651 or anomalous subdiffusion in the nucleus, either due to physical obstruction, transient DNA-  
652 binding events protein or protein complex formation (Dross et al., 2009; Kaur et al., 2013;  
653 Wachsmuth et al., 2000), as FCS only allows us to probe the speed of this complex.

654 However, upon pathway activation through WNT3A, CHIR99021 or S45F mutation we see a  
655 consistent increase in the fraction and absolute levels of this slow-diffusing nuclear CTNNB1  
656 complex (Figure 5E, Figure 8E-F), compatible with increased CTNNB1 binding to its target  
657 sites. For WNT stimulation, we measured that the concentration of bound SGFP2-CTNNB1 in  
658 the nucleus increased to a 89 nM, which corresponds to something in the order of 20,000  
659 bound CTNNB1 molecules in one nucleus, assuming a small nuclear volume of 0.36 pL (Tan et  
660 al., 2012). Published CHIPseq studies report many CTNNB1 DNA binding sites, ranging from  
661 several hundred to several thousand sites in mammalian cells (Cantù et al., 2018; Doumpas  
662 et al., 2019; Schuijers et al., 2014). It is therefore highly likely that at least part of the slow-  
663 diffusing CTNNB1 particles we measure indeed represents CTNNB1 that is associated with the  
664 WNT enhanceosome.

665 Regulation of CTNNB1 nuclear accumulation

666 In HAP1 cells, endogenous CTNNB1 is excluded from the nucleus in the absence of WNT. Our  
667 live imaging data reveal an immediate and preferential increase in nuclear CTNNB1 upon  
668 WNT3A stimulation, until an equilibrium is reached between the cytoplasmic and nuclear  
669 levels (Figure 3D). This is consistent with previous observations in HEK293 cells stably  
670 overexpressing low levels of YFP-CTNNB1 (Kafri et al., 2016).

671 Intriguingly, CTNNB1 does not contain nuclear import or export signals and can translocate  
672 independently of classical importin and exporter pathways (Fagotto et al., 1998; Wiechens  
673 and Fagotto, 2001; Yokoya et al., 1999). Hence, the molecular mechanism of CTNNB1  
674 subcellular distribution remains incompletely understood. Evidence from Fluorescence  
675 Recovery After Photobleaching (FRAP) studies suggest that the increase in nuclear CTNNB1 is  
676 due to changes in binding to its interaction partners in the cytoplasm and nucleus (retention)  
677 rather than active changes in nuclear import and export rates (shuttling) (Jamieson et al.,  
678 2011; Krieghoff et al., 2006). We argue that the two are not mutually exclusive, as our  
679 experimental data and computational model show that WNT regulates both  
680 nucleocytoplasmic shuttling and nuclear retention of CTNNB1. Indeed, we see an increase of  
681 nuclear CTNNB1 complexes in the nucleus (Figure 5C-D) and the dissociation of CTNNB1 from  
682 TCF is reduced almost 10-fold in WNT signaling conditions in our computational model (Table  
683 5). Our model predicts that this increased nuclear retention indeed also increases the  
684 nuclear/cytoplasmic ratio (Figure 8H). However, to reconcile our computational prediction  
685 with our experimental observations we additionally need to include a shift from nuclear  
686 export to nuclear import upon pathway activation (Figure 6, Figure 8). Our integrated  
687 experimental biology and computational modelling approach thus reveals that WNT signaling

688 not only regulates the absolute levels of CTNNB1 through destruction complex inactivation,  
689 but also actively changes its subcellular distribution through nuclear retention and shuttling.  
690 The fact that direct inhibition of GSK3 mediated phosphorylation of CTNNB1 results in the  
691 same behavior, indicates that the phosphorylation status of CTNNB1 plays a critical role. This  
692 further emphasizes the importance of posttranslational modifications and conformational  
693 changes in CTNNB1 for its subcellular localization and function (Gottardi and Gumbiner, 2004;  
694 Sayat et al., 2008; Valenta et al., 2012; van der Wal and van Amerongen, 2020; Wu et al.,  
695 2008) .

#### 696 Challenges and opportunities for fluorescence fluctuation spectroscopy techniques

697 Using fluorescence fluctuation spectroscopy techniques (FCS and N&B) we have quantified  
698 endogenous CTNNB1 concentrations and complexes in living cells for the first time, which  
699 provided novel and long-awaited biophysical parameters for computational modelling.  
700 Moreover, our approach has also yielded novel insights into CTNNB1 regulation that challenge  
701 current dogmas in the field. If we are correct, this has important consequences. First, if only  
702 part of the cytoplasmic CTNNB1 pool is uncomplexed (i.e. free or monomeric), regardless of  
703 whether the WNT/CTNNB1 pathway is off or on (either via physiological WNT3A stimulation  
704 or via oncogenic activation), this is a rewrite of the textbook model. Second, if the slow-  
705 diffusion cytoplasmic CTNNB1 complex indeed represents a proteasome-associated  
706 destruction complex, this would fuel a debate that has remained unresolved for many years  
707 (Li et al., 2012; Verkaar et al., 2012). As more studies will use these image-based techniques  
708 to determine biophysical properties of WNT/CTNNB1 signaling components (Ambrosi et al.,  
709 2020; Eckert et al., 2020), the field will undoubtedly learn how to interpret these findings.

710 As with any technique, there are several limitations to consider. First of all, for the  
711 determination of the absolute concentration by FCS it should be noted that a small portion of  
712 SGFP2-CTNNB1 could be in a non-fluorescent state. Although our FCS analysis model already  
713 accounts for dynamic dark states such as the triplet state, non-matured fluorophores could  
714 lead to a slight underestimation of our concentrations. However, this is expected to be a very  
715 small fraction as SGFP2 has very good maturation kinetics (Kremers et al., 2007). Secondly,  
716 our findings concerning the diffusion kinetics are limited by the assumptions we make in the  
717 FCS fitting model. Although obvious mistakes in underlying assumptions immediately become  
718 clear due to bad fitting results and can therefore be excluded, not every wrong assumption  
719 will stand out accordingly. Our data clearly shows that assuming only one diffusion speed for  
720 CTNNB1 in HAP1 cells would be incorrect (Figure 4). However, whether with the second  
721 diffusion speed we measure a single distinct, large complex, or rather an average of multiple  
722 different CTNNB1 containing complexes cannot be determined in our current set-up. In  
723 addition, we assume that CTNNB1 is present as a free-floating monomer (as fixed for our first  
724 component), based on previous observations (Gottardi and Gumbiner, 2004; Maher et al.,  
725 2010). However, at least one report suggests that CTNNB1 is not present as a monomer but  
726 rather in small cytoplasmic complexes of ~200 kDa (Gerlach et al., 2014). As diffusion speed  
727 is relatively insensitive to differences in size (e.g. an 8-fold increase in protein mass is  
728 expected to result in only a 2-fold reduction of the diffusion coefficient for a spherical  
729 particle), it is possible that we do not measure truly free-floating CTNNB1, but rather smaller  
730 complexes. In addition, point FCS is limited to a single position in the cell. Therefore, in  
731 addition to the intercellular differences in the WNT signaling response of individual cells, our  
732 measurements also sample intracellular heterogeneity caused by the presence of organelles  
733 and molecular crowding. Notwithstanding these limitations, we have been able to show that

734 a large portion of CTNNB1 is present in a very large complex in both stimulated and  
735 unstimulated conditions and that this complex has a statistically and biologically significant  
736 different speed after WNT3A treatment.

737 The biophysical parameters we obtained from point FCS and N&B have allowed us to learn  
738 more about the speed and occupancy of the SGFP2-CTNNB1 complexes in living cells.  
739 Moreover, using different stimuli and perturbations of the pathway we have been able to link  
740 this to the phosphorylation status of CTNNB1. However, FCS and N&B do not provide  
741 conclusive evidence on the identity and composition of these complexes. An exciting  
742 possibility would be to label additional components presumed to be present in the CTNNB1-  
743 containing complexes at the endogenous level to uncover the precise composition and  
744 stoichiometry of protein complexes involved in WNT signaling. For instance, Fluorescence  
745 Cross Correlation Spectroscopy (FCCS) could be employed to test if two proteins reside within  
746 the same complex (Elson, 2011; Hink, 2014; Macháň and Wohland, 2014). Ultimately, a  
747 combination of such quantitative functional imaging techniques, biochemical and proteomic  
748 approaches, together with additional perturbations will need to be employed to further our  
749 understanding of the dynamic composition of endogenous CTNNB1 complexes, as well as to  
750 help us resolve the molecular mechanism underlying nucleocytoplasmic shuttling and nuclear  
751 retention. As both genome editing and live cell imaging techniques continue to improve,  
752 additional possibilities will open up to address longstanding questions in cellular signaling in  
753 a physiological context with high spatial and temporal resolution. New opportunities and  
754 challenges await as these investigations extend to 3D organoid cultures, developing embryos  
755 and living organisms.

## 756 Material and Methods

### 757 DNA Constructs

758 The following constructs were used: pSpCas9(BB)-2A-Puro (PX459) V2.0 ((Ran et al., 2013), a  
759 kind gift from Feng Zhang, available from Addgene, plasmid #62988), MegaTopflash ((Hu et  
760 al., 2007), a kind gift from Dr. Christophe Fuerer and Dr. Roel Nusse, Stanford University),  
761 CMV Renilla (E2261, Promega, Madison, WI), pSGFP2-C1 ((Kremers et al., 2007), a kind gift  
762 from Dorus Gadella, available from Addgene, plasmid #22881), pmScarlet-i\_C1 (Bindels et al.,  
763 2017), a kind gift from Dorus Gadella, available from Addgene, plasmid # 85044), pSYFP2-C1  
764 ((Kremers et al., 2006), a kind gift from Dorus Gadella, available from Addgene, plasmid  
765 #22878), mTurquoise2-C1 ((Goedhart et al., 2012), a kind gift from Dorus Gadella, available  
766 from Addgene, plasmid # 54842), pEGFP (Clontech, Mountain View, CA), pEGFP<sub>2</sub> and pEGFP<sub>3</sub>  
767 ((Pack et al., 2006), a kind gift from Masataka Kinjo) and pBluescript II KS(+) (Stratagene, La  
768 Jolla, CA).

769 The gRNA targeting the start codon in exon2 of human *CTNNB1* was designed using the MIT  
770 webtool ([crispr.mit.edu](http://crispr.mit.edu)) and cloned into pX459. Oligos RVA567 and RVA568 (Table 6)  
771 encoding the gRNA were annealed, and ligated into BbsI-digested pX459 plasmid as  
772 previously described (Ran et al., 2013) to obtain pX459-CTNNB1-ATG. The gRNA targeting  
773 codon 3 of *CTNNB1* for mutagenesis of Serine 45 to Phenylalanine (S45F) was similarly  
774 designed and cloned by introducing RVA561 and RVA562 (Table 6) into pX459, yielding pX459-  
775 CTNNB1-S45.

776 The repair plasmid for SGFP2-CTNNB1 (pRepair-SGFP2-CTNNB1) was cloned using Gibson  
777 cloning (Gibson et al., 2009). First, a repair plasmid including the Kozak sequence from the  
778 pSGFP2-C1 plasmid was generated (pRepair-Kozak-SGFP2 -CTNNB1). For this, 5' and 3'



779 homology arms were PCR amplified from genomic HEK293A DNA with primers RVA618 and  
780 RVA581 (5' arm) or RVA619 and RVA584 (3' arm). SGFP2 was amplified with Gibson cloning  
781 from pSGFP2-C1 with primers RVA582 and RVA583 and the backbone was amplified from SacI  
782 digested pBlueScript KS(+) with primers RVA622 and RVA623. The final repair construct  
783 (pRepair-SGFP2-CTNNB1) contains the endogenous *CTNNB1* Kozak sequence before the  
784 SGFP2 ATG. To obtain (pRepair-SGFP2-CTNNB1), the backbone and homology regions were  
785 amplified from pRepair-SGFP2-Kozak-CTNNB1 with primers RVA1616 and RVA1619 and an  
786 SGFP2 without the Kozak sequence was amplified from pSGFP2-C1 with primers RVA1617 and  
787 RVA1618. To generate color variants of the repair plasmid SYFP2, mScarlet-i and mTurquoise2  
788 were also amplified from their respective C1 vectors with primers RVA 1617 and RVA 1618.  
789 PCR products were purified and assembled with a Gibson assembly master mix with a 1:3  
790 (vector:insert) molar ratio. Gibson assembly master mix was either purchased (E2611S, NEB)  
791 or homemade (final concentrations: 1x ISO buffer (100mM Tris-HCL pH 7.5, 10mM MgCl<sub>2</sub>,  
792 0.2M dNTPs (R0181, Thermo Scientific), 10mM DTT (10792782, Fisher), 5% PEG-8000  
793 (1546605, Sigma-Aldrich, St Louis, MO), 1mM NAD<sup>+</sup> (B9007S, NEB)), 0.004 U/μl T5  
794 exonuclease (M0363S, NEB), 0.5 U/μl Phusion DNA Polymerase (F-530L, Thermo Scientific)  
795 and 4 U/μl Taq DNA ligase (M0208S, NEB)).

796 The following plasmids are available from Addgene: pX459-CTNNB1-ATG (#153429), pX459-  
797 CTNNB1-S45 (#164587), pRepair-SGFP2-CTNNB1 (#153430), pRepair-mSci-CTNNB1  
798 (#153431), pRepair-SYFP2-CTNNB1 (#153432), pRepair-mTq2-CTNNB1 (#153433)).

799

800 Primers used

801 **Table 6:** primers/oligonucleotides used in this study

|         |   |
|---------|---|
| RVA24   | CAAGTTTGTGTAGGATATGCC   |
| RVA25   | CGATGTCAATAGGACTCCAGA   |
| RVA124  | AGTGTGAGGTCCACGGAAA   |
| RVA125  | CCGTCATGGACATGGAAT  |
| RVA555  | GCCAAACGCTGGACATTAGT  |
| RVA558  | AGACCATGAGGTCTGCGTTT  |
| RVA561  | CACCGTTGCCTTTACCACTCAGAGA   |
| RVA 562 | AAACTCTCTGAGTGGTAAAGGCAAC   |
| RVA567  | CACCGTGAGTAGCCATTGTCCACGC   |
| RVA568  | AAACGCGTGGACAATGGCTACTCAC   |
| RVA581  | tgctcaccatggtggGATTTTCAAACAGTTGTATGGTATACTTC  |
| RVA582  | actgttttgaaaatcCCACCATGGTGAGCAAGGGC   |
| RVA583  | agtagccattgtccaCTTGACAGCTCGTCCATGCCG  |
| RVA584  | gacgagctgtacaagTGGACAATGGCTACTCAAGGTTTG   |
| RVA618  | atacgactcactatagggcggaattggagctGATGCAGTTTTTTTCAATATGTC  |
| RVA619  | ttctagagcggccgccaccgcggtggagctCTCTCTTTTCTTACCACAACATTTTATTTAAAC   |
| RVA622  | AAGAGAGAGCTCCACCGCGGTGGCGGCCG   |
| RVA623  | TGCATCAGCTCCAATTCGCCCTATAGTGAGTCG   |
| RVA1616 | tgtccacgctgGATTTTCAAACAGTTGTATGG  |
| RVA1617 | atacaactgttttgaaaatccagcgtggacaATGGTGAGCAAGGCGAG  |
| RVA1618 | cacaaaccttgagtagccatCTTGACAGCTCGTCCATGC   |
| RVA1619 | ATGGCTACTCAAGGTTTGTGTCATTAAATC  |
| RVA2540 | CTTACCTGGACTCTGGAATCCATTCTGGTGCCACTACCACAGCTCCTTTCTGTCCGGTAAAGGCAATCCTGAGGAAGA<br>GGATGTGGATACCTCCCAAGT |

802

803 Cell Culture, Treatment and Transfection

804 HAP1 cells (a kind gift from Thijn Brummelkamp, NKI) were maintained in full medium  
805 (colorless IMDM (21056023, Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented  
806 with 10% FBS (10270106, Gibco) and 1X Glutamax (35050061, Gibco)) under 5% CO<sub>2</sub> at 37°C  
807 in humidifying conditions and passaged every 2-3 days using 0.25% Trypsin-EDTA (25200056,  
808 Gibco). Cells were routinely tested for mycoplasma. We verified the haploid identity of the  
809 parental HAP1<sup>WT</sup> by karyotyping of metaphase spreads. To maintain a haploid population,  
810 cells were resorted frequently (see below) and experiments were performed with low  
811 passage number cells.

812 Where indicated, cells were treated with CHIR99021 (6mM stock solution in DMSO) (1677-5,  
813 Biovision, Milpitas, CA) or Recombinant Mouse Wnt-3a (10µg/ml stock solution in 0.1% BSA  
814 in PBS) (1324-WN-002, R&D systems, Bio-Techne, Minneapolis, MN) with DMSO and 0.1% BSA  
815 in PBS as vehicle controls, respectively.

816 Cells were transfected using Turbofect (R0531, ThermoFisher, Thermo Fisher Scientific,  
817 Waltham, MA), X-tremeGene HP (6366546001, Roche, Basel, Switzerland) or Lipofectamine  
818 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA) in Opti-MEM (Gibco) according to  
819 the manufacturer's instructions.

#### 820 HAP1<sup>SGFP2-CTNNB1</sup> and HAP1<sup>SGFP2-CTNNB1(S45F)</sup> generation

821 800.000 HAP1 cells/well were plated on 6-well plates. The following day, cells were  
822 transfected with Turbofect and 2000 ng DNA. pX459-CTNNB1-ATG and pRepair-SGFP2-  
823 CTNNB1 were transfected in a 2:1, 1:1 or 1:2 ratio. pSGFP2-C1, pX459 or pX459-CTNNB1-ATG  
824 were used as controls. From 24 to 48 hours after transfection cells were selected with 0.75  
825 µg/ml puromycin (A1113803, Gibco). Next, cells were expanded and passaged as needed until  
826 FACS sorting at day 9. For FACS analysis and sorting cells were washed, trypsinized,  
827 resuspended with full medium and spun down at 1000 rpm for 4 minutes. For sorting, cells  
828 were stained with 1 µg/ml Dapi (D1306, Invitrogen) in HF (2 % FBS in HBSS (14175053, Gibco)),  
829 washed with HF and resuspended in HF. To determine the haploid population, a separate  
830 sample of cells was stained with 5 µM Vybrant® DyeCycle™ Violet Stain (V35003, Invitrogen)  
831 in full medium for 30 minutes and kept in vibrant containing medium. Cells were filtered with  
832 a 70 µm filter and then used for FACS sorting and analysis on a FACSARIA3 (BD, Franklin Lanes,  
833 NJ). Vybrant-stained cells were analyzed at 37° and used to set a size gate only containing  
834 haploid cells. Dapi-stained cells were single cell sorted at 4°C into 96-well plates, that were

835 previously coated overnight with 0.1 % gelatin (G9391, Sigma-Aldrich) in MQ and contained  
836 full medium supplemented with 1 % penicillin/streptomycin (15140122, Gibco) and 0.025 M  
837 HEPES (H3375 Sigma-Aldrich, 1 M stock solution, pH 7.4, filter sterilized). The 3 independent  
838 clones used in this study were obtained from separate transfections of the same parental cell  
839 line. Clones were genotyped and sanger sequenced using primers RVA555 and RVA558 (Table  
840 6).

841 HAP1<sup>SGFP2-CTNNB1(S45F)</sup> were generated from HAP1<sup>SGFP2-CTNNB1</sup> clone 1. The same procedure as  
842 above was followed with slight adaptations; Cells were transfected 1000 ng  
843 pX459-CTNNB1-S45 or pX459 with 2 or 4 µl 10mM repair oligo (RVA 2540) with Turbofect,  
844 selected with puromycin and expanded as described above. Haploid single cells were sorted  
845 after 11 days as described above. For haploid size discrimination Vybrant™ DyeCycle™ Ruby  
846 Stain (V10273) was used. The 5 clones used in this study were obtained from 2 separate  
847 transfection (clone 2,3,16,24 from the same transfection, clone 27 from a second  
848 transfection). Clones were genotyped using primers RVA555 and RVA558 (Table 6), followed  
849 by HpaII (ER0511, ThermoFisher) restriction as per the manufacturer's instruction. RVA555  
850 was used for sanger sequencing.

851 Resorting of the cell lines was also performed with the same FACS procedure, with collection  
852 of cells in 15 mL tubes containing full medium with 1 % penicillin and 0.025 M HEPES.

853 FACS data were analyzed and visualized with FlowJo™.

#### 854 Luciferase Assay

855 For luciferase assays, 100.000 cells per well were seeded on a 24-well plate. Cells were  
856 transfected with 1µl X-tremeGene HP and 400 ng MegaTopflash reporter and 100 ng CMV-  
857 Renilla or 500 ng SGFP2-C1 as a negative control 24 hours later. Cells were treated with the

858 indicated concentration of CHIR99021 24 hours after transfection and after another 24 hours  
859 medium was removed and the cells were harvested with 50  $\mu$ l Passive Lysis Buffer (E1941,  
860 Promega). Luciferase activity was measured on a GloMax Navigator (Promega) using 10 $\mu$ l  
861 lysate in a black OptiPlate 96-well plate (6005279, Perkin Elmer, Waltham, MA) and 50  $\mu$ L  
862 homemade firefly and luciferase reagents (according to (Fuerer et al., 2014; Hampf and  
863 Gossen, 2006)).

864 For luciferase assays, three technical replicates (i.e. three wells transfected with the same  
865 transfection master mix) were pipetted and measured for each sample in each experiment.  
866 For each technical triplicate, the average MegaTopflash activity was calculated and depicted  
867 as a single dot in Figure 2C and Figure 7 supplement 1G. Three independent biological  
868 experiments, each thus depicted as an individual dot, were performed. To calculate  
869 MegaTopflash activity, Renilla and Luciferase luminescence values were corrected by  
870 subtracting the average background measured in the SGFP2-transfected control.  
871 MegaTopflash activity was calculated as the ratio of corrected Firefly and Renilla  
872 luminescence and normalized to the average reporter activity of the relative DMSO control.

### 873 Western Blot

874 The remaining lysates from the technical triplicates of the luciferase assay were combined  
875 and they were cleared by centrifugation for 10 minutes at 12.000 g at 4°C. Western blot  
876 analysis was performed and quantified as previously described (Jacobsen et al., 2016).  
877 Antibodies were used with the following dilutions, 1:1000 Non-phosphorylated (Active)  $\beta$ -  
878 catenin clone D13A1 (8814S, Cell Signaling, Danvers, MA), 1:2000 total  $\beta$ -catenin clone 14  
879 (610153, BD), 1:1000  $\alpha$ -Tubulin clone DM1A (T9026, Sigma-Aldrich), 1:1000 GFP polyclonal  
880 (A-6455, Invitrogen), 1:20.000 IRDye 680LT Goat anti-Rabbit IgG (926-68021, LI-COR, Lincoln,

881 NE), 1:20.000 IRDye 800CW Donkey anti-Mouse IgG (926–32212, LI-COR). Raw data for all  
882 blots have been made available at <https://osf.io/vkexg/>.

### 883 qRT-PCR

884 For qRT-PCR analysis, 100.000 HAP1 cells per well were seeded on a 24-well plate. After 48  
885 hours, cells were treated with indicated concentrations of CHIR99021. Cells were harvested  
886 24 hours after treatment. RNA was isolated with Trizol (15596018, Invitrogen) according to  
887 the manufacturer's instructions. cDNA was synthesized using SuperScriptIV (18090010,  
888 Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed with  
889 SyberGreen (10710004, Invitrogen). The endogenous WNT target gene *AXIN2* was amplified  
890 using primers RVA124 and RVA125, and *HPRT* housekeeping control was amplified using  
891 primers RVA24 and RVA25.

892 For qRT-PCR experiments, three technical replicates (i.e. three reactions with the same cDNA)  
893 were pipetted and measured for each sample in each experiment. For each technical  
894 triplicate, the mean fold-change in *AXIN2* expression was calculated and depicted as a single  
895 dot in Figure 2D and Figure 7 supplement 1H. Three independent biological experiments, each  
896 thus depicted as an individual dot, were performed. Relative expression levels of *AXIN2* were  
897 calculated using the comparative Delta-Ct method (Livak and Schmittgen, 2001; Schmittgen  
898 and Livak, 2008). Briefly, *AXIN2* expression was normalized for *HPRT* expression and then the  
899 relative fold-change to a WT DMSO sample was calculated for all clones and conditions.

### 900 Time-lapse imaging

901 The day before imaging, 88.000 cells/well were seeded on an 8 well chamber slide with glass  
902 bottom (80827-90, Ibidi, Gräfelfing, Germany). HAP1<sup>SGFP2-CTNNB1</sup> clone 2 was used for the main  
903 Figure 3, all 3 clones were used for Figure 3 supplement 1. HAP1<sup>SGFP2-CTNNB1(S45F)</sup> clone 2 was

904 imaged for Figure 8A. Approximately 6 hours before imaging, medium was replaced with full  
905 medium supplemented with 1% penicillin/streptomycin, 0.025M HEPES and 500nM SiR-DNA  
906 (SC007, Spirochrome, Stein am Rhein, Switzerland). Time lapse experiments were performed  
907 on an SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) at 37°C with a HC PL  
908 APO CS2 63x/1.40 oil objective (15506350, Leica), 488 and 633 lasers, appropriate AOBS  
909 settings, using HyD detectors for fluorescent signal with a 496-555 for SGFP2-CTNNB1 and  
910 643-764 bandpass for SiR-DNA, and a transmission PMT. Using multi-position acquisition, up  
911 to 24 images were captured every 5 minutes. Focus was maintained using AFC autofocus  
912 control on demand for every time point and position. Automated cell segmentation and  
913 intensity quantification was performed using a custom CellProfiler™ pipeline (made available  
914 at <https://osf.io/6pmwf/>). Output data was further analyzed in R/RStudio. Cells with a  
915 segmented cytoplasmic area of less than 10 pixels were excluded. Intensities were normalized  
916 per position to the average intensity in the cellular compartment (nucleus or cytoplasm) for  
917 that position before the addition of the compounds. The imaging settings resulted in low  
918 signal in regions not occupied by cells (~10% of the nuclear intensity, and ~5% of the  
919 cytoplasmic intensity in untreated cells), and the data was therefore not background-  
920 corrected. The nuclear cytoplasmic ratio was calculated by dividing the raw nuclear intensity  
921 by the raw cytoplasmic intensity. Movies and still images were extracted with FIJI/ImageJ.

#### 922 FCS and N&B cell preparation and general settings

923 Two days before FCS and N&B experiments, 44.000 cells/well were seeded on an 8-well  
924 chamber slide with a glass bottom (80827-90, Ibidi). For low, FFS-compatible expression of  
925 control samples, HAP1<sup>WT</sup> cells were transfected with ~5 ng pSGFP2-C1, pEGFP (monomer),  
926 pEGFP<sub>2</sub> (dimer) or pEGFP<sub>3</sub> (trimer) and ~200 ng pBlueScript KS(+) per well with Turbofect, X-

927 tremeGene HP or Lipofectamine 3000 the day before the experiment. Lipofectamine 3000  
928 yielded the best transfection efficiency. For Figure 4, 5, 8K and accompanying supplements,  
929 HAP1<sup>SGFP2-CTNNB1</sup> clone 2 was used. For Figure 7, 8 and accompanying supplements, CHIR99021  
930 data was recorded and pooled for all three HAP1<sup>SGFP2-CTNNB1</sup> clones, and S45F data was  
931 recorded and pooled from HAP1<sup>SGFP2-CTNNB1(S45F)</sup> clones 2, 24 and 27 and HAP1<sup>SGFP2-CTNNB1</sup> clone  
932 1 (the parental line for these S45F mutant clones) was used as the wild-type control.

933 FCS and N&B measurements were performed on an Olympus FV-1000 equipped with Sepiall  
934 and PicoHarp 300 modules (Picoquant, Berlin, Germany) at room temperature. An Olympus  
935 60x water immersed UPLS Apochromat (N.A. 1.2) objective was used for FCS acquisition and  
936 Figure 3 supplement 1E, and an Olympus 60x silicon immersed UPLS Apochromat (N.A. 1.4)  
937 objective was used for N&B measurements. Green fluorophores were excited with a 488 nm  
938 diode laser (Picoquant) pulsing at 20 MHz and detected through a 405/480-488/560/635 nm  
939 dichroic mirror (Chroma, Bellows Falls, VT) and 525df45 nm bandpass filter (Semrock,  
940 Rochester, NY) with an Avalanche Photodiode (APD) (MPD, Bolzano, Italy). For, figure 2  
941 supplement 1E and for FCS and N&B reference images the same laser and dichroic were used,  
942 but the signal was detected through a 505-540 bandpass filter with an internal PMT of the FV-  
943 1000 Olympus microscope.

#### 944 FCS data acquisition and analysis

945 For FCS measurements, a confocal image was recorded. In this reference image, a single pixel  
946 was set as region of interest (ROI), based on its localization in the cytoplasm or nucleus as  
947 judged by the transmission image. In this ROI, the fluorescence intensity was measured over  
948 time using an APD, for typically 120 seconds.



949 FCS measurements were analyzed in FFS Dataprocessor version 2.3 (SSTC, Minsk, Belarus).  
950 The autocorrelation curve ( $G(\tau)$ ) was calculated from the measured intensity ( $I$ ) according to  
951 equation 1. Intensity traces with significant photobleaching, cell movement or focal drift were  
952 excluded from further analysis (see supplementary file 1 – tab FCS measurements and fitting).  
953 From other traces a portion of the trace with minimal (less than 10%) intensity drift or  
954 bleaching was selected to generate autocorrelation curve (AC).

$$G(\tau) = 1 + \frac{\langle \delta I(t) * \delta I(t + \tau) \rangle}{\langle I \rangle^2} \quad \text{Eq. 1}$$

955  
956 The resulting AC was fitted with a Triplet-state-diffusion model, described in equation 2.  $G_\infty$   
957 accounts for offset in the AC for example by intensity drift.  $N$  is the average of the number of  
958 particles that reside in the confocal volume.  $F_{trip}$  and  $\tau_{trip}$  describe the fraction of molecules in  
959 the dark state and the relaxation of this dark state respectively. Of note, in this case,  $F_{trip}$  and  
960  $\tau_{trip}$  account both for blinking of the fluorescent molecules and for the afterpulsing artefact of  
961 the APD.  $\tau_{diff,i}$  describes the diffusion rate of the fluorescent molecules with the corresponding  
962 fraction,  $F_i$ . This diffusion time depends on the structural parameter ( $sp$ ), which is defined as  
963 the ratio of the axial ( $\omega_z$ ) over the radial axis ( $\omega_{xy}$ ) of the observation volume.

$$G(\tau) = G_\infty + \frac{1}{\langle N \rangle} * \frac{F_{trip}}{1 - F_{trip}} e^{\frac{-\tau}{\tau_{trip}}} * \sum_j \frac{F_i}{\left(1 + \frac{\tau}{\tau_{diff,i}}\right) \sqrt{1 + \frac{\tau}{\tau_{diff,i}} * sp^2}} \quad \text{Eq. 2}$$

964 The apparent particle numbers ( $N_{apa}$ ) for SGFP2-CTNNB1 were corrected for autofluorescence  
965 and bleaching (equation 3). The autofluorescence ( $I_{autofluorescence}$ ) of HAP1 cells in the  
966 nucleus and cytoplasm was measured in untransfected HAP1 cells using the same settings as  
967 for FCS measurements. The correction for moderate bleaching is based on the intensity of the

968 selected portion of the intensity trace for AC calculation ( $I_{ana}$ ) and the intensity at the start of  
969 the measurement ( $I_{start}$ ).

970 The size and shape of the observation volume was calibrated daily by measuring Alexa Fluor™  
971 488 NHS Ester (A20000, Molecular probes, Thermo Scientific, stock dilution in MQ) in PBS in  
972 a black glass-bottom cell imaging plate with 96 wells (0030741030, Eppendorf, Hamburg,  
973 Germany). From the FCS measurements of Alexa488, the  $\tau_{diff}$  and  $sp$  were determined by  
974 fitting with a single diffusion and blinking component. The diffusion coefficient (D) of  
975 Alexa488 in aqueous solutions at 22.5 °C is  $435 \mu\text{m}^2\text{s}^{-1}$  (Petrášek and Schwille, 2008). From  
976 these parameters, the axial diameter can be determined with equation 4 and the volume can  
977 be approximated by a cylinder (equation 5). This allows for transformation of particle  
978 numbers to concentrations (equation 5) and diffusion times to diffusion coefficients (equation  
979 4) that are independent of measurement settings and small daily changes in alignment of the  
980 microscope.

$$N_{corr} = N_{apa} * \left[1 - \frac{I_{autofluorescence}}{I_{total}}\right]^2 * \left[\frac{I_{start}}{I_{ana}}\right] \quad \text{Eq. 3}$$

$$\tau_{diff} = \frac{\omega_{xy}^2}{4D} \quad \text{Eq. 4}$$

$$V = 2\pi\omega_{xy}^3 * sp \quad \text{Eq. 5}$$

$$C = \frac{N_{corr}}{V * N_A} \quad \text{Eq. 6}$$

981 The model to fit SGFP2-CTNNB1 measurements contained 2 diffusion components. The first  
982 diffusion component was fixed to the speed of monomeric SGFP2-CTNNB1. To estimate the

983 speed of monomeric SGFP2-CTNNB1, the speed of free floating SGFP2, transfected in HAP1  
984 cells, was measured to be  $24.1 \mu\text{m}^2\text{s}^{-1}$  using FCS. Subsequently, this speed was used to  
985 calculate the speed of monomeric SGFP2-CTNNB1 with Einstein-Stokes formula (Equation 7).  
986 As the temperature (T), dynamic viscosity ( $\eta$ ) and Boltzmann's constant ( $k_B$ ) are equal  
987 between SGFP2 and SGFP2-CTNNB1 measurements, the expected difference in diffusion  
988 speed is only caused by the radius (r) of the diffusing molecule assuming a spherical protein.  
989 The difference in radius was approximated by the cubic root of the ratio of the molecular  
990 weight of the SGFP2-CTNNB1 fusion protein (88 + 27=115 kDa) and the size of the SGFP2  
991 protein (27 kDa), thus expecting a 1.62 times lower diffusion coefficient (compared to free  
992 floating SGFP2) of  $14.9 \mu\text{m}^2\text{s}^{-1}$  for SGFP2-CTNNB1. It must be noted that, especially for larger  
993 protein complexes, the linearity between the radius of the protein and the speed is not  
994 ensured, if the shape is not globular, and due to other factors such as molecular crowding in  
995 the cell and hindrance from the cytoskeletal network. We therefore did not estimate the size  
996 of the measured CTNNB1 complexes, but rather compared them to measurements from other  
997 FCS studies.

$$D = \frac{k_B T}{6\pi\eta r} \quad \text{Eq. 7}$$

998 In the fitting model, the structural parameter was fixed to the one determined by the  
999 Alexa488 measurements of that day. To ensure good fitting, limits were set for other  
1000 parameters;  $G_\infty$  [0.5-1.5], N [0.001, 500],  $\tau_{\text{trip}}$  [ $1 \cdot 10^{-6}$ -0.05 ms],  $\tau_{\text{diff2}}$  [10-150 ms]. This model  
1001 was able to fit most Autocorrelation Curves from FCS measurements. In case of clear misfits,  
1002 as judged by the distribution of residuals around the fitted curves, the measurement was  
1003 excluded (see supplementary file 1 – tab FCS measurements and fitting).

1004 N&B data acquisition and analysis

1005 For N&B analysis, 50 images were acquired per measurement with a pixel time of 100  $\mu\text{s}$ /pixel  
1006 and a pixel size of 0.138-0.207  $\mu\text{m}$ . The fluorescent signal was acquired with the APD  
1007 described above for the FCS measurements. As a control and to optimize acquisition settings,  
1008 HAP1 cells transfected with SGFP2, EGFP monomer, dimer or trimer were measured alongside  
1009 HAP1<sup>SGFP2-CTNNB1</sup> cells treated with BSA, WNT3A, DMSO or CHIR99021, or HAP1<sup>SGFP2-CTNNB1(S45F)</sup>  
1010 cells. APD readout was converted to a TIF stack using a custom build *.ptu* converter (Crosby  
1011 et al., 2013). This TIF stack was further analyzed using an ImageJ macro script (modified from  
1012 (Crosby et al., 2013), made available at <https://osf.io/ys5qw/>) based on Digman, Dalal,  
1013 Horwitz, & Gratton, 2008. Within the script, average brightness and particle numbers were  
1014 calculated for nuclear or cytoplasmic ROIs, which were set based on transmission image (see  
1015 Figure 5F). Static or slow-moving particles, including membrane regions, were excluded by  
1016 thresholding and/or ROI selection, since they can severely impact the brightness measured.  
1017 Data were further analyzed in R/RStudio. Brightness was normalized to the median value of  
1018 the EGFP-monomer brightness measured on the same day in the same cellular compartment  
1019 (nucleus/cytoplasm). Our FCS and N&B analysis assume a different confocal volume. In FCS  
1020 we assume a cylinder with factor  $\gamma=1$ , whereas in N&B we assume a 3D-Gauss with factor  
1021  $\gamma=0.3536$ . To be able to compare particle numbers obtained with both techniques, particle  
1022 numbers obtained with N&B were divided by the factor  $\gamma=0.3536$ .

1023 Data representation and statistical analysis

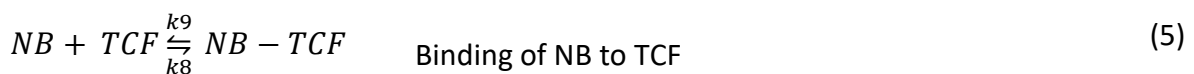
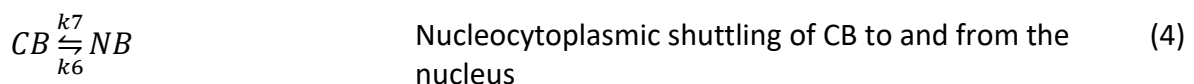
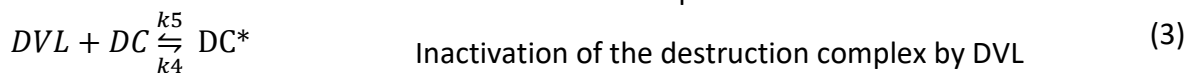
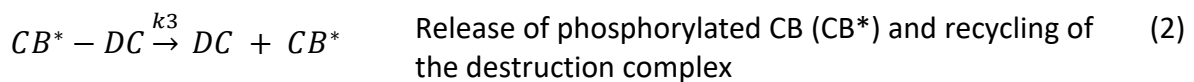
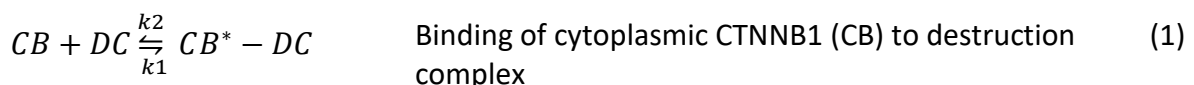
1024 Data processing and representation were performed in RStudio (version 1.1.456 running R  
1025 3.5.1 or 3.6.1). 95% confidence intervals of the median mentioned in the text and shown in  
1026 Table 1-2 and Supplementary file 1 were calculated using PlotsOfDifferences (Goedhart,

1027 2019). The P-values in Table 3 and Supplementary file 1 were also calculated using  
 1028 PlotsOfDifferences, which uses a randomization test and makes no assumption about the  
 1029 distribution of the data. Representation of the imaging data in Figure 4 supplement 2 and in  
 1030 supplementary movies 3-4 were generated in RStudio using a script based on  
 1031 PlotsOfDifferences (made available at <https://osf.io/sxakf/>).

### 1032 Model description

1033 We developed a minimal model for WNT signaling based on a previous model from the  
 1034 Kirschner group (Lee et al., 2003). The model is available as an interactive app at  
 1035 [https://wntlab.shinyapps.io/WNT\\_minimal\\_model/](https://wntlab.shinyapps.io/WNT_minimal_model/) and the R source code of the model is  
 1036 available at <https://osf.io/jx29z/> (WNT\_minimal\_model\_v2.3.R).

1037 Our minimal model comprises the following reactions:



1038 Below, we show the differential equations that govern the concentrations of the different  
 1039 compounds over time for the reactions described above. Table 4 in the main text gives the  
 1040 correspondence between the variables (i.e.  $x_1$ ) in the differential equations and the model  
 1041 name (i.e. CB) in the reactions. The parameter  $w$  in equations (7) and (8) is  $w = 0$  in the  
 1042 absence of WNT and  $w = 1$  if WNT is present, i.e. in our minimal model the inactive form of

1043 the destruction complex (DC\*) is only present if WNT is present. The parameter  $b$  in equation  
1044 (6) represents the constant production of CTNNB1, corresponding to  $v_{12}$  in Lee et al., 2003.

$$\frac{dx_1}{dt} = -k_1x_1x_2 + k_2x_3 - k_6x_1 + k_7x_5 + b \quad (6)$$

$$\frac{dx_2}{dt} = -k_1x_1x_2 + (k_2 + k_3)x_3 - w(k_4x_2 - k_5x_4) \quad (7)$$

$$\frac{dx_3}{dt} = k_1x_1x_2 - (k_2 + k_3)x_3 \quad (8)$$

$$\frac{dx_4}{dt} = w(k_4x_2 - k_5x_4) \quad (9)$$

$$\frac{dx_5}{dt} = k_6x_1 - k_7x_5 - k_8x_5x_6 + k_9x_7 \quad (10)$$

$$\frac{dx_6}{dt} = -k_8x_5x_6 + k_9x_7 \quad (11)$$

$$\frac{dx_7}{dt} = k_8x_5x_6 - k_9x_7 \quad (12)$$

1045

1046 **Equilibrium conditions without WNT**

1047 The parameters in our model can in part be determined from our measurements of the  
1048 equilibrium concentrations of CB, NB and their complexes, see Table 4-5 in the main text.  
1049 Where we could not determine the parameters from our measurements, we used published  
1050 values as indicated.

1051 Under equilibrium conditions, the concentrations of the compounds do not change with time  
1052 and the left-hand side of equations (6) - (12) is zero. From equations (10) and (11) we can  
1053 determine the ratio of the rate constants  $k_6$  and  $k_7$  from the measured values of  $x_1$  and  $x_5$ :

$$k_6x_1 = k_7x_5 \Leftrightarrow \frac{k_6}{k_7} = \frac{x_5}{x_1} = \frac{87}{91} = 0.96 \quad (13)$$

1054

1055 From equations (6), (8), (10) and (11) we have:

$$-k_3x_3 + b = 0 \Leftrightarrow k_3 = \frac{b}{x_3} = \frac{0.423}{62.5} = 0.0068 \text{ min}^{-1} \quad (14)$$

1056 Our reaction (1) corresponds closely to step 8 in Lee et al. therefore, we use the value of the

1057 dissociation constant  $K_8 = 120 \text{ nM}$  from Lee et al. for our dissociation constant  $K_1 = \frac{k_2}{k_1}$ .

1058 The concentration of the destruction complex is obtained from equation (1) under

1059 equilibrium conditions using equations (6), (8), (10), (11) and (14)

$$1060 \quad -k_1x_1x_2 + k_2x_3 + b = 0$$

1061 The value of  $b$  is assumed to be small compared to the two other terms, so we calculate the

1062 concentration of the destruction complex as:

$$1063 \quad x_2 = K_1 \frac{x_3}{x_1} = 120 \frac{62.5}{91} = 82.4 \text{ nM. It was then verified in our interactive app that this value}$$

1064 for the destruction complex is indeed consistent with the equilibrium conditions without WNT  
1065 stimulation.

1066 To calculate the dissociation constant for the NB-TCF complex, we estimate an equilibrium

1067 concentration for free TCF ( $x_6$ ) from Tan et al. (2012). From their Figure 11 it is seen that the

1068 bound TCF concentration in equilibrium in the presence of WNT has about the same value as

1069 the initial free TCF concentration and that no initial bound TCF is present. However, we

1070 measured NB-TCF also in the initial state. Therefore, we consider the free TCF concentration

1071 value from Tan et al. as a lower bound for the estimate of total TCF. Also, from Figure 11 of

1072 Tan et al. (2012) we estimate that of the initial free TCF, a fifth remains in the nucleus as free

1073 TCF after WNT is turned on. We measured 86 nM NB-TCF in the nucleus after the application

1074 of WNT. This leads to an estimate of the total concentration of TCF,  $\text{TCF}^0$ , in the nucleus of:

1075  $[TCF^0] = 86 + 0.2 \times 86 = 103$  nM. If we assume that the total TCF concentration does  
 1076 not change by the application of WNT, we calculate the dissociation constant of the NB-TCF  
 1077 complex from equation (12):

$$k_8 x_5 (TCF^0 - x_7) = k_9 x_7 \Rightarrow \frac{k_9}{k_8} = K_2 = \frac{x_5 (TCF^0 - x_7)}{x_7} = \frac{87 \cdot 81}{22} = 320 \text{ nM} \quad (15)$$

1078

1079 **Equilibrium conditions with WNT**

1080 We model the action of WNT by deactivation of the destruction complex by DVL through  
 1081 reaction 3 by setting  $w = 1$  in equations (7) and (9). The dissociation constant of CB\*-DC,  $K_1$ ,  
 1082 is assumed not to change in the presence of WNT. The measurements of free CB and NB in  
 1083 equilibrium (see Table 2) give for the ratio of  $k_6$  and  $k_7$ :

$$k_6 x_1 = k_7 x_5 \Leftrightarrow \frac{k_6}{k_7} = \frac{x_5}{x_1} = \frac{170}{145} = 1.17 \quad (16)$$

1084 The value of the rate of decay of the phosphorylated complex CB\*-DC,  $k_3$ , is found to be the  
 1085 same for the "without WNT" situation:

$$-k_3 x_3 + b = 0 \Leftrightarrow k_3 = \frac{b}{x_3} = \frac{0.423}{62.5} = 0.0068 \text{ min}^{-1} \quad (17)$$

1086 To uniquely determine the ratio of  $k_4$  and  $k_5$ , we need the concentrations of the destruction  
 1087 complex DC and DC\* neither of which we have access to. We can, however, fit this ratio with  
 1088 our model to the measured values of  $x_1$  and  $x_7$  and find  $k_4/k_5 = 1.7$ .

1089 We again calculate the dissociation constant of the NB-TCF complex from equation (12), using  
 1090 the concentrations for NB and NB-TCF obtained with FCS.

$$k_8 x_5 (TCF^0 - x_7) = k_9 x_7 \Rightarrow \frac{k_9}{k_8} = K_2 = \frac{x_5 (TCF^0 - x_7)}{x_7} = \frac{170 \cdot 17}{86} = 33.6 \text{ nM} \quad (18)$$



1091 Notice that we determined the ratios of the rate constants from the measured equilibrium  
1092 values of free and bound CTNNB1 in the cytoplasm and the nucleus. This means that our rate  
1093 constants are determined up to a multiplicative factor: the equilibrium equations do not  
1094 change if all rate constants  $k_i$  and the parameter  $b$  are multiplied by the same factor,  $Rate$ .  
1095 The factor  $Rate$  determines how fast our model system reaches equilibrium. By comparing  
1096 the times equilibrium was reached by the cytoplasmic and nuclear SGFP2-CTNNB1 signals  
1097 (Figure 4 C, D) of about 4.5 hours, we fitted a factor  $Rate = 20$  for our model.

1098 Our model shows that the ratios of  $k_6/k_7$  and  $k_9/k_8$  are different for the conditions without  
1099 and with WNT stimulation, suggesting a change in mechanism for nuclear shuttling of CTNNB1  
1100 and nuclear retention of CTNNB1 in going from the WNT 'off' situation to the WNT 'on'  
1101 situation. It seems likely that such changes do not occur instantaneously. In our model we  
1102 therefore allow a gradual rise in  $k_5/k_4$  and a gradual transition of the ratios of  $k_6/k_7$  and  
1103  $k_9/k_8$  from WNT 'off' to the WNT 'on'. In our model this is included by setting a parameter  
1104 ("Steep") that indicates the time after application of WNT the transition from WNT 'off'  
1105 parameter values to WNT 'on' parameter values is complete. The value that gives a good  
1106 approximation of the experimentally observed concentration curves is  $Steep = 150$  minutes  
1107 (Figure 6 panels B-F).

## 1108 Acknowledgements

1109 We thank the van Leeuwenhoek Centre for Advanced Microscopy (LCAM, Section Molecular  
1110 Cytology, Swammerdam Institute for Life Sciences, University of Amsterdam) for the use of  
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1112 Jasmijn Span for cloning the color variants of the repair plasmid as a student in our lab, Marten  
1113 Postma and Joachim Goedhart for assistance with data handling and analysis, Dorus Gadella  
1114 for carefully reading the manuscript, and all colleagues for stimulating discussions and  
1115 suggestions.

1116 **Figure supplements**

1117 **Figure 1 - supplement 1:** FACS Gating strategy for haploid HAP1 cells.

1118 **Figure 1 - supplement 2:** *SGFP2-CTNNB1* locus

1119 **Figure 2 - supplement 1:** Verification of the WNT/CTNNB1 responsiveness of HAP1 cells.

1120 **Figure 3 - supplement 1:** Verification of imaging results with WNT3A three independent  
1121 HAP1<sup>SGFP2-CTNNB1</sup> clones.

1122 **Figure 3 - supplement 2:** Difference analysis of SGFP2-CTNNB1 fluorescence.

1123 **Figure 3 supplement 3:** Unnormalized nuclear and cytoplasmic intensity measurements.

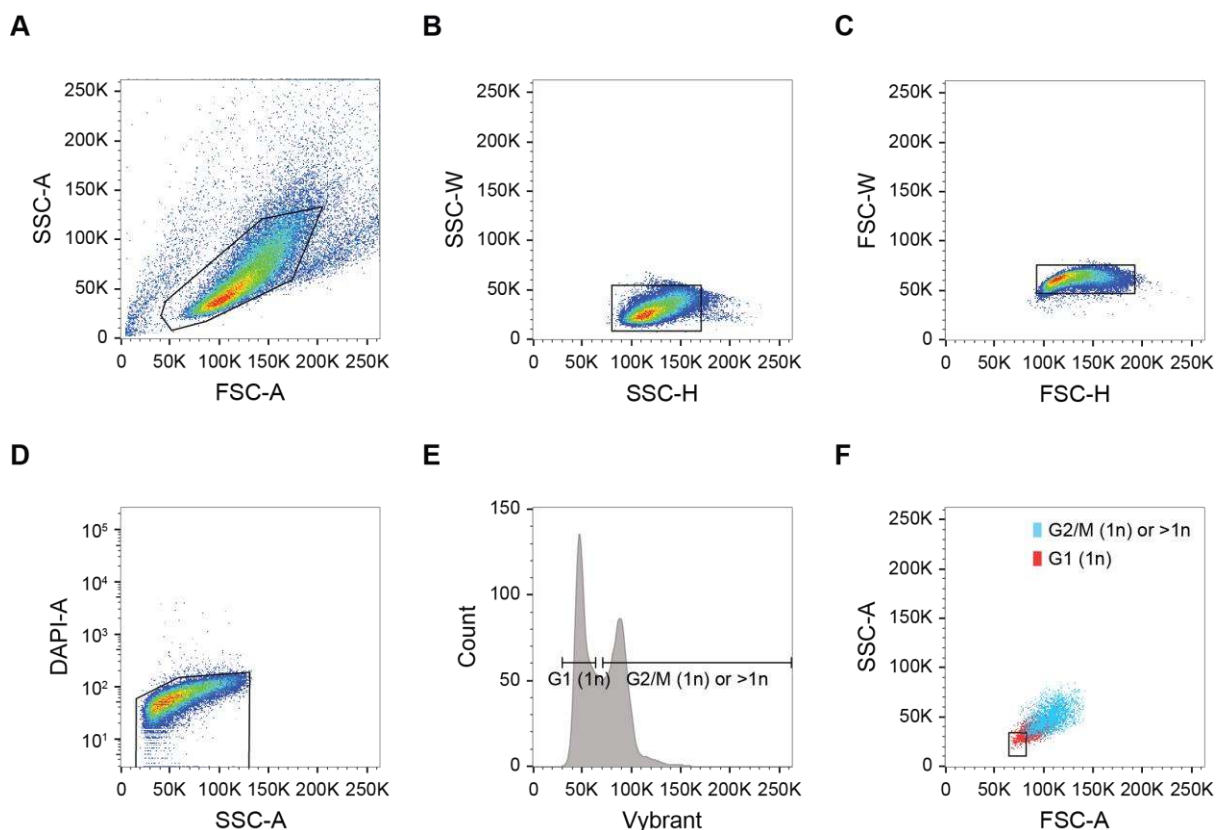
1124 **Figure 5 - supplement 1:** Quantification of SGFP2-CTNNB1 particles, fluorescence and fluorescence  
1125 lifetime.

1126 **Figure 5 supplement 2:** Number and Brightness analysis

1127 **Figure 7 supplement 1** Generation and characterization of a S45F mutant cell line  
1128 (HAP1<sup>SGFP2-CTNNB1(S45F)</sup>).

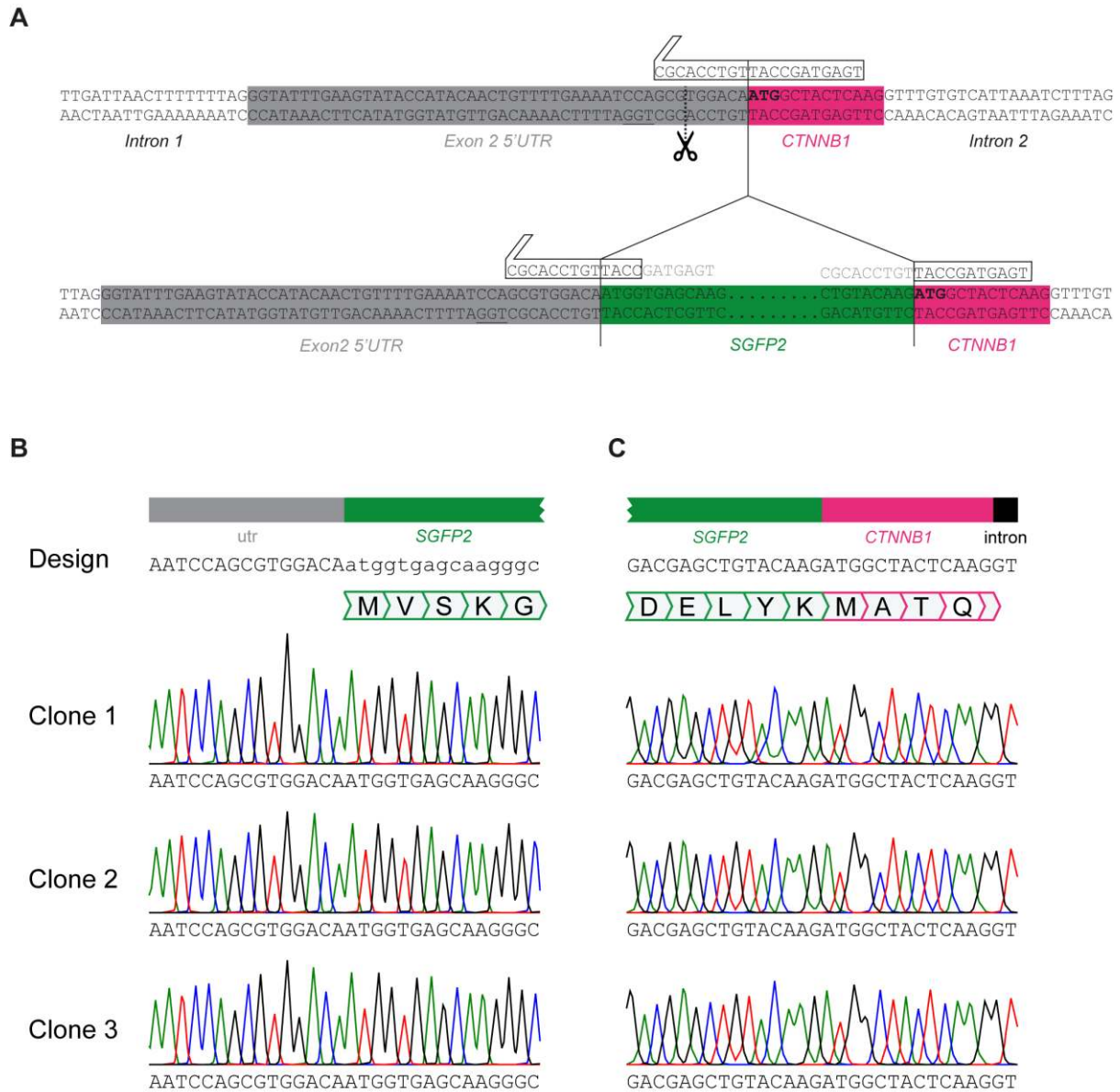
1129 **Figure 8 supplement 1** Live imaging of HAP1<sup>SGFP2-CTNNB1</sup> upon CHIR99021 stimulation.

1130 **Figure 8 supplement 2** additional biophysical properties of SGFP2-CTNNB1<sup>S45F</sup> and SGFP2-CTNNB1  
1131 under CHIR99021 stimulation.



1132

1133 **Figure 1 – supplement 1:** FACS Gating strategy for haploid HAP1 cells. A-C) Single-cell gating based on forward scatter (FSC) and side scatter  
1134 (SSC). D) Live cell gating based on DAPI exclusion. E-F) Haploid cell sorting based on Vybrant live-cell DNA dye. E) Haploid cell cycle profile.  
1135 Only cells in G1 can be confidently identified as haploid (1n). The second peak contains both G2/M haploid cells, as well as diploid (2n) and  
1136 polyploid events. Of note, the depicted HAP1<sup>WT</sup> population is mainly haploid. F) Back-gating of the haploid G1 population from E onto the  
1137 forward and side scatter plot. A stringent gate is set based on cell size to ensure only G1 (1n) cells qualify for sorting.



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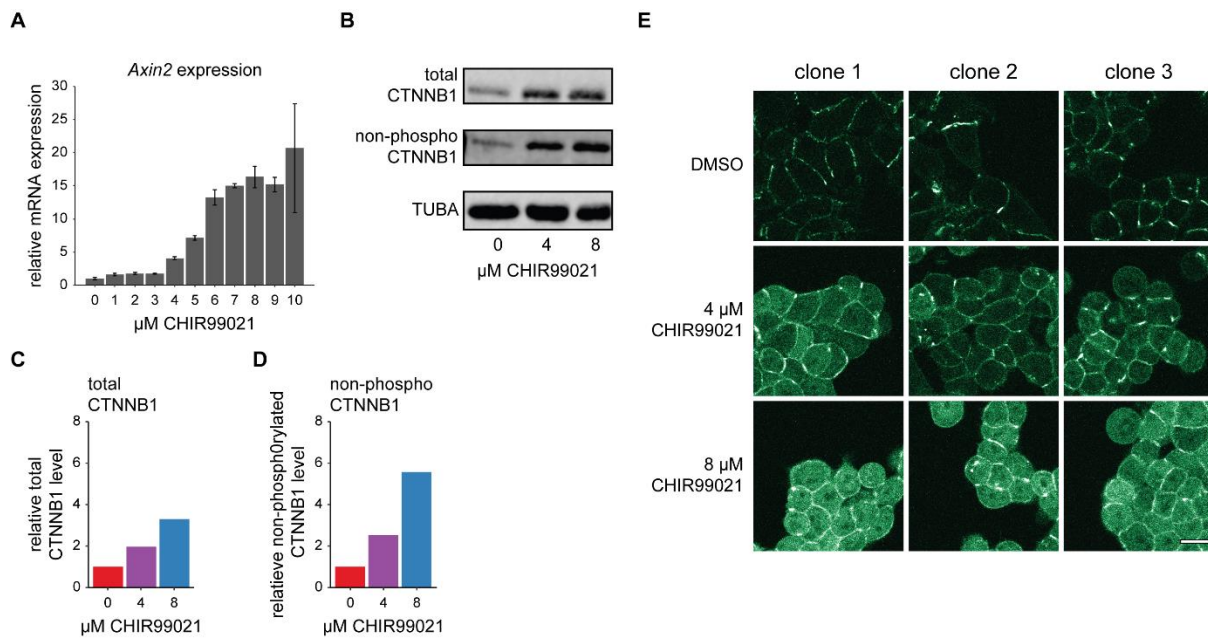
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**Figure 1 – supplement 2: SGFP2-CTNNB1 locus** A) Detailed view of *CTNNB1* exon 2 depicting gRNA design relative to the wildtype (top) and repaired (bottom) *CTNNB1* allele. Note that the repair template contains the same sequence as the repaired allele depicted here. *CTNNB1* sequences are shown in capital letters, *SGFP2* sequences shown in lowercase. 5' UTR, *SGFP2* and *CTNNB1* and intron regions are indicated below the colored boxes. The gRNA (white arrow box above sequence) overlaps the start codon (depicted in bold), resulting in a Cas9-mediated double-strand break in the 5' UTR (predicted cut site indicated by dotted line and scissors, PAM site underlined). After successful homologous recombination, most of the gRNA binding site is destroyed, thus minimizing the chance of cutting the repair template or re-cutting the repaired allele. PCR based screening confirmed that 22/23 single-cell sorted clones indeed showed an integration of the expected size at the *CTNNB1* locus. Complete sequence coverage of the insertion site in exon 2 was obtained for 9/11 sequenced clones, of which 8 showed the desired repair, and 1 clone showed an additional point mutation in the repaired locus. B-C) Sequencing of three independent HAP1<sup>SGFP2-CTNNB1</sup> clones on the 5' (B) and the 3' (C) end of *SGFP2* integration in exon 2 of *CTNNB1*. Sanger sequencing of the endogenous *CTNNB1* locus of clone 1, clone 2 and clone 3 shows an exact match to the design and thus correct homology directed repair.



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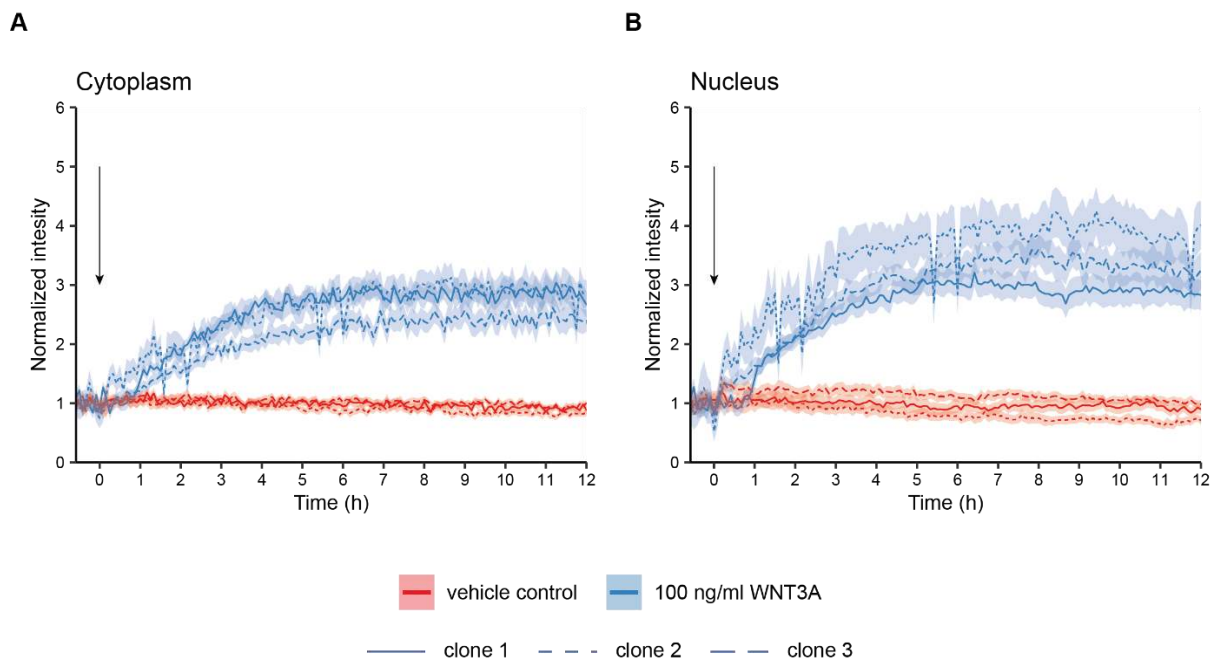
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**Figure 2 supplement 1:** Verification of the WNT/CTNNB1 responsiveness of HAP1 cells. A) Graph depicting *AXIN2* qRT-PCR results from HAP1<sup>WT</sup> cells treated with the indicated range of CHIR99021 (1-10 μM) or DMSO vehicle control (0 μM) for 24 hours. *HPRT* was used as a reference gene. Error bars represent standard deviation within technical triplicates from n=1 biological experiment. Based on this, we selected 4μM and 8 μM as intermediate and high levels of WNT/CTNNB1 pathway induction for follow up experiments. B) Western blot, showing the increase in total (top) and non-phosphorylated (i.e. active) CTNNB1 levels (middle) in response to pathway stimulation. HAP1<sup>WT</sup> cells were treated for 24 hours with 4 or 8 μM CHIR99021, or DMSO vehicle control (0 μM). Alpha-Tubulin (TUBA, bottom) serves as a loading control. C-D) Quantification of the western blot from (B) depicting the relative fold change of total CTNNB1 (C) or non-phosphorylated CTNNB1 (D) to DMSO control corrected for Tubulin loading. E) Representative confocal microscopy images of three independent HAP1<sup>SGFP2-CTNNB1</sup> clones, treated for 24 hours with 4 or 8 μM CHIR99021, or DMSO vehicle control. Scalebar is 10 μm.



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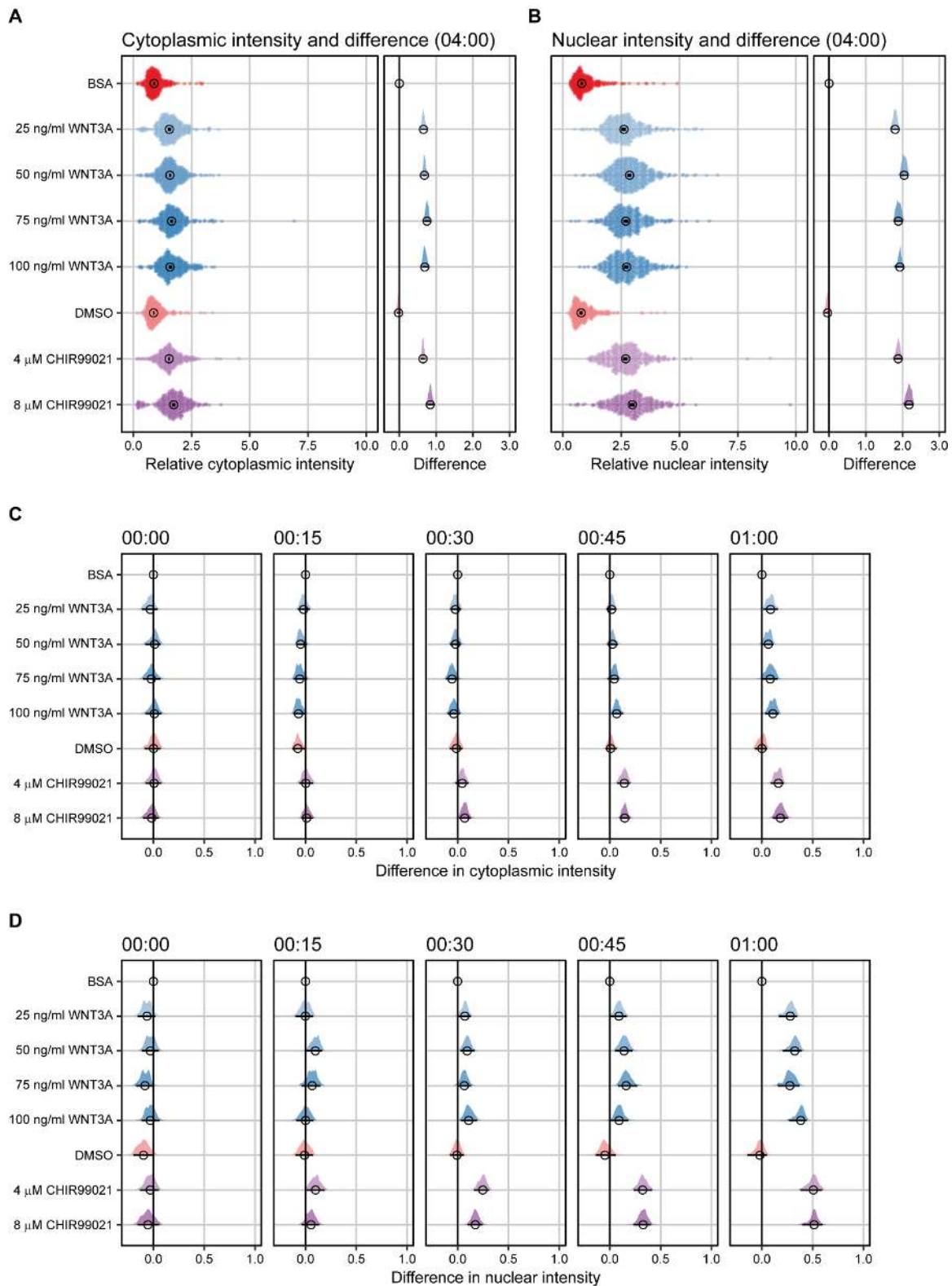
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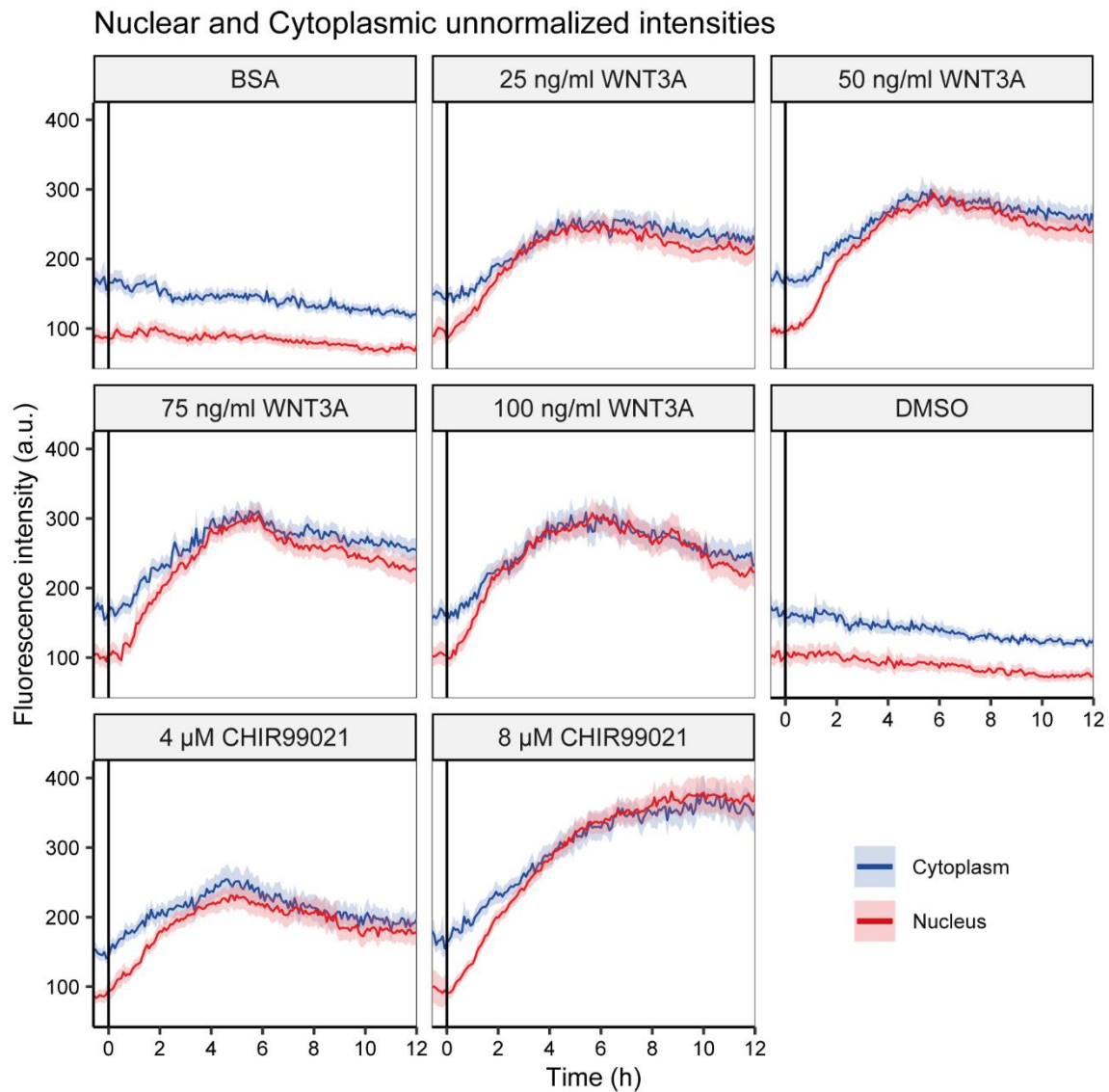
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**Figure 3 supplement 1:** Graphs showing quantification of time-lapse microscopy experiments with three independent HAP1<sup>SGFP2-CTNNB1</sup> clones. Stills of this experiment are shown in Figure 3C. Segmentation was performed as described in Figure 4. Arrow indicates the moment of starting the different treatments (BSA in red or 100 ng/ml WNT3A in blue). Solid lines represent the mean normalized intensity and shading the 95% confidence interval in the cytoplasm (A) or nucleus (B). Line pattern indicates the three different clones. n=13-158 cells for each condition and time point for n=1 biological experiment.



1168

1169 **Figure 3 supplement 2:** Difference analysis of SGFP2-CTNNB1 fluorescence. A-B) Plots depicting the relative intensity (left) and the difference  
 1170 in relative intensity to BSA treated cells (right) in the cytoplasm (A) and nucleus (B) after 4 hours of treatment. Circles indicate the median  
 1171 value and bars indicate the 95% CI. In the relative intensity plot (left) the distribution is built from individual data points in a violin-type  
 1172 fashion to faithfully represent the distribution of data. In the difference plot (right) the distribution of differences is represented in a half  
 1173 violin plot. If the 95% CI in the difference plot does not overlap the zero line, which indicates no difference, the sample is significantly  
 1174 different from BSA control condition. C-D) Plots depicting the difference in relative intensity in the cytoplasm (C) and nucleus (D) between  
 1175 the moment of addition and 1 hour of treatment. Titles indicate the time (hh:mm). The distribution of differences is represented in a half  
 1176 violin plot. Circles indicate the median value and bars indicate the 95% CI. If the 95% CI does not overlap the zero line, which indicates no  
 1177 difference, the sample is significantly different from the BSA control condition.



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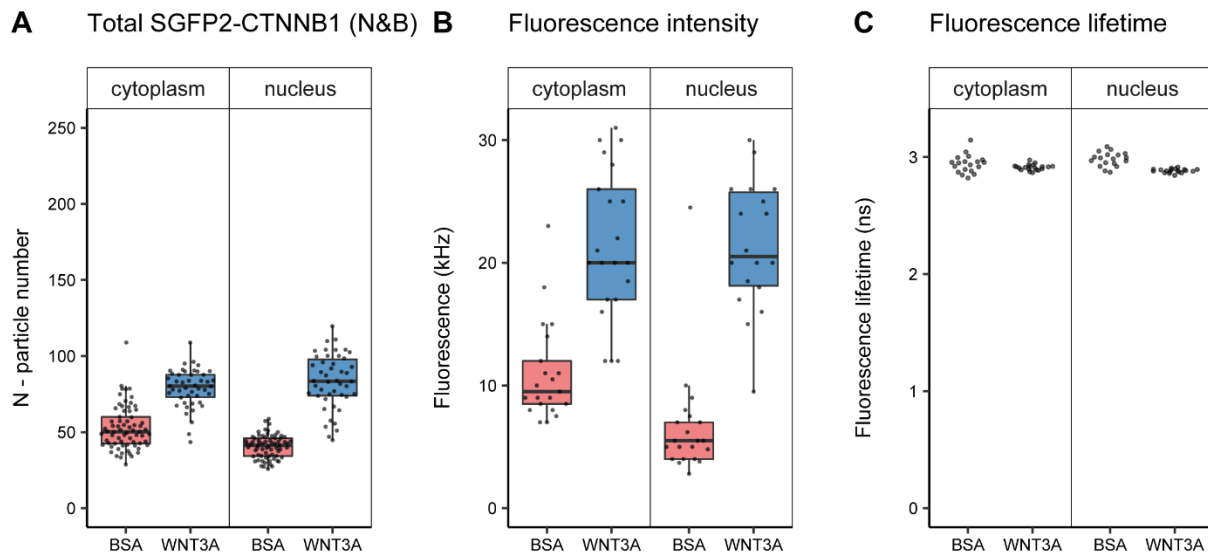
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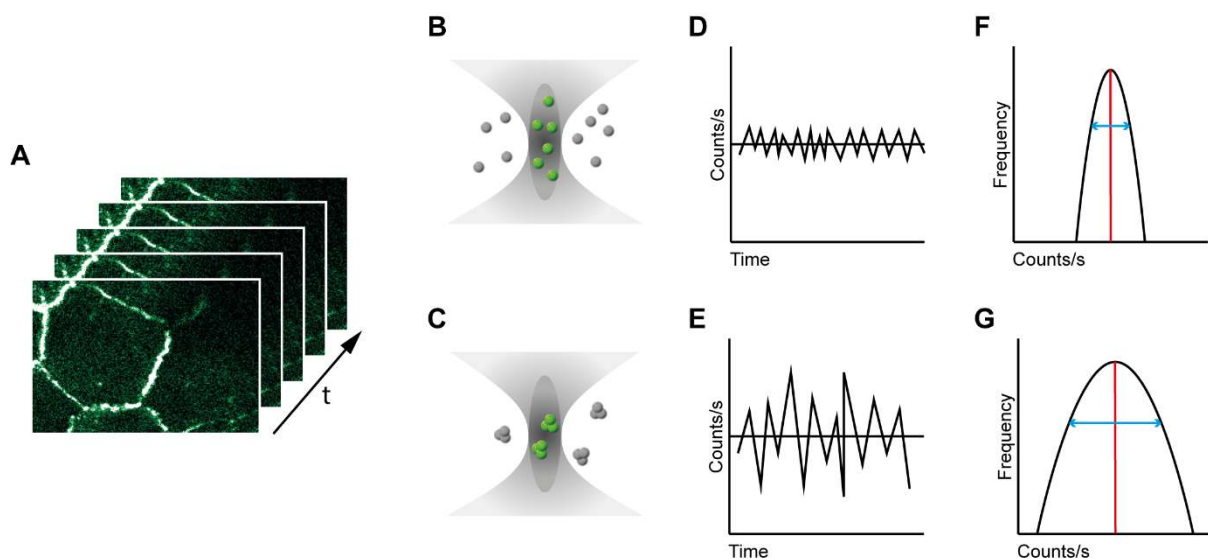
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**Figure 3 supplement 3:** Unnormalized nuclear and cytoplasmic intensity measurements. Graphs showing the unnormalized fluorescence intensities quantified for a single biological replicate shown in Figure 3. The vertical black lines indicate the moment of starting the different treatments (as indicated on top of each graph). Solid lines represent the mean normalized intensity and shading the 95% confidence interval in the cytoplasm (blue) or nucleus (red). n=64-148 cells for each condition and time point.



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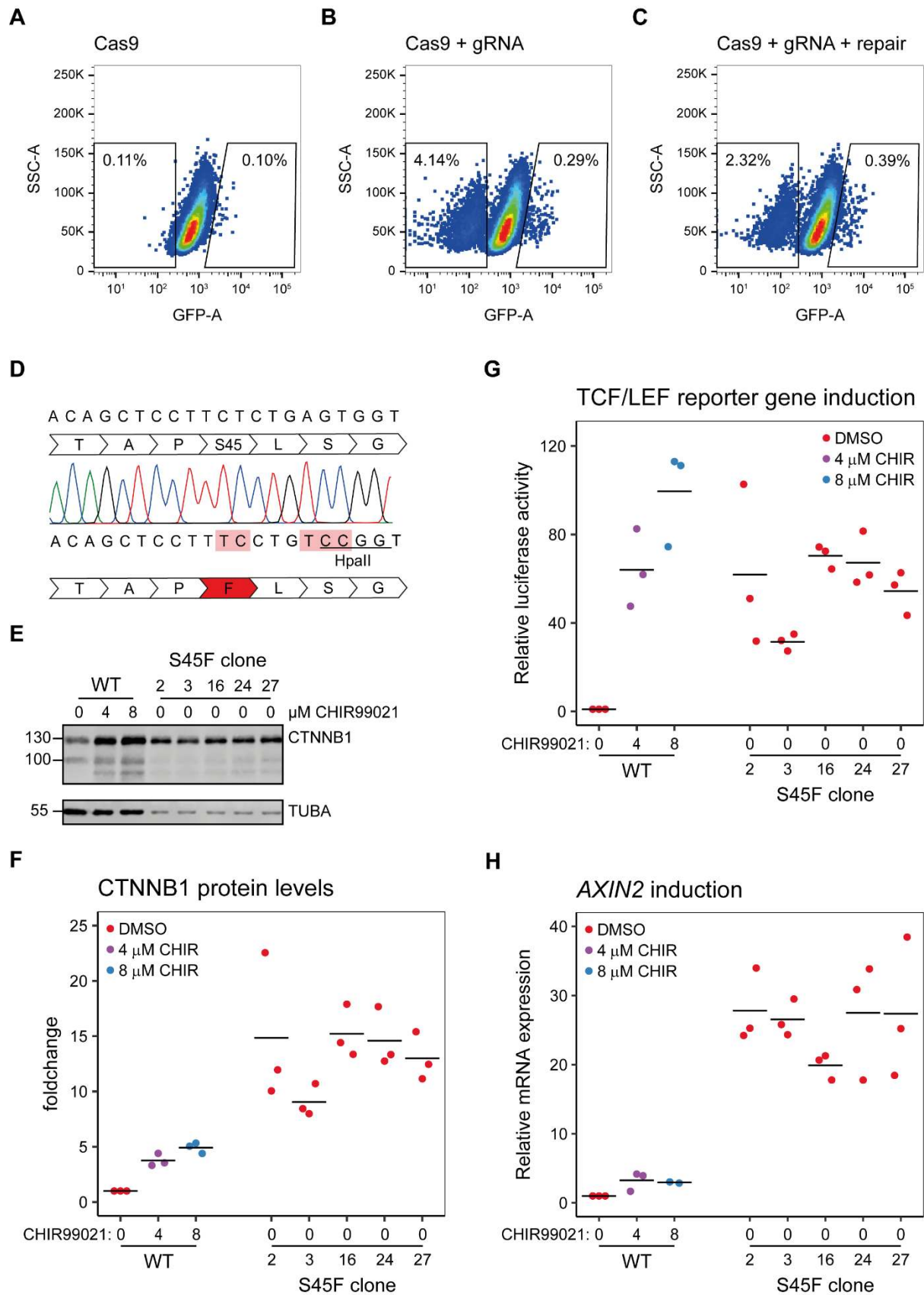
1184 **Figure 5 supplement 1** Quantification of SGFP2-CTNNB1 particles, fluorescence and fluorescence lifetime. Details on sample size and  
 1185 statistics can be found in supplementary file 1. A) Graph depicting the total number of SGFP2-CTNNB1 particles as measured with N&B. B)  
 1186 Graph depicting the average fluorescence intensity at the start of the FCS measurement. The increase in SGFP2-CTNNB1 fluorescence in the  
 1187 cytoplasm (2.1-fold) exceeds the increase in the SGFP2-CTNNB1 concentration (1.1-fold, Figure 5A), but does correspond to the relative  
 1188 increase measured by time-lapse imaging (1.7-fold, Figure 4C). C) Graph depicting fluorescence lifetimes calculated from the FCS  
 1189 measurements. The Fluorescence lifetime of SGFP2-CTNNB1 is independent of the subcellular compartment and treatment. Therefore,  
 1190 photophysical effects are not the cause for the difference between the fold-change in fluorescence and concentrations of the FCS  
 1191 measurements as described in (B).



1192

1193 **Figure 5 supplement 2:** Number and Brightness analysis A) A series of images are acquired over time (t) B-C) Representation of the confocal  
 1194 volume in a single pixel of the image in (A) representing monomers with a brightness of 1 (B) and trimers with a brightness of 3 (C) both  
 1195 confocal volumes emit a fluorescence of 6. D-E) Graph depicting the variation in fluorescence, measured by an APD in counts/s, in a single  
 1196 pixel over time for the monomer (D) and trimer (E). With a higher brightness the variation around the average becomes larger. Note that  
 1197 the time-scale in N&B is much larger than in FCS (Figure 4 A-C) due to the use of images rather than a single point and therefore the variation  
 1198 is no longer dependent on diffusion kinetics. F-G) Graph depicting the distribution of fluorescence observed in a single pixel. The average  
 1199 (red line) in our case is the same for 6 monomers (F) or 2 trimers (G), but the standard deviation (blue line) is much larger for the trimer.  
 1200 The average and standard deviation are used to calculate the number





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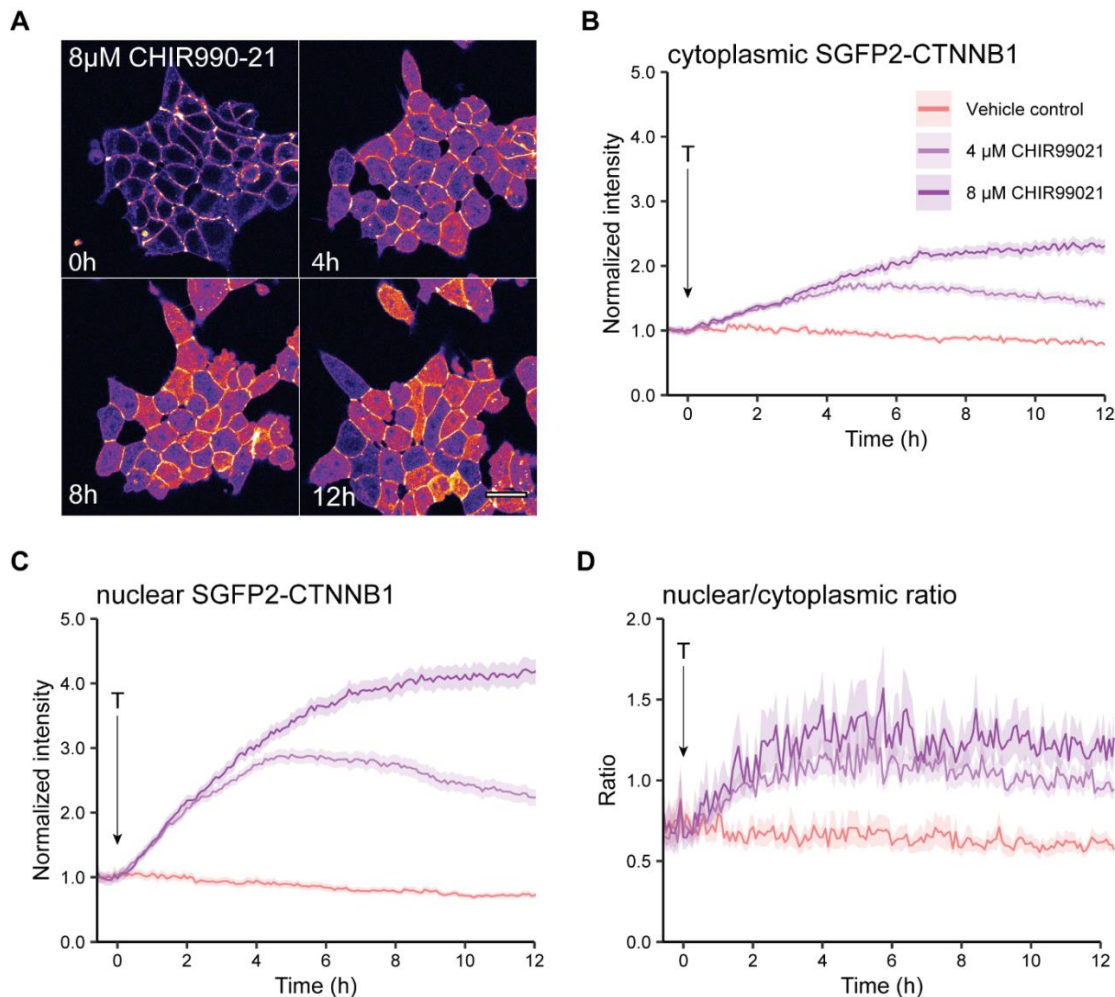
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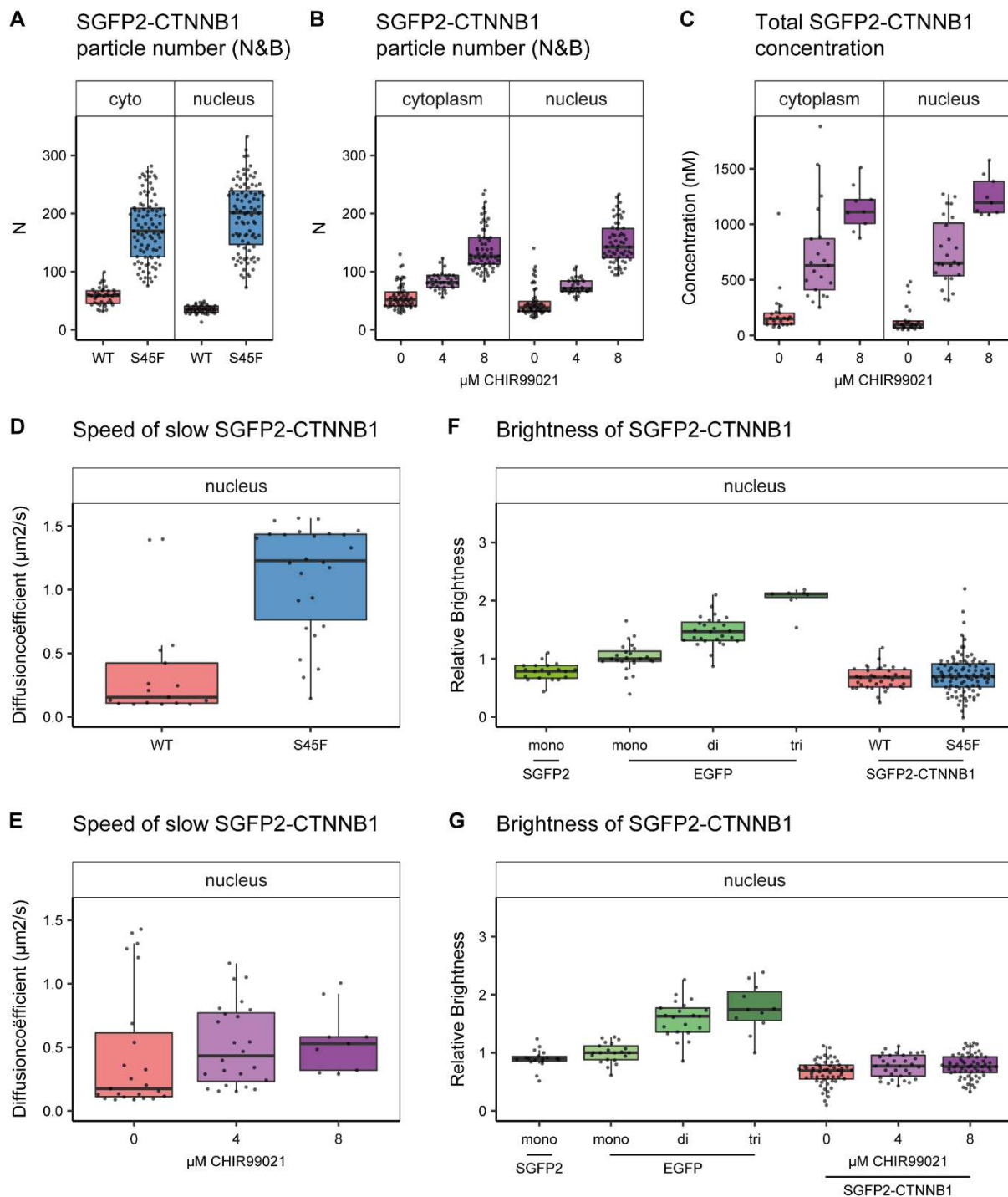
**Figure 7 supplement 1** Generation and characterization of a S45F mutant cell line (HAP1<sup>SGFP2-CTNNB1(S45F)</sup>). A-C) FACS graphs showing the selection strategy for HAP1<sup>SGFP2-CTNNB1(S45F)</sup> cells. HAP1<sup>SGFP2-CTNNB1</sup> clone 1 was transfected with CRISPR constructs similar to figure 1C, see material and methods for details. Transfection with a guide RNA in the third exon of CTNNB1 resulted in a population with reduced SGFP2 fluorescence and a population with increased fluorescence (B) compared to a Cas9 only control (A). Co-transfection of a single stranded

1206 oligo repair, led to a reduction in the population with decreased SGFP2 fluorescence (representing cells repaired with NHEJ that resulted in  
 1207 a loss of the SGFP2-CTNNB1 fusion protein) and an increase in the population with increased SGFP2 fluorescence (representing cells repaired  
 1208 with NHEJ that resulted in a stabilizing SGFP2-CTNNB1 truncation, mutation, insertion or deletion as well as cells with the correct HDR  
 1209 resulting in the stabilizing S45F mutation). Cells with increased fluorescence were sorted as single haploid cells (see Figure 1 Supplement 1)  
 1210 and expanded for further analysis D) Graphical representation of part of the 3<sup>rd</sup> exon of SGFP2-CTNNB1 before (top) and after (bottom)  
 1211 CRISPR/Cas9-mediated mutagenesis. Sanger sequencing is shown for one correctly targeted clone. The mutation changes the 45<sup>th</sup> serine to  
 1212 a phenylalanine (S45F) and additionally removes the gRNA binding site and a silent mutation that introduces a HpaII restriction site for  
 1213 screening purposes. Mutated bases are indicated in light red. 14/32 single cell clones showed the expected genotyping fragment size and  
 1214 7/32 showed large insertions or deletions. 7/14 clones showed the expected genotyping band contained the HpaII restriction site, 3/14 did  
 1215 not. Sanger sequencing coverage for the exon3 locus was obtained for 6/7 clones and all showed correct targeting (as shown for 1 clone  
 1216 here). Five clones were further analyzed; Clone 2,3,16 and 24 are from one single transfection, clone 27 from an independent transfection.  
 1217 E) Western blot, showing SGFP2-CTNNB1<sup>WT</sup> (WT) and SGFP2 CTNNB1<sup>S45F</sup> (S45F clone) protein levels. All panels are from one blot that was  
 1218 cut at the 70 kDa mark. Top: HAP1<sup>SGFP2-CTNNB1(WT)</sup> and HAP1<sup>SGFP2-CTNNB1(S45F)</sup> clones express the SGFP2-CTNNB1 protein at the expected height.  
 1219 Bottom: alpha-Tubulin (TUBA) loading control. A representative image of n=3 independent experiments is shown. F) Quantification of  
 1220 Western blots from n=3 independent experiments, including the one in, normalized to the SGFP2-CTNNB1<sup>WT</sup> control (E), showing that the  
 1221 S45F clones express higher levels of SGFP2-CTNNB1 than their HAP1<sup>SGFP2-CTNNB1(WT)</sup> parental line, even after 24h treatment with 8 $\mu$ M  
 1222 CHIR99021. Horizontal bar indicates the mean. G) Graph depicting the results from a MegaTopflash dual luciferase reporter assay, showing  
 1223 increased levels of TCF/LEF reporter gene activation for and HAP1<sup>SGFP2-CTNNB1(S45F)</sup>, comparable to HAP1<sup>SGFP2-CTNNB1(WT)</sup> cells treated for 24 hours  
 1224 with 8 $\mu$ M CHIR99021 treatment. Data points from n=3 independent experiments are shown. Horizontal bar indicates the mean. Values are  
 1225 depicted relative to the DMSO control, which was set to 1 for each individual cell line. H)



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1227 **Figure 8 supplement 1** Live imaging of HAP1<sup>SGFP2-CTNNB1</sup> upon CHIR99021 stimulation. A) Representative stills from confocal time-lapse  
 1228 experiments corresponding to Supplementary Movies 3, showing an increase of SGFP2-CTNNB1 after treatment with 8 $\mu$ M CHIR99021. Scale  
 1229 bar = 20  $\mu$ m. B-D) Quantification of time-lapse microscopy series, using the segmentation pipeline shown in Figure 3E. Arrow indicates  
 1230 the moment of starting the different treatments (T, see legend in B for details). B-C) Graph depicting the normalized intensity of SGFP2-CTNNB1  
 1231 in the cytoplasm (B) or nucleus (C) over time. Solid lines represent the mean normalized fluorescence intensity and shading indicates the  
 1232 95% confidence interval. n=166-400 cells for each condition and time point, pooled data from n=3 independent biological experiments. D)  
 1233 Graph depicting the nuclear/cytoplasmic ratio of SGFP2-CTNNB1 over time, calculated from raw intensity values underlying (B) and (C). This  
 1234 experiment was recorded simultaneously with the data shown in Figure 3. Additional data representation of this experiment is found in Figure  
 1235 3 supplement 2 and Supplementary movies 4-6.



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**Figure 8 supplement 2** additional biophysical properties of SGFP2-CTNNB1<sup>S45F</sup> and SGFP2-CTNNB1 under CHIR99021 stimulation. The S45F mutant was introduced using CRISPR (see Figure 7 supplement 1) and CHIR treated and control cells were measured after 24 hours. Details on sample size and statistics can be found in supplementary file 1. A-B) Graph depicting the total number of SGFP2-CTNNB1 particles as measured with N&B upon S45F mutation (A) or CHIR99021 stimulation (B). C) Graph depicting the total concentration of SGFP2-CTNNB1 particles (monomeric plus complexed) as measured with FCS upon CHIR99021 treatment. D-E) Graphs depicting the speed of the second diffusion component (i.e. SGFP2-CTNNB1 containing complex) in the nucleus measured by FCS for S45F mutant (D) or 24 hour CHIR99021 treatment (E). E-F) Graph depicting the molecular brightness of SGFP2-CTNNB1 in the nucleus relative to controls as measured with N&B. EGFP monomer was used for normalization and EGFP dimer and trimer as controls for N&B measurements.

## 1245 Supplementary Movie legends

1246 **Supplementary Movie 1-3:** Representative movies of confocal time-lapse experiments, showing  
1247 SGFP2-CTNNB1 (left, green), SiR-DNA staining (middle, magenta) and transmission image (right, grey)  
1248 after treatment with vehicle control (BSA) (**Supplementary Movie 1**), 100 ng/ml WNT3A  
1249 (**Supplementary Movie 2**) or 8  $\mu$ M CHIR99021 (**Supplementary Movie 3**). Time of addition is at  
1250 00:00:00 (indicated at the top left). Scale bar in the lower right represents 20 $\mu$ m.

1251 **Supplementary Movie 4-6:** Movies showing the quantification of time-lapse microscopy series (from  
1252 Figure 4 and Supplementary Movie 1-3) at each time point showing all individual cells from 3 biological  
1253 experiments. Time of addition of the indicated substances is at 00:00:00 (indicated at the top left).  
1254 The left graph represents the raw data (colored dots, each dot is one cell, n=155-400 cells for each  
1255 condition and time point), the median (black circle) and the 95% CI of the median (black bar). The right  
1256 graph represents the median difference (black circle) from the treatments to the control (BSA). When  
1257 the 95% CI (black bar) does not overlap 0, the difference between the two conditions is significant.

1258 **Supplementary Movie 4:** Quantification of the normalized intensity of SGFP2-CTNNB1 in the  
1259 cytoplasm. Significant changes in intensity can first be observed after 40 minutes of 8  $\mu$ M CHIR99021,  
1260 and after 70-80 minutes of 4  $\mu$ M CHIR99021 or 25-100ng/ml WNT3A treatment.

1261 **Supplementary Movie 5:** Quantification of the normalized intensity of SGFP2-CTNNB1 in the nucleus.  
1262 Significant changes in intensity can be observed for all treatments (but not controls) after 20-50  
1263 minutes.

1264 **Supplementary Movie 6:** Quantification of the nuclear-cytoplasmic ratio of SGFP2-CTNNB1, calculated  
1265 from raw intensity values underlying Supplementary Movies 4 and 5. Significant changes in the  
1266 nuclear-cytoplasmic ratio can be observed for all treatments (but not controls) after 20-50 minutes.

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## 1268 Supplementary Files

1269 **Supplementary File 1:** Tables of all summary statistics (mean, median, 95% confidence intervals,  
1270 differences, p-values) of the FCS and N&B parameters show in Figure 5, 7 and 8 and accompanying  
1271 supplements.

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