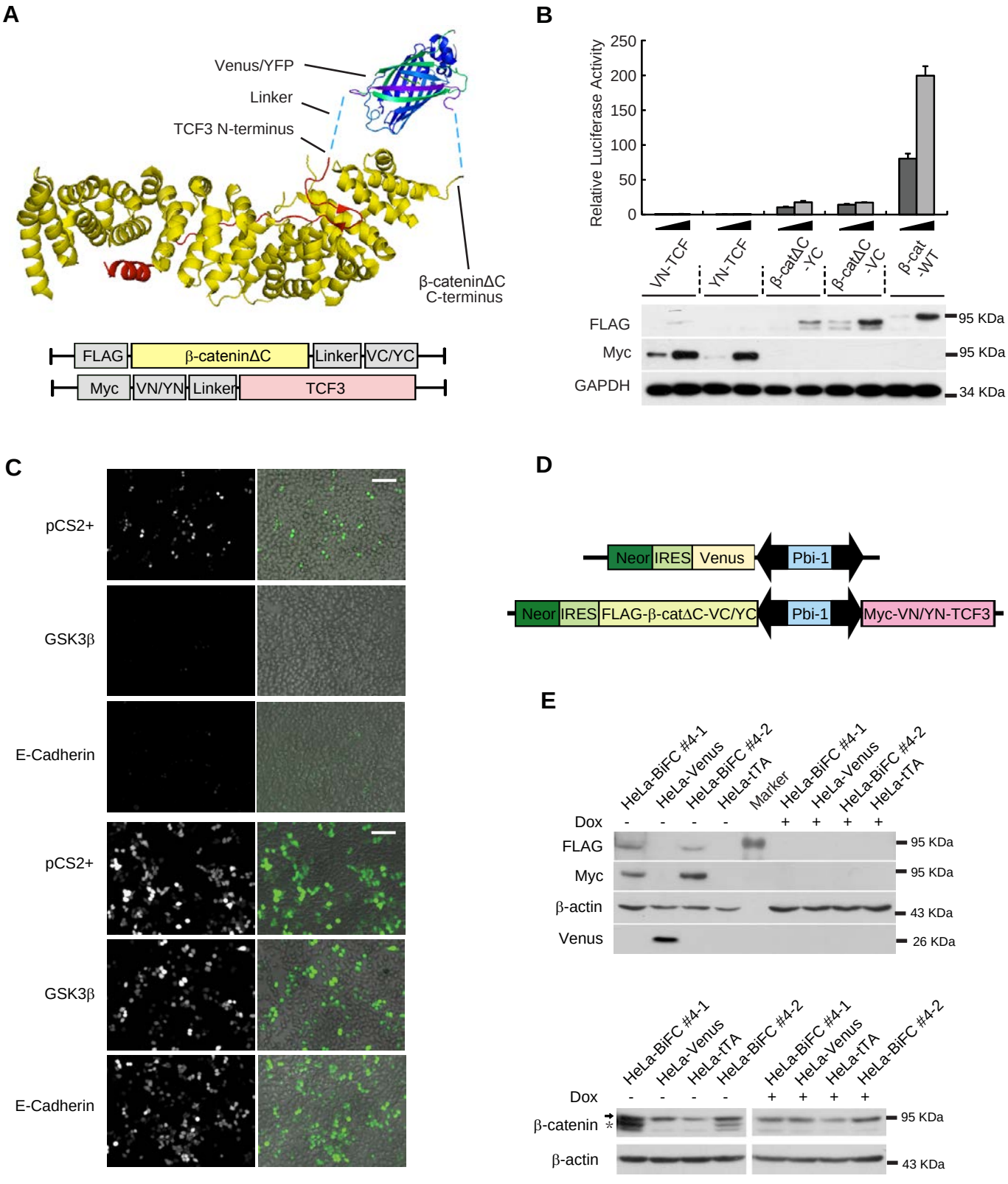
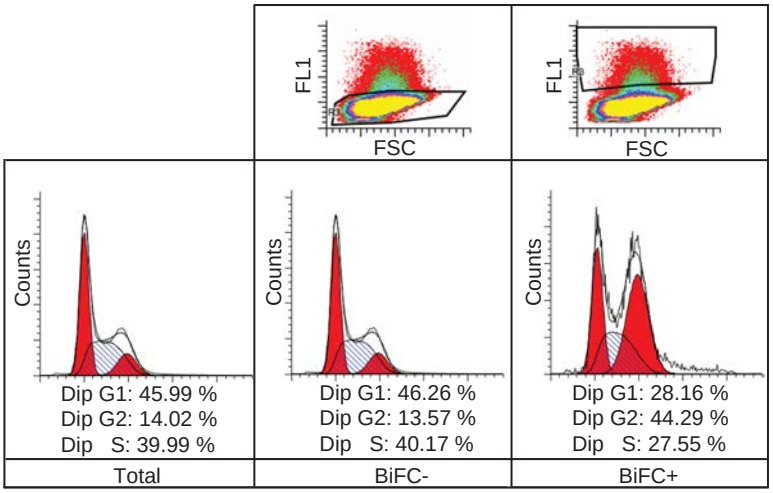
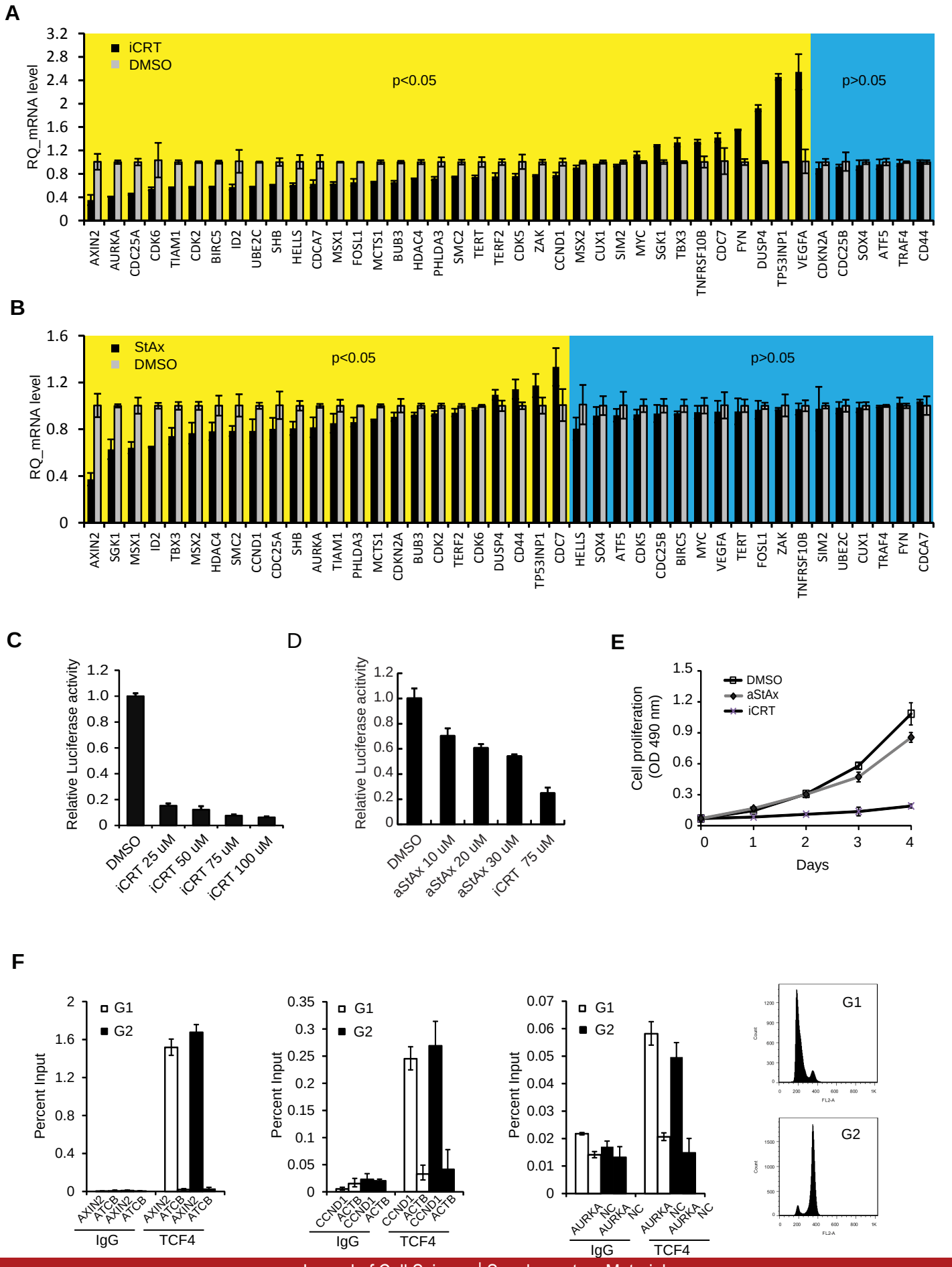
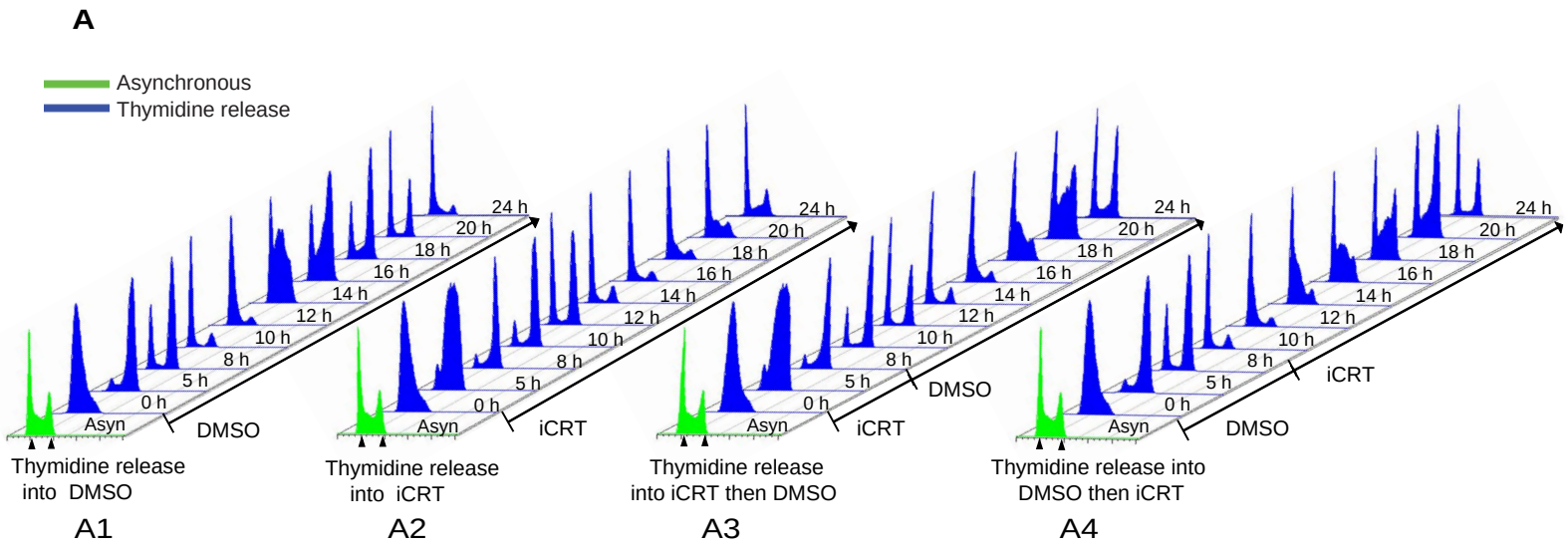
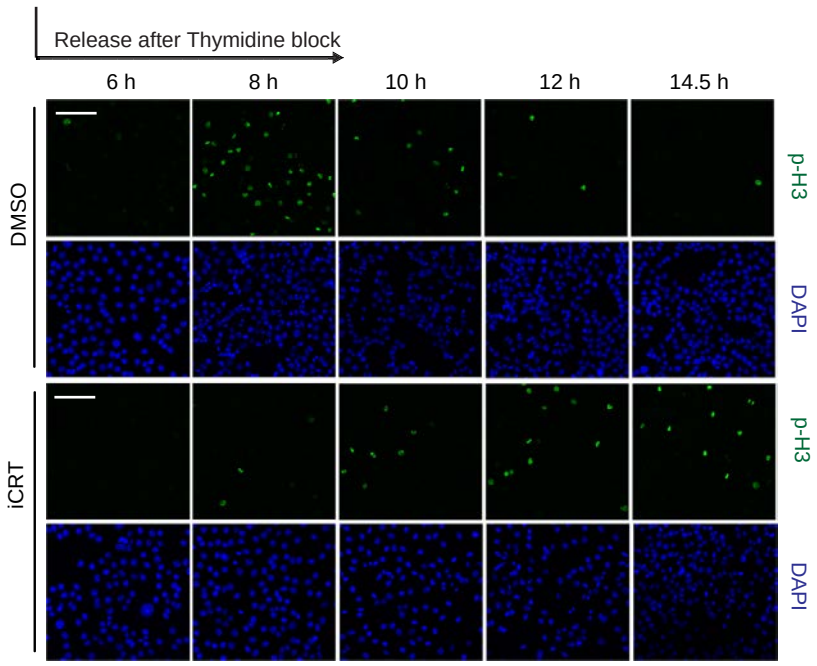
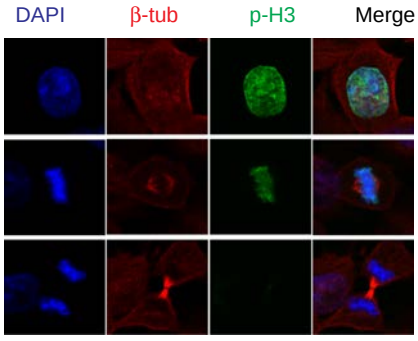

A
Venus/YFP
Linker
TCF3 N-terminus
β-cateninΔC
C-terminus
FLAG
β-cateninΔC
Linker
VC/YC
Myc
VN/YN
Linker
TCF3
B
Relative Luciferase Activity
250
200
150
100
50
0
VN-TCF
YN-TCF
β-catΔC -YC
β-catΔC -VC
β-cat -WT
FLAG
Myc
GAPDH
95 KDa
95 KDa
34 KDa
C
pCS2+
GSK3β
E-Cadherin
pCS2+
GSK3β
E-Cadherin
D
Neor
IRES
Venus
Pbi-1
Neor
IRES
FLAG-β-catΔC-VC/YC
Pbi-1
Myc-VN/YN-TCF3
E
HeLa-BiFC #4-1
HeLa-Venus
HeLa-BiFC #4-2
HeLa-tTA
Marker
HeLa-BiFC #4-1
HeLa-Venus
HeLa-BiFC #4-2
HeLa-tTA
Dox
- - - - + + + +
FLAG
Myc
β-actin
Venus
95 KDa
95 KDa
43 KDa
26 KDa
HeLa-BiFC #4-1
HeLa-Venus
HeLa-tTA
HeLa-BiFC #4-2
HeLa-BiFC #4-1
HeLa-Venus
HeLa-tTA
HeLa-BiFC #4-2
Dox
- - - - + + + +
β-catenin
*
β-actin
95 KDa
43 KDa

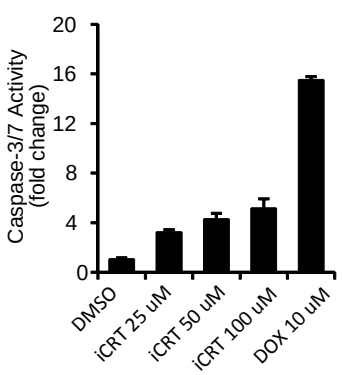

FL1
FSC
FL1
FSC
Counts
Dip G1: 45.99 %
Dip G2: 14.02 %
Dip S: 39.99 %
Total
Counts
Dip G1: 46.26 %
Dip G2: 13.57 %
Dip S: 40.17 %
BiFC-
Counts
Dip G1: 28.16 %
Dip G2: 44.29 %
Dip S: 27.55 %
BiFC+

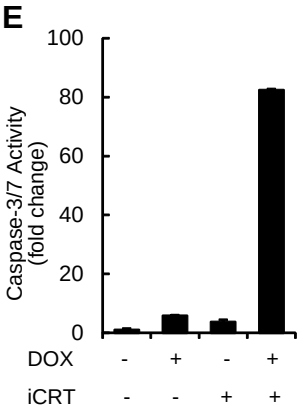

A
RQ_mRNA level
iCRT
DMSO
p<0.05
p>0.05
AXIN2 AURKA CDC25A CDK6 TIAM1 CDK2 BIRC5 ID2 UBE2C SHB HELLS CDCA7 MSX1 FOSL1 MCTS1 BUB3 HDAC4 PHLDA3 SMC2 TERT TERF2 CDK5 ZAK CCND1 MSX2 CUX1 SIM2 MYC SGK1 TBX3 TNFRSF10B CDC7 FYN DUSP4 TP53INP1 VEGFA CDKN2A CDC25B SOX4 ATF5 TRAF4 CD44
B
RQ_mRNA level
StAx
DMSO
p<0.05
p>0.05
AXIN2 SGK1 MSX1 ID2 TBX3 MSX2 HDAC4 SMC2 CCND1 CDC25A SHB AURKA TIAM1 PHLDA3 MCTS1 CDKN2A BUB3 CDK2 TERF2 CDK6 DUSP4 CD44 TP53INP1 CDC7 HELLS SOX4 ATF5 CDK5 CDC25B BIRC5 MYC VEGFA TERT FOSL1 ZAK TNFRSF10B SIM2 UBE2C CUX1 TRAF4 FYN CDCA7
C
Relative Luciferase activity
DMSO
iCRT 25 uM
iCRT 50 uM
iCRT 75 uM
iCRT 100 uM
D
Relative Luciferase acitivity
DMSO
aStAx 10 uM
aStAx 20 uM
aStAx 30 uM
iCRT 75 uM
E
Cell proliferation (OD 490 nm)
DMSO
aStAx
iCRT
Days
F
Percent Input
G1
G2
AXIN2 ACTB AXIN2 ACTB AXIN2 ACTB AXIN2 ACTB
IgG
TCF4
CCND1 ACTB CCND1 ACTB CCND1 ACTB CCND1 ACTB
IgG
TCF4
AURKA NC AURKA NC AURKA NC AURKA NC
IgG
TCF4
G1
G2

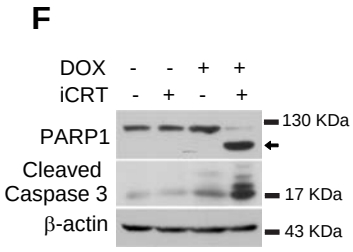

A
Asynchronous
Thymidine release
Asyn
0 h
5 h
8 h
10 h
12 h
14 h
16 h
18 h
20 h
24 h
DMSO
iCRT
Thymidine release into DMSO
Thymidine release into iCRT
Thymidine release into iCRT then DMSO
Thymidine release into DMSO then iCRT
A1
A2
A3
A4

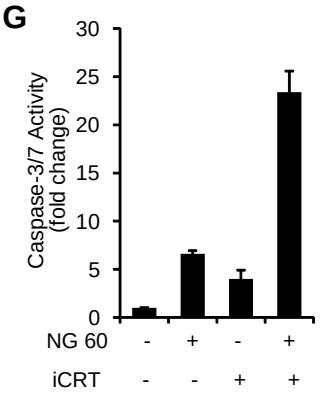

B
Release after Thymidine block
6 h
8 h
10 h
12 h
14.5 h
DMSO
iCRT
p-H3
DAPI

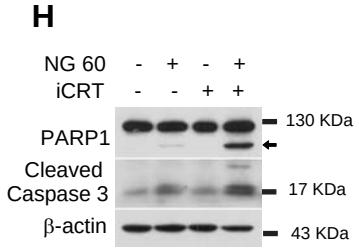

C
DAPI
β-tub
p-H3
Merge


D
Caspase-3/7 Activity (fold change)
0
4
8
12
16
20
DMSO
iCRT 25 uM
iCRT 50 uM
iCRT 100 uM
DOX 10 uM


E
Caspase-3/7 Activity (fold change)
0
20
40
60
80
100
DOX - + - +
iCRT - - + +


F
DOX - - + +
iCRT - + - +
PARP1
Cleaved Caspase 3
β-actin
130 KDa
17 KDa
43 KDa


G
Caspase-3/7 Activity (fold change)
0
5
10
15
20
25
30
NG 60 - + - +
iCRT - - + +


H
NG 60 - + - +
iCRT - - + +
PARP1
Cleaved Caspase 3
β-actin
130 KDa
17 KDa
43 KDa

Supplementary Figure legends

Fig. S1. Visualization of the β-catenin/TCF complex by BiFC.

A. Top: Schematic of the BiFC biosensor. The BiFC biosensor comprises β-catenin (yellow) and TCF3 (red) proteins, each was fused with a half of Venus protein (VC and VN) or YFP protein (YC and YN) at their C- and N-terminus, respectively. Bottom: Schematic of the BiFC constructs. FLAG or Myc tag was added at their N-terminus for protein detection. A C-terminal deleted β-catenin (β-catenin ΔC) was used in all cases to minimize the Wnt activity when these constructs were over-expressed.

B. Top: Wnt-responsive TOP-FLASH reporter assay in HEK293T cells transfected with BiFC components. Five or ten ng plasmid DNA per well in 96 well plate were transfected, respectively. Bottom: The expression levels of the BiFC components in these samples were determined by Western blot. FLAG antibody was used to detect FLAG-β-cateninΔC-VC/YC and Myc antibody was used to detect Myc-VN/YN-TCF3. GAPDH was used as a loading control.

C. BiFC signals (upper panels) or GFP signals (lower panels) in HEK293T cells after co-transfection with the indicated plasmids. Fluorescence and phase-contrast merged fluorescence images are shown. Bar = 100 μm.

D. Schematic of the constructs for the doxycycline-repressible BiFC stable lines. The pBI vector allows the simultaneous regulation of both β-cat-VC and VN-TCF3 by one central, bidirectional TRE (Tet responsive element). The internal ribosome entry site (IRES) permits the translation of two open reading frames from one messenger RNA. These constructs were introduced into HeLa-tTA cells to produce stable cell lines. A doxycycline-repressible Venus stable line was also established as a control.

E. Western blot analysis of the expression levels of the biosensor components and Venus in doxycycline-repressible BiFC and Venus stable cell lines. Withdraw of Dox (doxycycline) induced the expression of the proteins. The relative expression level was compared in lower panel, which indicated that the β-cat-VC (*) was at a similar

level to endogenous β-catenin (arrow). Since the C-terminal domain of β-catenin was deleted, it is still slightly smaller than the wild type, even though it is fused to half of the Venus protein (VC).

Fig. S2. The β-catenin/TCF BiFC signal accumulates during the S-G2 phases.

Analysis of the DNA content profile and YFP signal intensity of BiFC-YFP stable cells by flow cytometry. The BiFC-YFP signal could only be detected after incubation for 4 h at 30℃. The cells were then fixed by paraformaldehyde, stained with propidium iodide and analyzed by FACS. The DNA content profiles of the total, BiFC-positive, and -negative gated populations are shown.

Fig. S3. Identification of β-catenin/TCF target genes during the S-G2 phases.

A, B. Effects on Wnt target genes expression in S-G2 phase HCT116 cells upon Wnt inhibition by (A) iCRT or (B) aStAx . HCT116 cells were synchronized and treated as in Fig. 3B. Forty two genes in the cell death and cell cycle categories were analyzed using real-time PCR. The results are the average of triplicate data; the SD and p-values were calculated in a two-tailed student t-test.

C. Wnt-responsive TOP-FLASH reporter assay in HCT116 cells with the indicated treatments. iCRT or vehicle was introduced 24 h before cell lysis and detection.

D. Wnt-responsive TOP-FLASH reporter assay in HCT116 cells to compare the inhibitory effects of aStAx and iCRT. iCRT, aStAx, or vehicle was introduced 24 h before cell lysis and detection.

E. MTT assay in HCT116 cells to compare the inhibitory effect of aStAx and iCRT on cell proliferation. The cells were treated with DMSO, iCRT (50 μM) or aStAx (20 μM) for 4 days and the MTT assay was performed every day. The results presented are the mean ± SD from quintuplicate samples.

F. Chromatin immunoprecipitation assays in G2- or G1-enriched HCT116 cells (5 h and 12.5 h after release from the thymidine block, using TCF4 or a mouse IgG antibodies as indicated. 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. S4. β-catenin/TCF complex-mediated target gene expression ensures G2/M progression and cell survival.

A. Delay of cell cycle progression by iCRT treatment. Synchronized HCT116 cells were released into medium containing either DMSO (control) or 75 μM iCRT. (A1) DMSO; (A2) iCRT; (A3) iCRT for the first 8 h and then replaced by DMSO containing medium; (A4) DMSO for the first 9 h and then replaced by iCRT containing medium. Cells were harvested at the indicated time points and their cellular DNA content was determined by propidium iodide staining and flow cytometry. Asyn, asynchronous cells.

B. HCT116 cells after release from a thymidine block were treated with DMSO or iCRT for the indicated time periods and then fixed and stained with p-H3 antibody (green) or DAPI (blue). Bar = 100 μm.

C. Closer views of representative iCRT-treated HCT116 cells at different stages of mitosis. Cells were stained with DAPI (blue), p-H3 (green) or β-tubulin (β-tub, red). Bar = 10 μm.

D. Caspase-3/7 activation in response to Wnt inhibition by iCRT. HCT116 cells were incubated with DMSO, iCRT or DOX (Doxorubicin, as a positive control) for 24 h. Effects on Caspase-3/7 activity were assessed using the Apo-ONE homogeneous Caspase-3/7 assay. Results show the fold increase of Caspase-3/7 activity relative to the DMSO-treated cells. Each data point represents the mean ± SD of the experiment performed in triplicate.

E, F. Sensitivity of iCRT-treated (50 μM) HCT116 cells to Doxorubicin-triggered (DOX, 1 μM) apoptosis. Cells were treated for 24 h. Apoptosis was detected by measurement of (E) Caspase-3/7 activity and (F) cleavage of Caspase-3 and PARP1 by Western blot. The arrow indicates the cleaved PARP1.

G, H. Sensitivity of iCRT-treated (50 μM) HCT116 cells to NG 60-triggered (7.5 μM) apoptosis. Cells were treated for 24 h. Apoptosis was detected by measurement of (G)

caspase-3/7 activity and (H) cleavage of Caspase-3 and PARP1 by Western blot. The arrow indicates the cleaved PARP1.

Movie 1: Example 1 of a dynamic β-catenin/TCF-BiFC signal during the cell cycle. HeLa cells expressing the BiFC biosensor were traced for 40 h. Images were recorded every 30 min by a high content screening platform (HCS, ArrayScan VTI 700, ThermoFisher Cellomics).

Movie 2: Example 2 of a dynamic β-catenin/TCF-BiFC signal during the cell cycle. HeLa cells expressing the BiFC biosensor were traced for 40 h. Images were recorded by HCS platform.

Movie 3: HeLa cells expressing the BiFC biosensor were released from a thymidine block and then traced for 26 h. Images were recorded every 20 min by a Nikon Ti-E A1Rsi inverted confocal microscope (Nikon Instruments).

Movie 4: Example 1 of a Venus fluorescence signal during the cell cycle. HeLa cells expressing the Venus fluorescence proteins were traced for 40 h. Images were recorded by HCS platform.

Movie 5: Example 2 of a Venus fluorescence signal during the cell cycle. HeLa cells expressing the Venus fluorescence proteins were traced for 40 h. Images were recorded by HCS platform.

Movie 1.

Movie 2.

Movie 3.

Movie 4.

Movie 5.

Tables S1 to S7.

Download Supplementary Tables