The S-G2 phase enriched β-catenin/TCF complex ensures cell survival and cell cycle progression

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Abstract

Wnt/β-catenin signaling via the β-catenin/TCF complex plays crucial roles in tissue homeostasis. Wnt stimulated β-catenin/TCF complex accumulation in the nucleus regulates cell survival, proliferation, and differentiation through the transcription of target genes. Compared with those in G1, LRP6 receptor activation and cytosolic β-catenin are both up-regulated in G2 cells. However, accumulation of the Wnt pathway negative regulator, AXIN2, also occurs in this phase. Therefore, it is unclear whether Wnt signaling is active in G2 phase cells. Here, we established a bimolecular fluorescence complementation (BiFC) biosensor system for the direct visualization of β-catenin/TCF interaction in living cells. Using the BiFC biosensor and co-immunoprecipitation experiments, we demonstrated the nucleus-localized β-catenin/TCF complex increases during the S and G2 phases, and declines in the next G1 phase. Accordingly, a subset of Wnt target genes was transcribed by the β-catenin/TCF complex during both S and G2 phases. In contrast, transient inhibition of this complex disturbed both cell survival and G2/M progression. Our results suggest that in S-G2 phase cells, Wnt/β-catenin signaling is highly active and functions to ensure cell survival and cell-cycle progression.

Keywords: cell cycle/β-catenin/TCF/BiFC/cell survival

Introduction

Canonical Wnt/β-catenin signaling functions both in multiple developmental events during embryogenesis and in adult tissue homeostasis and carcinogenesis (Logan and Nusse, 2004; Moon et al., 2004; Klaus and Birchmeier, 2008; Clevers and Nusse, 2012). In the absence of Wnt ligands, levels of cytoplasmic β-catenin are fine tuned by the "destruction complex". This complex includes tumor suppressor adenomatous polyposis coli (APC), the central scaffold protein Axin, glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (CK1) as its core components (MacDonald et al., 2009). β-catenin is phosphorylated by CK1 and GSK3β at N-terminal residues S45 and S33/37/T41, respectively, followed by its degradation via the β-TrCP-ubiquitin pathway (Valenta et al., 2012; Stamos and Weis, 2013). However, In the presence of Wnt ligands, the sequestration of Dishevelled and Axin by the Frizzled/LRP5/6 receptor complex reduces the availability of the cytoplasmic destruction complex and results in the accumulation of cytosolic β-catenin (Mao et al., 2001; Zeng et al., 2005; Bilic et al., 2007; Li et al., 2012; Kim et al., 2013). Subsequently, β-catenin enters the nucleus and engages lymphoid enhancing factor/T-cell factor (LEF/TCF) family transcription factors to initiate target gene transcription (Cadigan and Waterman, 2012; Valenta et al., 2012). Canonical Wnt/β-catenin signaling functions primarily by triggering target gene transcription in which the formation of the β-catenin/TCF complex is considered a key step of Wnt signal transduction.

Since the identification of the first transcriptional Wnt/β-catenin target gene, *engrailed*, multiple approaches have been developed to explore Wnt targets, which have resulted in a Wnt targetome sketch containing hundreds of genes (DiNardo et al., 1988; Vlad et al., 2008). Notably, genes involved in cell proliferation are over-presented in the target list. In agreement with this, the promotion of cell proliferation is considered one of the most typical cellular functions of Wnt/β-catenin signaling (Logan and Nusse, 2004). Indeed, Wnt stimulates G1/S transition by activating its direct targets MYC and CCND1 (cyclin D1) as well as by down-regulating molecules that inhibit the cell-cycle such as CDKN2A (also known

as p21) (Niehrs and Acebron, 2012). Numerous studies have also demonstrated that Wnt signaling can promote proliferation in various tissues and cell types, such as neural stem cells, endothelial cells, and pancreatic β-cells (Masckauchan et al., 2005; Rulifson et al., 2007; Pei et al., 2012). In particular, Wnt signaling is required for the routine renewal cycle of intestinal and colonic tissues (Gregorieff and Clevers, 2005; Clevers, 2006). Moreover, hyper-activation of Wnt signaling resulting from APC/AXIN/β-catenin mutations triggers uncontrolled cell proliferation and eventually leads to cancer (Clevers and Nusse, 2012). Conversely, inhibition of Wnt signaling by dominant-negative TCF expression induces G1 or G2 arrest (van de Wetering et al., 2002; Vijayakumar et al., 2011).

In addition to promoting cell proliferation, components of the Wnt pathway, like AXIN2, β-catenin, GSK3β, and APC, also participate in mitotic program execution. This includes spindle assembly, microtubule rearrangement, and centrosome division (Niehrs and Acebron, 2012). During these processes, AXIN2, GSK3β, and β-catenin localize at the centrosome while APC and AXIN2 accumulate on the spindle to maintain their normal function (Huang et al., 2007; Hadjihannas et al., 2010; Chilov et al., 2011).

Cell death and survival are also regulated by Wnt signaling (Pecina-Slaus, 2010). In most reported cases, inhibition of Wnt signaling increases apoptosis while its activation protects cells from agent-induced and intracellular apoptosis, although there have been a few exceptions where Wnt activation led to cell death (Chen et al., 2001; You et al., 2002; Olmeda et al., 2003; Zimmerman et al., 2013). Many genes involved in anti-apoptotic processes, such as the IAP family members *BIRC5/7* (namely survivin and livin) were also identified as Wnt targets (Zhang et al., 2001; Huang et al., 2006; Yuan et al., 2007). These data thus establish that Wnt signaling has a key role in cell survival.

Interestingly, Wnt signaling fluctuates throughout the cell cycle. For example, the level of β-catenin protein along the course of the cell cycle is not constant, but instead is elevated in S-G2 (Olmeda et al., 2003). Moreover, the Wnt receptor LRP6 is highly phosphorylated during mitosis by cyclin Y and its related cyclin-dependent kinase (CDK14). Consistently, Wnt-responsive reporter expression was found to be higher in G2/M cells than those in G1 (Davidson et al., 2009). These data indicate that there are distinct Wnt signaling activities at different cell-cycle stages. Paradoxically, mRNA levels of Wnt target genes, such as *AXIN2*, a negative feedback regulator also known as conductin/axil, and *LGR5*, a co-receptor for R-spondin1 and a positive modulator of β-catenin signaling (de Lau et al., 2011; Glinka et al., 2011), peak at the G2/M phase, whereas mRNA of *MYC* accumulates in the G1/S phase (Rabbitts et al., 1985; Hadjihannas et al., 2012). In the same report, it was also shown that phospho-β-catenin, which should be fated to ubiquitination and degradation, was enriched during the S-G2 phase (Hadjihannas et al., 2012). From these results, it was concluded that Wnt/β-catenin signaling is relatively low in the G2 phase. These observations emphasize the complexity of the interplay between Wnt signaling and the cell cycle, furthering the controversy over which cell-cycle phase possesses the highest level of Wnt signaling. Notably, previous studies that observed the influences of cell cycle on Wnt signaling focused on upstream regulation of the Wnt pathway, while the status of the core transcriptional activation complex, β-catenin/TCF, has not yet been deciphered in the cell-cycle context. Thus, the precise dynamics of Wnt activity during the cell cycle, particularly the transcriptional regulation of Wnt targets, require further study.

To gain insight into the regulation of Wnt signaling during cell cycle, especially the status of the core transcriptional activation complex, we developed a bimolecular fluorescence complementation (BiFC) biosensor to visualize the β-catenin/TCF interaction in living cells. With the assistance of this tool, we showed that the amount of nuclear-localized β-catenin/TCF complex increases during S-G2 (S-G2 denotes S and G2 phases in this article, while, G2-M denotes G2 and M phases) and declines

when cells re-enter the G1 phase. Accordingly, a subset of Wnt target genes was found to be transcriptionally regulated by the β-catenin/TCF complex during the S-G2 phase. These results may help answer the long-standing question of why the Wnt signal is highly stimulated at this cell-cycle stage.

Results

Visualization of the β-catenin/TCF complex by BiFC

To detect β-catenin-TCF interaction in living cells, we designed a BiFC biosensor system using fragments derived from fluorescent protein Venus or yellow fluorescent protein (YFP) (Kerppola, 2006; Shyu et al., 2006). According to the crystal structure of the β-catenin/TCF3 complex (Graham et al., 2000), we fused either VN (the N-terminal half of Venus) or YN (the N-terminal half of YFP) to the N-terminus of TCF3, and either VC (the C-terminal half of Venus) or YC (the C-terminal half of YFP) to the C-terminus of β-catenin (Fig. S1A). Interaction between β-catenin and TCF3 thereby enabled reconstitution of an intact bimolecular fluorescent complex (Fig. S1A). Because aberrant activation of Wnt/β-catenin signaling may trigger apoptosis (Kim et al., 2000; Olmeda et al., 2003), the transactivation domain of β-catenin (the C-terminal 113 amino acids) was truncated to keep the transcriptional activity of β-catenin/TCF at a moderately low, if not null, level (Fig. S1B). By transient transfection, we were able to over-express our Venus or YFP biosensor systems in HeLa cells and observed BiFC signal specifically in the nucleus (left column, Fig. 1A), where the β-catenin/TCF complex forms. The Venus system enabled the visualization of protein-protein interaction directly under physiological conditions (37°C), whereas the YFP system required that the cells were pre-incubated at a lower temperature (30°C) to stabilize BiFC signals (Kerppola, 2006).

Next, we tested the specificity of the BiFC signal system. To do this, two mutant forms of TCF3, TCF3ΔNLS and TCF3ΔN, were incorporated into the BiFC system instead of wild type (WT). In TCF3ΔNLS, the nuclear localization signal was deleted, thus it was expected to be retained in the cytosol. In TCF3ΔN, the N-terminal

β-catenin binding domain was largely eliminated, thus it was expected to be unable to bind β-catenin. As shown in Fig. 1A, the β-catenin/TCF3ΔNLS complex localized exclusively in the cytoplasm, while BiFC signals derived from β-catenin/TCF3ΔN were barely detected. Moreover, consistent with their roles in β-catenin degradation and TCF binding competition (Orsulic et al., 1999), the BiFC signal derived from β-catenin and WT TCF3 was strongly reduced by co-expression of either GSK3β or E-Cadherin, respectively (upper panels, Fig. S1C). Of note, GFP expression was not changed by either treatment thus further supporting that the reduction of BiFC signal was specific (lower panels, Fig. S1C). Collectively, these results suggest that the nuclear BiFC signal originated specifically from β-catenin and TCF interactions.

β-catenin/TCF BiFC signals accumulate in the S and G2 phases of the cell cycle

Next, HeLa cell lines stably expressing doxycycline-repressible β-catenin/TCF-BiFC were generated, in which FLAG-β-catenin-VC and Myc-VN-TCF3 constructs were simultaneously induced using the pBI system (Fig. S1D) (Baron et al., 1995). A control cell line expressing inducible Venus was generated using the same strategy. Both the FLAG-β-catenin-VC and the Myc-VN-TCF3 proteins were specifically induced upon the withdrawal of doxycycline (upper panel, Fig. S1E). The level of induced β-catenin was comparable to that of endogenous β-catenin (lower panel, Fig. S1E). In these cell lines, nuclear localized BiFC signals were specifically induced by withdrawing doxycycline, but only in about 10% of cells (data not shown).

To exclude variations in integration sites and copy numbers among cells, the stable cell lines were amplified from single cells and further sorted into single clones by FACS repeatedly. Surprisingly, the BiFC signal in these cells became heterogeneous after one passage and reached a stable ratio of ~10% positivity in two to three passages. This dynamic change was not observed in the Venus stable cell line expressing the intact fluorescent protein. Thus, we speculated that this heterogeneity resulted from BiFC dynamics, and that the cell cycle might be involved.

To this end, real-time polymerase chain reaction analysis of the β-catenin/TCF-BiFC-positive and -negative cell populations revealed that the mRNA level of cyclin B1 (*CCNB1)*, a G2/M phase marker (Penelova et al., 2005), was significantly higher in the β-catenin/TCF-BiFC-positive fraction (Fig. 1B). Consistent with this observation, using DNA content profiling, an enrichment of G2/M cells was detected in the β-catenin/TCF-BiFC-positive fraction when compared with those in either the total or negative populations. Notably, both the BiFC-Venus and the BiFC-YFP β-catenin/TCF biosensor stable lines showed similar results (Figs. 1C and S2). Therefore, we hypothesized that the β-catenin/TCF-BiFC signal was enriched in G2/M cells.

To test this hypothesis, time-lapse analyses of the BiFC signals were performed. The BiFC signals were continuously monitored for 40 h by high content screening (HCS), which allows large-scale and real-time scanning. A sharp increase of the average intensity of the BiFC signal was observed before mitosis in most BiFC-positive cells (Fig. 1D, E and supplementary material Movies 1, 2). The fluorescent signals were then released from the nucleus into the whole cell with the collapse of the nuclear envelope. During anaphase and cytokinesis, BiFC signals were evenly distributed into two daughter cells, but as cells entered into the next G1 phase, the BiFC signals gradually decreased to almost undetectable levels (Fig. 1D and supplementary material Movies 1, 2). Approximately 81.6% of the BiFC-positive dividing cells exhibited ascending signal intensities before mitosis and 68.9% exhibited descending fluorescent signals after mitosis as quantified with IMARIS software (Fig. 1E). However, a few daughter cells were observed behaving differently. In these cells, upon separation the BiFC signal in one daughter cell declined gradually, while in the other cell, the signal became even stronger. Many of these brighter cells eventually died. The phenomenon that BiFC signals increased during the S-G2 phase was also confirmed using cells released from a thymidine block regime (supplementary material Movie 3). In contrast, similar changes were not observed in the Venus stable control cell line. Indeed, the average intensity of the Venus signal remained steady

during interphase and only increased when cells were rounded up for mitosis (Fig. 1D and supplementary material Movies 4, 5). As the BiFC signal originates specifically from the β-catenin/TCF complex, these results suggest that the amount of β-catenin/TCF complex fluctuates with cell-cycle progression. Specifically, it accumulates in the S phase, peaks at the G2 phase, and declines in the G1 phase.

The endogenous nuclear β-catenin/TCF4 complex is enriched during the S and G2 phases in colon cancer cells

Based on the observation that β-catenin/TCF-generated BiFC signals are dynamic during the cell cycle, we further hypothesized that there was an oscillation of the interaction between endogenous β-catenin and TCF during cell cycle. Colon cancer HCT116 cells were chosen to test our hypothesis as they bear a wild-type APC gene but have a Ser45 deletion in one β-catenin allele (Li et al., 2012). Therefore, they exhibit constitutive Wnt/β-catenin signaling and do not require ligand stimulation. This cell line was also confirmed to have an intact Wnt signaling cascade upstream of β-catenin (Li et al., 2012). TCF4 was analyzed because it is the most predominantly expressed and functional member of the TCF genes in the colonic and intestinal epithelium and is important for the proliferation of colon cancer cells (Korinek et al., 1997; Batlle et al., 2002; Najdi et al., 2009).

To test endogenous protein-protein interactions at different cell-cycle stages, we first used a thymidine block to synchronize cells at the early S phase, and then released them to progress through the full cell cycle. Cells were harvested at different time points and the degree of synchronization was analyzed by flow cytometry (Fig. 2A). Cells were then separated into two fractions: a nuclear fraction and a detergent soluble cytosolic/membrane fraction. Consistent with a previous report (Olmeda et al., 2003), western-blot experiments indicated that the amount of nuclear-localized β-catenin increased during S-G2, but quickly dropped to basal levels once the cells returned to G1 (Fig. 2B). Meanwhile, the amount of β-catenin in the cytosolic/membrane fraction stayed constant throughout the cell cycle. TCF4, conversely, was exclusively nuclear

localized; its level slightly increased when cells were released from the early S phase and thereafter kept steady (Fig. 2B).

Next, co-immunoprecipitation experiments were applied to detect interactions between β-catenin and TCF4 in the nuclear fraction during cell cycle. Significant enrichment of the β-catenin-TCF4 complex was detected during the S-G2 phase while a decline was observed as cells entered the next G1 phase (Fig. 2C). Therefore, consistent with the dynamics of the BiFC signal, the amount of endogenous β-catenin/TCF complex in HCT116 cells increases during the S phase, peaks at G2, and decreases in the G1 phase of the cell cycle.

Transcriptional targets of the β-catenin/TCF complex during the S and G2 phases of the cell cycle

Because β-catenin interacts with the LEF/TCF family of transcription factors in the nucleus to initiate target gene expression, the accumulation of the β-catenin/TCF complex in the nucleus during the S-G2 phase may imply that Wnt target genes are transcribed in this time window. Therefore, we next investigated Wnt target gene expression in S-G2 phase cells.

First, the expression levels of 515 genes that were reported to be responsive to Wnt stimulation were profiled in HCT116 cells through microarray experiments. Of these, 342 were confirmed to be expressed (Tables S1 and S2). These genes were analyzed for GO term classification using the DAVID program (http://david.abcc.ncifcrf.gov/) and were found to be mainly linked to six biological processes (Fig. 3A and Tables S3–S5). Consistent with typical functions of Wnt signaling in colon cancer cells, genes related to cell proliferation were significantly enriched in this pool. Genes related to cell death and cell cycle also exhibited enrichment greater than 10% (Fig. 3A) and were selected for further analysis due to their strong implications with the S-G2 phase. Consequently, the list was simplified to 42 genes whose promoters

contained consensus LEF/TCF binding sites and their occupancy by β-catenin or TCF/LEF was experimentally verified (Table S6).

We then set out to investigate whether the transcription of these genes was controlled by the β-catenin/TCF complex in the S-G2 phase. To do this, specific inhibitors were used to block the formation of the complex during these phases. The inhibitors needed to be supplied and effective in a relatively short time period in HCT116 cells, starting at G1/S transition and finishing at G2/M transition, approximately 8 h in total. Dominant negative TCF4 is a well-characterized inhibitor of β-catenin binding to DNA-associated TCF4 (van de Wetering et al., 2002); however, it is impossible for the Tet-On/Tet-Off inducible system to produce enough protein in such a short time window. Instead, we employed other types of inhibitors, including the small molecular inhibitor iCRT and the stapled peptide inhibitor aStAx, both of which are reported to disrupt β-catenin/TCF4 association (Gonsalves et al., 2011; Grossmann et al., 2012). For inhibitor assays, synchronized HCT116 cells were released and treated simultaneously with vehicle, iCRT, or aStAx for 8 h such that the interaction of β-catenin and TCF4 was inhibited exactly during the S-G2 phase, as shown in Fig. 3B. Importantly, upon treatment of iCRT, the mRNA expression levels of 23 of the 42 genes were significantly down-regulated ($P<0.05$) (Figs. 3C and S3A). The down-regulation of 10 of these 23 genes was further confirmed by aStAx treatment (Fig. S3B). The slight inconsistency between the two inhibitors may reflect their different capacity to inhibit Wnt-mediated transcription as measured by the TOP-Flash reporter and cell proliferation assays. Indeed, aStAx was a much weaker inhibitor than iCRT in our system (Fig. S3C–E). Collectively, our data suggest that the transcription of a subset of Wnt target genes during the S-G2 phase is at least partially mediated by the β-catenin/TCF complex.

To confirm that these genes were indeed transcribed in S-G2 cells, we also performed nascent RNA capture assays to detect newly synthesized transcripts during this phase. Briefly, HCT116 cells were synchronized and then released into iCRT- or

vehicle-containing medium for 6 h prior to harvest. EU labeling reagent was introduced at the 4-h time point to label newly synthesized mRNAs. In the presence of iCRT, statistically significant decreases in nascent transcript levels were observed for 21 of the 23 genes when compared with those in the vehicle control (Fig. 3D), indicating that the transcription of these 21 genes at the S-G2 phase is Wnt dependent. To further confirm that the β-catenin/TCF complex indeed triggers gene transcription in S-G2, chromatin immunoprecipitation (ChIP) assays were performed with G1- or G2-enriched cells. Interestingly, more β-catenin were detected bound to the promoter regions of several Wnt target genes, such as *AXIN2*, *AURKA*, and *CCND1*, in G2 phase cells when compared with those from G1, as analyzed by real-time PCR using ChIP-enriched DNA fragments (Fig. 3E). In contrast, the amount of bound TCF4 remained constant at the two phases (Fig. S3F). These results are consistent with previous reports that TCF binds to target genes constantly while β-catenin binding determines gene transcription (Cadigan and Waterman, 2012). Together, these results strongly suggest that the β-catenin/TCF complex triggers the expression of a subset of Wnt target genes at the S-G2 phase during cell-cycle progression.

β-catenin/TCF complex-mediated target gene expression ensures G2/M progression

We observed that some genes required for G2/M progression, such as *CDC25A*, *AURKA*, and *SMC2*, were down-regulated by Wnt inhibition. This suggests that Wnt signaling is essential for cell-cycle progression through the G2/M phase. To test this hypothesis, HCT116 cells were released from a G1/S block into fresh medium or medium supplemented with iCRT and cell-cycle progression was monitored for the following 24 h. The vast majority of control cells transited into G2 phase after 4 h; exited mitosis and returned to the next G1 phase at the 10-h time point; and then entered the S phase after 13.5 h (left, Fig. 4A). Accordingly, the levels of p-H3 (phospho-Histone 3), a specific mitotic marker, and CCNB1, peaked as the cells entered mitosis and fell when the process was completed (left, Fig. 4B). The phosphorylation level of CDK1 at Tyr15 (p-Tyr15), which represents the inactivation

of CDK1 (Norbury et al., 1991), decreased after 8 h during the G2/M transition without affecting the abundance of CDK1 (left, Fig. 4B). However, cells that were released into iCRT-containing medium progressed through S phase with a slight delay and transited into G2 phase after 6 h. Division proceeded after 10 h and reentry into the next G1 phase happened after just 13.5 h of incubation. The cells were then mostly arrested in G1 phase until 23.5 h (right, Fig. 4A). Consistent with the DNA content changes, lagged p-H3 and weakened CCNB1 accumulation during G2 phase were observed. Moreover, CDK1 phosphorylation only started to decrease after 12 h in iCRT-treated cells (right, Fig. 4B).

To more pertinently verify the effects of iCRT on G1 and G2 cells, they were treated at either the S-G2 phase or after cells entered the next G1 phase. As shown in Fig S4A, when iCRT was added to the released cells immediately after the thymidine block and then removed 8 h later, a delay in G2/M progression was observed (compare 10-h time points in S4A1 and S4A3), however, many cells reentered the next S and M phases after iCRT was removed (compare 20-h time points in S4A3 and S4A2). When iCRT was added only 9 h after the release (to coincide with S-G2 phase entry), the entry of cells into S phase was significantly delayed (compare 14- to 18-h time points in S4A1 and S4A4). These results suggest that, 1) the iCRT treatment did not cause any permanent damage to the cell-cycle machinery in S-G2 cells; 2) the Wnt inhibition of S-G2 phase cells induced a delay of G2/M transition; 3) the Wnt inhibition of G1 phase cells induced a delay of G1/S transition; and 4) the G1 arrest observed in S4A2 required Wnt inhibition at both the G1 and G2 phases.

Further studies focused on monitoring how iCRT-treated cells entered and proceeded through mitosis. At 7.5 h, delayed centrosome separation was observed together with later mitosis entry (Fig. 4C). Unlike control cells that entered mitosis simultaneously, iCRT-treated cells exhibited a much broader time window of mitosis entry, starting anywhere from the 8- to 14-h time point (Fig. S4B). The amount of p-H3 remained at a high level during this period (right, Fig. 4B). Notably, despite the delayed entry, the

crucial mitotic processes including DNA condensation, chromosome alignment and segregation, and cytokinesis proceeded successfully (Fig. S4C). Thus, these results suggest that iCRT-treatment delays G2/M transition but does not affect mitosis. Similarly, aStAx treatment also induced a delay of G2/M progression concomitant with a lag in p-H3 accumulation, as well as delayed G1 entry (Fig. 4D).

Taken together, these observations suggest that the disruption of the β-catenin/TCF complex in HCT116 cells results in a significant delay of G2/M transition, in accompany with a lag in accumulation of CCNB1, H3 phosphorylation, activation of Cdk1, and delayed centrosome separation. These results indicate that β-catenin/TCF-mediated transcription is essential for G2/M progression in Wnt-dependent cancer cells.

β-catenin/TCF complex-mediated target gene expression ensures cell survival during S and G2/M phases

One important function of Wnt/β-catenin signaling in cancer cells is to protect against apoptosis (Pecina-Slaus, 2010). We observed that the transcription of some anti-apoptotic genes was controlled by the β-catenin/TCF4 complex during the S-G2 phase, thus we hypothesized that in addition to cell-cycle progression, another function of β-catenin/TCF signaling during the S-G2 phase is to maintain inhibition of apoptosis.

To test this, caspase-3/7 activity, an indicator of cell apoptosis, was monitored in HCT116 cells following exposure to iCRT and was found to be enhanced in a dose-dependent manner (Fig. S4D). To further explore the relationship between apoptosis and β-catenin/TCF, doxorubicin (DOX, a typical chemotherapy drug that acts via the DNA damage process) and Purvalanol A (NG 60, a potential chemotherapy drug that functions via CDK1 inhibition) (Goga et al., 2007) were chosen to further induce apoptosis in HCT116 cells, and the effects of their combination with iCRT were examined. Under these settings, while caspase-3/7

activity and apoptosis-related PARP1 cleavage were modestly increased by DOX or NG 60 treatment alone, addition of iCRT greatly enhanced the apoptotic profile (Fig. S4E–H). These results therefore confirm that like other Wnt signaling inhibitors, iCRT sensitized HCT116 cells to apoptosis induced by either DOX or NG60 treatment at low concentrations.

Next, we addressed whether inhibition of the β-catenin/TCF complex could enhance apoptosis in S-G2 cells (Fig. 5A). Compared with asynchronous cells, incubation of synchronized cells during the S-G2 phase with iCRT resulted in stronger caspase-3/7 activation (Fig. 5B). This observation suggests that the cells at the S-G2 phase were highly sensitive to Wnt inhibition. Moreover, iCRT treated S-G2 phase HCT116 cells were more sensitive to apoptosis induced by DOX or NG 60, as indicated by increased caspase-3/7 activity and enhanced PARP1 cleavage (Fig. 5A, C–F).

It has been shown that Wnt/β-catenin signaling suppresses apoptosis by inhibiting cytochrome c release from the mitochondria (Chen et al., 2001). Because cytochrome c release is a more upstream and specific event during the apoptotic process, we wondered whether it could be affected by iCRT in S-G2 cells. As shown in Fig. 5G, synchronized S-G2 cells were treated with either DMSO or iCRT in combination with either DOX or NG 60, then, cytochrome c distribution was determined by immunofluorescence staining. In control (DMSO treated) cells, only punctate staining of cytochrome c was observed, indicative of mitochondrial localization. However, in iCRT-treated cells, diffuse staining was observed, indicating the release of cytochrome c from the mitochondria into the cytosol. Furthermore, iCRT significantly enhanced either DOX- or NG 60-induced cytochrome c release (Fig. 5G, H).

These data are therefore consistent with the analysis of target gene expression shown earlier, as anti-apoptotic genes such as *ID2, BIRC5, HELLS, MCTS1, TERT,* and *SMC2* were all significantly down-regulated upon the disruption of the β-catenin/TCF4 complex (Fig. 3C, D). Taken together, these results indicate that

Wnt/β-catenin signaling promotes cell survival during the S-G2 phase, and probably also in the following mitosis, and thereby contributes to Wnt-dependent cancer cell resistance to chemotherapeutic drug treatment (Chikazawa et al., 2010; Abdullah and Chow, 2013) through the transcriptional activation of anti-cell death-related genes.

Discussion

Using BiFC technology and co-IP of endogenous proteins, we discovered that the β-catenin/TCF complex accumulates during the S-G2 phases and declines in the G1 phase, of the cell cycle. As the transcriptional executor of Wnt signaling, the β-catenin/TCF complex may regulate the expression of target genes, either all of them or just a subset, in S-G2 cells. Indeed, by transiently blocking the formation of this complex with small molecule inhibitors during the S-G2 period, we observed down-regulation of a group of Wnt target genes. Moreover, newly synthesized mRNAs of these genes were detected at the S-G2 stages, and their levels were reduced upon inhibition of β-catenin/TCF complex. Finally, we showed that β-catenin and TCF indeed bound to the promoter regions of target genes at G2 phase. At the cellular level, transient inhibition of the β-catenin/TCF complex disturbed both cell survival and G2/M progression. Our results thus provide the first characterization of Wnt signaling-regulated target gene transcription in S-G2 phase cells. They also potentiate the future dissection of mechanisms involving cell-cycle-mediated regulation of Wnt signaling.

G1 *vs* G2 Wnt Signaling

Whether Wnt signaling reaches a peak in G1 or G2/M phase has been controversial. In 1999, the cytoplasmic level of β-catenin protein was found to increase from the G1 to the S phase and it was suggested that Wnt/β-catenin signaling might function during G1/S transition (Orford et al., 1999). CDC20-mediated degradation of the Wnt inhibitor AXIN2 during G1/S was also observed, further supporting a possible peak of Wnt during this time window (Hadjihannas et al., 2012). However, the level of soluble β-catenin, which is not associated with the cytoskeleton and plasma

membrane, in synchronized cells was found at the maximal level in G2/M cells (Olmeda et al., 2003). Furthermore, G2/M-specific cyclin Y/CDK14 imposed priming phosphorylation on the Wnt receptor LRP6, again suggesting a G2/M peaked Wnt signaling (Davidson et al., 2009). The discrepancy concerning the expression levels of Wnt target genes during different cell-cycle stages has further added complexity to this issue, for example, *MYC* was found to be elevated at G1 while *LGR5* and *AXIN2* were up-regulated at G2 (Hadjihannas et al., 2012). Here, we directly focused on the final executor of canonical Wnt signaling, the nuclear β-catenin/TCF complex, and observed its enrichment in S-G2 cells. We further confirmed that Wnt target genes were transcribed in this cell-cycle phase. Our results thus support the conclusion that Wnt/β-catenin signaling is highly active in G2 phase cells. Although these findings were obtained from a cell culture system, our conclusion is strongly supported by the reported observation that hyper-activation of Wnt/β-catenin signaling occurs in G2-arrested cells in the crypt region of mouse intestine (Lee et al., 2009).

In HeLa cells, in which we established the BiFC system, Wnt signaling is relatively low, whereas in colon cancer HCT116 cells, in which we verified the amount of endogenous β-catenin/TCF complex, Wnt signaling is over-activated, largely owing to the accumulation of mutant β-catenin proteins. In either case, Wnt ligand was not significantly involved, if at all. Moreover, hyper-activation of LRP6 phosphorylation by G2/M kinase seems to happen only in the G2/M phase while the accumulation of the β-catenin/TCF complex begins earlier. Therefore, it seems that cells have an intrinsic mechanism to control the oscillation of basal β-catenin/TCF complex levels, which is still maintained when the overall level of β-catenin is elevated in colon cancer cells. The capacity that cells produce or allow a higher level of β-catenin/TCF complex during the S-G2 phase may therefore provide a basis for the ligand stimulation during the G2/M phase, as observed by Davidson and colleagues (Davidson et al., 2009). The accumulation of cytosolic β-catenin during the S-G2 phase may thus result in the elevation of S33/37/T41 phosphorylated β-catenin in SW480 colon cancer cells (Hadjihannas et al., 2012). We also noted that the

oscillation pattern of the level of nuclear β-catenin and that of the β-catenin/TCF complex were similar, though not identical (Fig. 2B, C). This is likely because the two phenomena are inter-dependent, as TCF binding could protect β-catenin from being shuttled into the cytosol and subsequently degraded (Lee et al., 2001). In other words, increased TCF binding could lead to accumulation of β-catenin itself.

The mechanism leading to the oscillation of the β-catenin/TCF complex along the cell cycle is not clear but it seems more likely that the β-catenin/TCF complex *per se* possesses regulatory hot spots. Indeed, post-translational modifications of both β-catenin and TCF have been repeatedly reported to influence their localization, complex formation, and target gene activation (Cadigan and Waterman, 2012; Valenta et al., 2012; Gao et al., 2014).

S-G2 specific functions of the Wnt/β-catenin signaling

A role of Wnt/β-catenin signaling in triggering the G1/S transition and cell proliferation has been elaborated and believed to occur largely via induction of *MYC* and *CCND1* (Schweinfest et al., 1988; Resnitzky et al., 1994). Here, we show that the β-catenin/TCF transcriptional complex is enriched in G2 cells, thus pinpointing the existent of Wnt target gene transcription in this cell cycle phase. We further confirm that in colon cancer cells, in which Wnt signaling is highly active and important for cell-cycle progression, inhibition of β-catenin/TCF complex formation reduces the expression of key genes responsible for G2/M transition and causes cell-cycle delay. Among the significantly down-regulated genes were *AXIN2*, *CDC25A*, and *AURKA*, all of which are important in the G2/M phase of the cell cycle. *AXIN2*, the most dramatically down-regulated target gene, is required for centrosomal cohesion (Hadjihannas et al., 2010). *CDC25A* is essential for mitotic entry. Indeed, G2 delay is caused by small interfering RNA to *CDC25A*, and cells microinjected with small interfering RNA to *CDC25A* exhibit delayed commencement of centrosome separation (Lindqvist et al., 2005). *AURKA* is required for centrosome maturation and accurate chromosome segregation, whereas its depletion delays mitotic entry (Liu and

Ruderman, 2006). Together, our results suggest that β-catenin/TCF complex-triggered target gene expression in the S-G2 phase contributes to Wnt-mediated cell-cycle progression. Consistently, Wnt signal promotes cell survival by overcoming G2/M arrest induced by prolonged microtubule disruption (Aoki et al., 2007). Also, Wnt inhibition causes G2 arrest in osteosarcoma (Leow et al., 2010) and colorectal tumor cells (Sekiya et al., 2002; Shan et al., 2009).

Promoting cell survival is another profound function of Wnt signaling (Khan and Bendall, 2006; Lento et al., 2013). Here, we show that the cells in the S-G2 phase are more sensitive to Wnt inhibition-induced apoptosis than those unsynchronized. This was concomitant with the down-regulation of known genes related to cell survival, such as *ID2*, *BIRC5*, *HELLS*, *TIAM1*, *MCTS1*, *TERT*, and *SMC2*. TIAM1, a guanine nucleotide exchange factor, has been shown to enhance resistance to anoikis (apoptosis due to detachment) in SW480 colon cancer cells and thereby promotes cell survival and tumor metastasis (Minard et al., 2006). BIRC5 (commonly known as Survivin) is a typical anti-apoptotic factor in colon tissues and its increased expression positively correlates with tumor survival (Vaziri et al., 2005; Hernandez et al., 2011). Given that cells require more protection against DNA damage-caused apoptosis during the S-G2 phase (Bartek et al., 2004; Huang et al., 2005), promoting the expression of anti-apoptotic genes during this period may present a fundamental function of Wnt/β-catenin signaling.

Undoubtedly, Wnt signaling has many more cellular functions, for example, control of cell differentiation, cell migration, cancer metastasis, and even energy homeostasis (Rao and Kuhl, 2010; Elghazi et al., 2012; Ouyang et al., 2013). How these activities are coordinated with cell-cycle progression is largely unknown. A comprehensive understanding of β-catenin/TCF-regulated target gene expression along the cell cycle will be extremely important in the exploration of these relationships and ChIP-seq analysis in synchronized cells could be informative.

Understanding the functions of cellular signals at a particular cell-cycle stage is emerging as a field of considerable research interest. For example, a recent report demonstrated that complex regulation of the cell cycle impacts TGFβ-Smad signaling, which in turn influences stem cell differentiation (Dalton, 2013; Pauklin and Vallier, 2013). This study and our current work highlight restrictive activities of cells to the extracellular signals imposed on them. It seems that the cellular function of a particular cell signal is not only cell type-dependent but also cell cycle stage-dependent.

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Author Contributions

YD and SS performed most of the experiments and analyzed the data with contributions from XZ, GZ, and JL. WT and SC were involved in BiFC construction. W Wei suggested the use and provided the Tet and pBI systems. YG and LL synthesized the Stapled peptide aStAx-35R. YGC and W Wu supervised the study; and YD, SS and W Wu wrote the manuscript.

Conflict of Interest

All authors declare no conflicts of interest.

Materials and methods

Cell Culture, transfection and luciferase reporter assay

Colorectal cancer cell HCT116 was grown in MCCOY's 5A medium. HEK293T and HeLa-tTA cells were grown in Dulbecco's modified Eagle's medium. Transfections and Luciferase reporter assays were performed as described (Zhu et al., 2012).

Antibodies and Reagents

Primary antibodies β-tubulin (KM9003) and GAPDH (KM9002) were purchased from Sungene. β-actin (M20010) was from AbMart. FLAG M2 (F3165) and γ-tubulin (T3559) was from Sigma. Myc (sc-40), Cyclin B1 (sc-245), CDK1 (sc-54), PARP1 (sc-8007) were from Santa Cruz Biotechnology. β-catenin (610154) was from BD Biosciences. TCF4 (2953), Histone H3 (9715), Lamin A/C (4777), γ-H2AX (2577), Cleaved Caspase-3(9664), phospho-CDK1 (9111) were from Cell Signaling Technology. Phospho-histone H3 (Ser10) (06-570) was from Upstate. Cytochrome c (AC909) was from Beyotime.

Small molecules iCRT14 (SML0203), Doxorubicin hydrochloride (D1515, Sigma) and Purvalanol A (NG 60, P4484) were purchased from Sigma. Stapled peptides aStAx-35R (Referred as aStAx in this study) was synthesized accordingly (Grossmann et al., 2012).

BiFC

Bimolecular Fluorescence Complementation (BiFC) assays were constructed accordingly (Kerppola, 2006). Venus fluorescence protein was selected as reporter for complementation. Human β-catenin (amino acid 1-667) and *Xenopus* TCF3 (full length) were respectively fused with the carboxyl- (VC, amino acid 155-239) and amino- (VN, amino acid 1-173) terminal of Venus and subcloned into the MCS of pBI bidirectional vectors (Clontech). HeLa-tTA cells transfected with the bidirectional constructs were further selected under G418 treatment to get stable lines and applied as BiFC cells. For YFP-based BiFC, YC (amino acid 159-240) and YN (amino acid

1-158) were used. The linker sequence is GGT AGT GGT AGT GGT AGT.

Immnoprecipitation (IP), Immunofluorescence (IF) and Chromatin immunoprecipitation (ChIP)

Subcellular fractionation was performed according to GeneTex protocol with several necessary changes (GeneTex, 2011). Nuclear fraction obtained was incubated with corresponding antibody and protein A/G beads to enrich target proteins. The beads-bound proteins were then eluted and subjected to immunoblotting. Band intensities were quantified with Image J software (http://imagej.nih.gov/ij/)(Schneider et al., 2012).

Immunofluorescence was performed as described (Liang et al., 2011).

Chromatin immunoprecipitation were performed as described (Yochum et al., 2007) with minor modification. β-catenin antibody (9562), mouse TCF4 antibody (2953), normal Rabbit IgG (2729) and mouse IgG (5415) were purchased from Cell Signaling Technology. Unlabeled goat anti-rabbit IgG (01-15-06, KPL) was used in β-catenin ChIP. All precipitated DNA were quantified by real-time PCR. Data are presented as the percentage of input.

Flow Cytometry and Apoptosis assay

HCT116 cells were synchronized with 2 mM thymidine (Sigma) for 20 h and then released into fresh medium. FACS analysis was carried on BD FACS Calibur (BD Biosciences). Cell sorting was performed on BD FACS Aria III (BD Biosciences). Data were further analyzed with ModFit and FlowJo. H3P-FITC staining was performed as described (Widrow and Laird, 2000). Apoptosis level was detected using Apo-One Homogenous Caspase-3/7 Kit (Promega).

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated with EZ-10 DNAaway RNA Mini-Preps Kit (BS88133,

Sangon Biotech) and cDNA was made using the Reverse Transcription System (Promega). Nascent RNA capture was done with Click-iT Nascent RNA capture Kit (Invitrogen).

ChIP-enriched DNA fragment and cDNA were analyzed with real-time PCR on ABI StepOne Plus instrument (Life technology). Primer sequences were listed in supplementary material Table S7.

Wnt Target gene list and bioinformatics

Wnt target gene list was prepared through an extensive review of published papers and resources including Wnt homepage (http://Wnt.stanford.edu). For details, see supplementary material Table S1. Gene annotation was performed on DAVID (http://*david.abcc.ncifcrf.gov/*).

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Figure Legends:

Fig. 1. β-catenin/TCF-BiFC signal accumulates during the S-G2 phases

A. HeLa cells expressing the β-catenin/TCF BiFC biosensors. β-catenin-VC (β-catenin) was co-transfected with VN-TCF3 (TCF3), VN-TCF3ΔNLS (TCF3ΔNLS) or VN-TCF3ΔN (TCF3ΔN). The third row shows the enlarged view of the corresponding boxed regions. Bar = 50 μm.

B. Real-time PCR analysis of BiFC-positive and -negative cells with indicated genes. Values were normalized to RPLP0 mRNA. ACTB (β-actin) was used as a control. Error bars represent SD. (n = 3).

C. Flow cytometry analysis of DNA content and GFP signal intensity in live BiFC cells stained with the cell-permeable DNA dye Hoechst 33342. The DNA profiles of the total, BiFC-positive, or -negative gated populations are shown.

D. **Left**: Dynamics of the BiFC and Venus signal during the cell cycle progression. Images were acquired every 30 min for a total of 40 h. Images revealed by fluorescence and phase contrast microscopy are shown respectively. **Right**: Quantification of the average fluorescence intensity of the BiFC and Venus signal during the cell cycle progression. Ana. onset: the onset of anaphase. NEBD: nuclear envelop breakdown.

E. Statistics of the BiFC dynamics during cell cycle shown in (D). 81.6% of the BiFC-positive dividing cells showed ascending signals before mitosis (n=38) and 68.9% showed descending trends after mitosis (n=61). Quantification of the average florescence intensity was analyzed by IMARIS software.

Fig. 2. The endogenous nuclear β-catenin/TCF4 complex is enriched during the S-G2 phases

A. Flow cytometry analysis of HCT116 cells that were synchronized by thymidine block, and then released at the indicated time points. The stage of the cell cycle was analyzed by propidium iodide (PI) staining and flow cytometry.

B. **Left**, Western blot analysis of cell lysates from samples harvested in panel A. The nuclear and cytosol/membrane fractions were separated. **Right**, nuclear β-catenin,

TCF4 and cytosol/membrane β-catenin levels were normalized to that of PARP1 or GAPDH, respectively. Quantifications from the 6 time points were further normalized to that of time 0 h. Graphs shown in B and C are quantification results from corresponding Western blotting photos. Band intensities were quantified with Image J software.

C. **Left**, Western blot analysis of nuclear extracts (input) or immunoprecipitates (IP) with the TCF4 antibody or a mouse IgG as control from cell samples harvested in panel A. **Right**, Immunoprecipitated β–catenin levels were normalized to that of the corresponding immunoprecipitated TCF4. Quantifications from all 6 time points were further normalized to that of time 0 h.

Fig. 3. Identification of transcriptional targets of the β-catenin/TCF complex during the S-G2 phases

A. Major categories of biological processes enriched in Wnt target genes in HCT116 cells. Significantly enriched processes were selected by the raw p-value and false discovery rate method (FDR) corrected p-value at thresholds of 0.05 and 0.1, respectively. The items highlighted in red were selected for further verification.

B. The experimental scheme of Wnt target verification during the S-G2 phase. After a 20 h thymidine block, HCT116 cells were released into medium containing DMSO, iCRT or aStAx for a 8 h treatment (precisely in S-G2) and then harvested for the assay shown in panel C.

C. Down-regulation of Wnt target genes during the S-G2 phase upon iCRT treatment. The expression level of genes involved in cell death and cell cycle progression was determined by real-time PCR. Values were normalized to RPLP0 mRNA. The results were the average of triplicate data; the SD and p-values were calculated in a two-tailed student t-test. Genes shown here are those that could be repeatedly down-regulated by iCRT in three independent assays; the full dataset of 42 genes is shown in supplementary material Fig. S3A.

D. Inhibition of nascent mRNA synthesis of Wnt target genes upon iCRT treatment during the S-G2 phase. iCRT was added as shown in panel B. EU was added at the 4 h

time point. Labeling was terminated at the 6 h point and cells were harvested for total RNA isolation. On-bead cDNA were prepared and examined using quantitative PCR. The results are the average of triplicate data; the SD and p-values were calculated in a two-tailed student t-test.

E. Chromatin immunoprecipitation assays in G2- or G1-enriched HCT116 cells (5 h or 12.5 h after release from the thymidine block). Promoter regions of *AXIN2*, *CCND1* and *AURKA* were tested and the promoter of ACTB (β-actin) was used as a control. All graphs show the average with SD. (n = 3). The cell cycle status is also shown.

Fig. 4. β-catenin/TCF complex-mediated target gene expression ensures G2/M progression.

A. Delay of G2/M progression by iCRT treatment. Synchronized HCT116 cells were released into medium containing either DMSO (control) or 50 μM iCRT. Cells were harvested at the indicated time points and their cellular DNA content was determined by PI staining and flow cytometry. Asyn: asynchronous cells.

B. Western blot analysis of cell extracts from samples as shown in panel A, using indicated antibodies.

C. HCT116 cells 7.5 h after release as in panel A were stained with DAPI (blue), p-H3 (Ser10-histone H3 phosphorylation, red) or γ-tubulin (centrosome, green) after treatment with DMSO or iCRT. Bar = 10 μm.

D. Delay of G2/M progression by aStAx treatment. Synchronized HCT116 cells were treated with DMSO or aStAx following release. The mitotic index was assessed by p-H3(Ser10-histone H3 phosphorylation) and PI staining using FACS at the indicated time points.

E. Percentage of p-H3 positive cells (boxed in panel D) after DMSO or aStAx treatment at indicated time points.

Fig. 5. β-catenin/TCF complex-mediated target gene expression ensures cell survival during S and G2/M phases

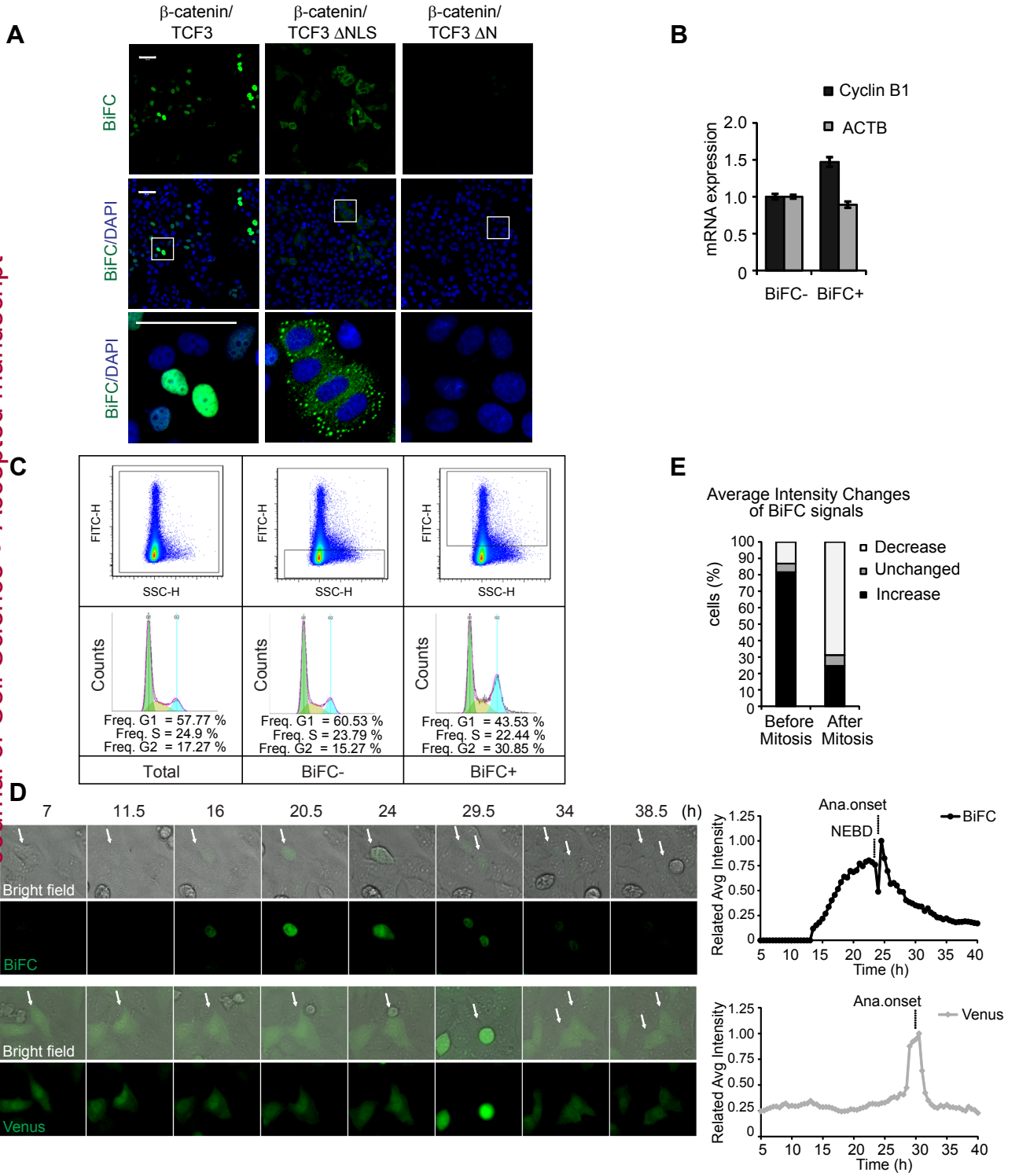
A. Schematic representation of the experiments shown in B-F. Thymidine-synchronized HCT116 cells were released and treated with DMSO or iCRT in combination with DOX or NG 60 for 8 h and then Caspase-3/7 activity and PARP1 cleavage were determined.

B. S-G2 phase cells were more sensitive to Wnt inhibition. iCRT treatment induced stronger intrinsic apoptosis in synchronized S-G2 cells in comparison to asynchronous cells. Caspase-3/7 activity was determined and the results are the average of triplicates. Asyn: asynchronous cells

C-F. iCRT (50 μM) sensitized S-G2 cells to Doxorubicin- (DOX, 1 μM) or NG 60- (5 μM) triggered apoptosis. Synchronized cells were treated during their S-G2 phase, as indicated. Apoptosis was detected by Caspase-3/7 activity and PARP1 cleavage. In C and E, the results are the average of triplicates. In D and F, arrows indicated the cleaved PARP1.

G. Thymidine-synchronized HCT116 cells were released into medium containing DMSO (for 8 h), iCRT (for 10 h), DOX (for 12 h), DOX + iCRT (for 12 h), NG 60 (for 12 h), NG 60 + iCRT (for 12 h) as indicated. The cells were then fixed and stained with DAPI (blue) and cytochrome c antibody (green). To ensure cells were treated during their S-G2 phases and harvested at G2 phase, different time periods were applied. Bar = 20 μm.

H. Quantification for cells with diffusing cytochrome c staining in panel G.



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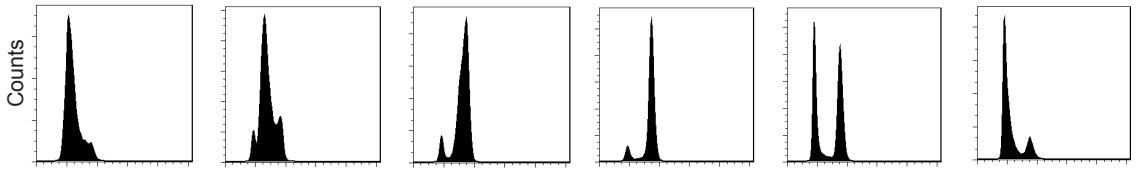
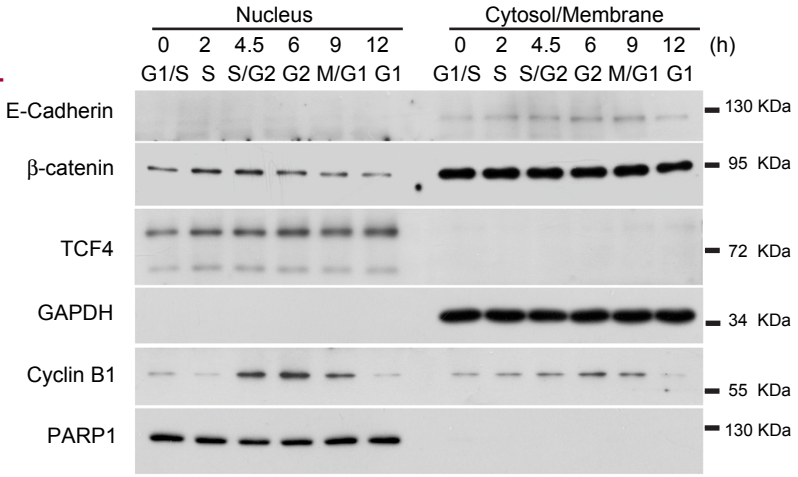
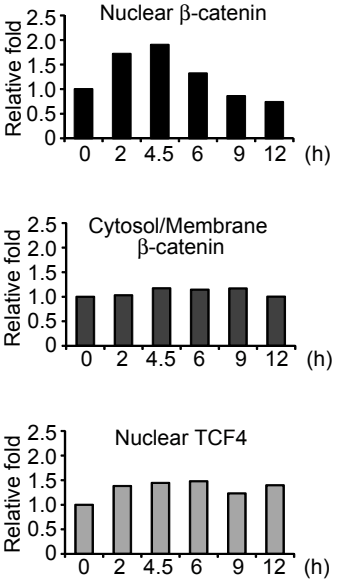


	0	2	4.5	6	9	12
G1	59.32%	8.07%	6.36%	7.11%	37.68%	66.26%
S	36.40%	84.90%	21.45%	13.06%	15.21%	22.45%
G2/M	4.28%	7.03%	72.19%	79.83%	47.11%	11.29%

B





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