Supplementary Materials for

Mechanical strain induces E-cadherin-dependent Yap1 and β-catenin activation to drive cell cycle entry

Blair W. Benham-Pyle, Beth L. Pruitt, and W. James Nelson

correspondence to: wjnelson@stanford.edu

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Materials and Methods

Application of Mechanical Strain

Very dense, quiescent monolayers of Madin-Darby canine kidney (MDCK) type II G cells were formed using a calcium switch method. Briefly, MDCK cells were plated on collagen I- or fibronectin-coated flexible PDMS substrates at ~1000 cells/mm^2 in low calcium (5μM) DMEM with low glucose (200 mg/L). After 60 minutes, media was removed and replaced by DMEM with low glucose and normal calcium levels (1.8mM). After 24 hours, cells had packed together and formed a very dense, quiescent monolayer. Strain was applied 24 hours after calcium switch for indicated time periods using an integrated strain array (ISA) *(16)* (Fig. S1). Briefly, cells were plated in PDMS wells on collagen I- or fibronectin-coated flexible PDMS substrates. Wells were placed over an acrylic pneumatic compartment, which automatically positioned a pillar beneath the center of each PDMS well; the width of the pillar varied between the wells so that different levels of in-plane strain (0 – 15%) were applied at the same time. Vacuum pressure applied using a microprocessor-based control system through the compartment deformed the PDMS substrate around the pillar, resulting in biaxial stretching accompanied by equi-biaxial in-plane strains within the monolayer and Poisson's contraction of the monolayer thickness (Fig. S1).

Cell Culture and Stable Cell Lines

Madin-Darby canine kidney (MDCK) type II G cells were grown in DMEM with low glucose and either 200 μM G418 (TOPdGFP, β-catenin-Engrailed) or 5 ug/mL puromycin (TBSmCherry) to maintain stable expression of reporter or dominant negative constructs of interest. Expression of the T151 E-cadherin mutant was induced by addition of 20 ng/mL Doxycycline to the medium (34). MDCK cell lines stably expressing the TBSmCherry reporter construct or the β-catenin-Engrailed dominant negative construct (βEng) were generated by selection with 200 μM G418 (βEng) or 5μg/ml puromycin (TBSmCherry) following transfection by Lipofectamine 2000 (11668019, SM-Invitrogen). YTIP-GFP, YTIP-RFP, TBSmCherry, and TOPdGFP transient transfections were also performed using the Lipofectamine 2000 reagent.

Inhibitor Studies

The small molecule inhibitors Verteporfin *(21)* (Sigma-Aldrich, SML0534) and iCRT3 *(24)* (Sigma-Aldrich, SML0211) were used to inhibit Yap1/TEAD and β-catenin/TCF interactions and transcriptional activity, respectively. Verteporfin (5μm), iCRT3 (25μM), and DMSO controls were added to strain array wells 1 hour prior to the application of strain. Fresh inhibitor was added every 6 hours subsequently until the end of the experiment (Yap1/TBS/Ki67 – 4 hours, β-catenin/TOPdGFP – 8 Hours, EdU – 24 Hours).

Immunofluorescence

MDCK cells, plated on collagen I-coated PDMS, were fixed in 4% (v/v) paraformaldehyde for 15 minutes, permeabilized in 0.5% Triton X-100 for 5 minutes, and blocked in PBS containing 0.2% BSA and 1% goat/donkey serum at room temperature. Primary antibodies used for immunofluorescence staining were: Ki67/rabbit (ab16667, Abcam), E-cadherin/rat (Decma-1, GTX11512, GeneTex), β-catenin/mouse (610154, BD Biosciences), Yap1/mouse (sc-101199, Santa Cruz Biotechnology), Myc/mouse (MMS-150P, Covance), HA/rabbit (ab-9110, Abcam), and GFP/mouse (11814460001, SM-Roche). EdU incorporation was performed using a Click-iT EdU Alexa Fluor 594 Imaging Kit (C10339, Molecular Probes/Invitrogen) as directed by the manufacturer. Immunofluorescence images were acquired with a Zeiss (Jena, Germany) Axiovert 200 inverted microscope equipped with a Mercury lamp and a 63X objective (Olympus, Tokyo, Japan), and acquired with AxioVision Microscopy Software (Zeiss). Yap1 confocal images were acquired with a Zeiss (Jena, Germany) Meta510 upright confocal microscope equipped with a Mercury lamp and a 63X objective, and acquired with LSM microscopy software (Zeiss).

Repetition of experiments, and number of cells analyzed

The design of the ISA enables the user to apply four different levels of strain to the same population of cells at the same time, in five separate wells *(16)* (Fig. S1). Additionally, the ISA has been parallelized to allow strain application on up to six replicate monolayers. Thus, three treatments can be run on the same baseplate at the same time, with two replicate monolayers per treatment. Two experiments were viewed as independent only if the monolayers were strained on separate ISA baseplates. For each monolayer, 3-5 images were taken from the top of the strain pillar, with each image containing 75-100 cells. For our studies the number of independent experiments ranged from 2-8 and the total number of cell analyzed ranged from 116 – 7390, with the lower number of cells occurring in experiments where transfected cells (YTIP, TOPdGFP, TBSmCherry) were analyzed. A full documentation of the number of experiments performed and the number of cells analyzed is provided in Table S1.

Selection of Representative Images in Figures

Representative images were one of at least eight images taken for each treatment in each of at least 3 independent experiment. Images were chosen because they represented a median intensity for the relevant biomarker, and images with the most or least apparent biomarker were eliminated from consideration. All chosen images used to compare treatments (ex. 0% vs. 15% strain, WT vs. βEng, each time-point in Figs. S2, 3, and 4) were chosen from the same experiment and were thus plated as a monolayer together, strained together on the same baseplate, fixed and stained with the same antibody mixes, and imaged at the same time.

Image Quantification

The chosen cell cycle markers, the transcriptional reporters, and (transcriptionally active) β-catenin and Yap1 localized in the cell nucleus. Therefore, the strategy for image quantification was nuclear segmentation (using Hoechst staining) followed by pixel intensity calculation for each nuclei; this method excluded cytoplasmic and plasma membrane staining. The nuclear pixel intensity of all biomarkers (Yap1, β-catenin, TBSmCherry, TOPdGFP, EdU, and Ki67) was analyzed on a single cell basis using a custom-written image-processing routine in MATLAB. Nuclei were identified and segmented by applying a series of thresh-holding and watershed algorithms to images of Hoechst stained cells, resulting in a binary nuclear mask. Nuclear objects with an area greater than 1.6 standard deviations above the mean or less than 1000 pixels were eliminated from further analysis to prevent over- or under-segmented nuclei from being included in the analysis. Binary masks for each image were used to produce connected component arrays of nuclear objects, and the mean pixel intensity for each relevant biomarker was calculated for each identified nuclear object in each image. The background intensity was calculated as the minimum pixel intensity of the entire image within the analyzed imaging channel, and was subtracted prior to export of the data for each image. The resulting data were exported from MATLAB to a text file for plotting and further analysis. Exported data for each nucleus included the object identifier, object area, and pixel intensity of up to 3 analyzed imaging channels.

Identification of Transfected/Positive Cells

Distributions of nuclear mean pixel intensities were plotted prior to determining intensity thresholds for identification of either transfected cells or biomarker positive cells (Fig. S6E-H). Thresholds for positive cells were chosen for each experiment based on the average minimum, median, and maximum pixel intensities across all images and strain treatments as well as the distribution of mean nuclear pixel intensities under no strain conditions. For experiments using transient transfection - YTIP dominant negative or TBSmCherry/TOPdGFP signal in T151 and β-Engrailed cell lines - thresholds for a transfected cell were at least 2 fold above the mean pixel intensity of a nucleus in the analyzed image. Example plots of mean nuclear intensities and

chosen thresholds for transfected cells and analyzed biomarkers are shown in Fig. S6E-H, using the example of Ki67 and TOPdGFP signal (green threshold lines) in YTIP-positive or – negative cells (red threshold line). Once chosen, thresholds were kept constant across all treatments within each experiment.

Statistical Analysis

Several methods for statistical analysis were considered. Distributions of nuclear mean pixel intensities were initially compared between treatments using an unpaired t-test, with the Holm-Sidak method to correct for multiple comparisons (alpha = 5.000%) not assuming a consistent SD. Because some nuclear pixel intensity distributions were not normally distributed, we converted all statistical analyses to the nonparametric Kolmogorov-Smirnoff (KS) test to compare intensity distributions between treatments. The t-test and KS test produced similar results. However, since cells within the monolayers are either positive or negative for the relevant biomarker (Ki67, EdU, etc.), we compared the distributions again using the Mann-Whitney test, also known as the Wilcoxon rank sum test, which has been extended to handle tied values and categorical data. Because the response to strain is heterogeneous within the monolayer population, signaling is represented as % cells positive for the relevant biomarker. Thus, the statistics reported in the paper compare the mean percentage of positive cells between multiple independent experiments using an unpaired t-test and the Holm-Sidak method to correct for multiple comparisons. Of note, Mann-Whitney tests of the raw mean intensity values in each experiment consistently identified the same results as statistically significant.

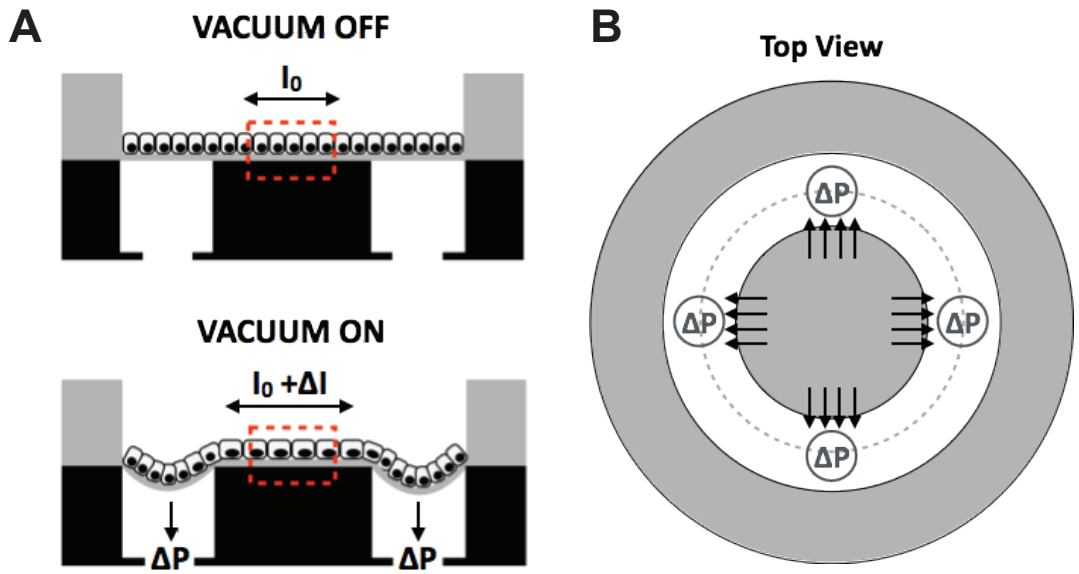

Fig. S1.

Schematic of integrated strain array (ISA) design for application of biaxial stretch *(15)*. Stretch creates equi-biaxial in-plane strains in the cell monolayer with a Poisson's contraction of the monolayer thickness. (**A**) Side view with and without vacuum pressure applied shows how distortion of flexible silicone substrate results in stretch of the adhered monolayer (**B**) Top view depicts biaxial stretch on a circular pillar resulting in equi-biaxial in-plane strains.

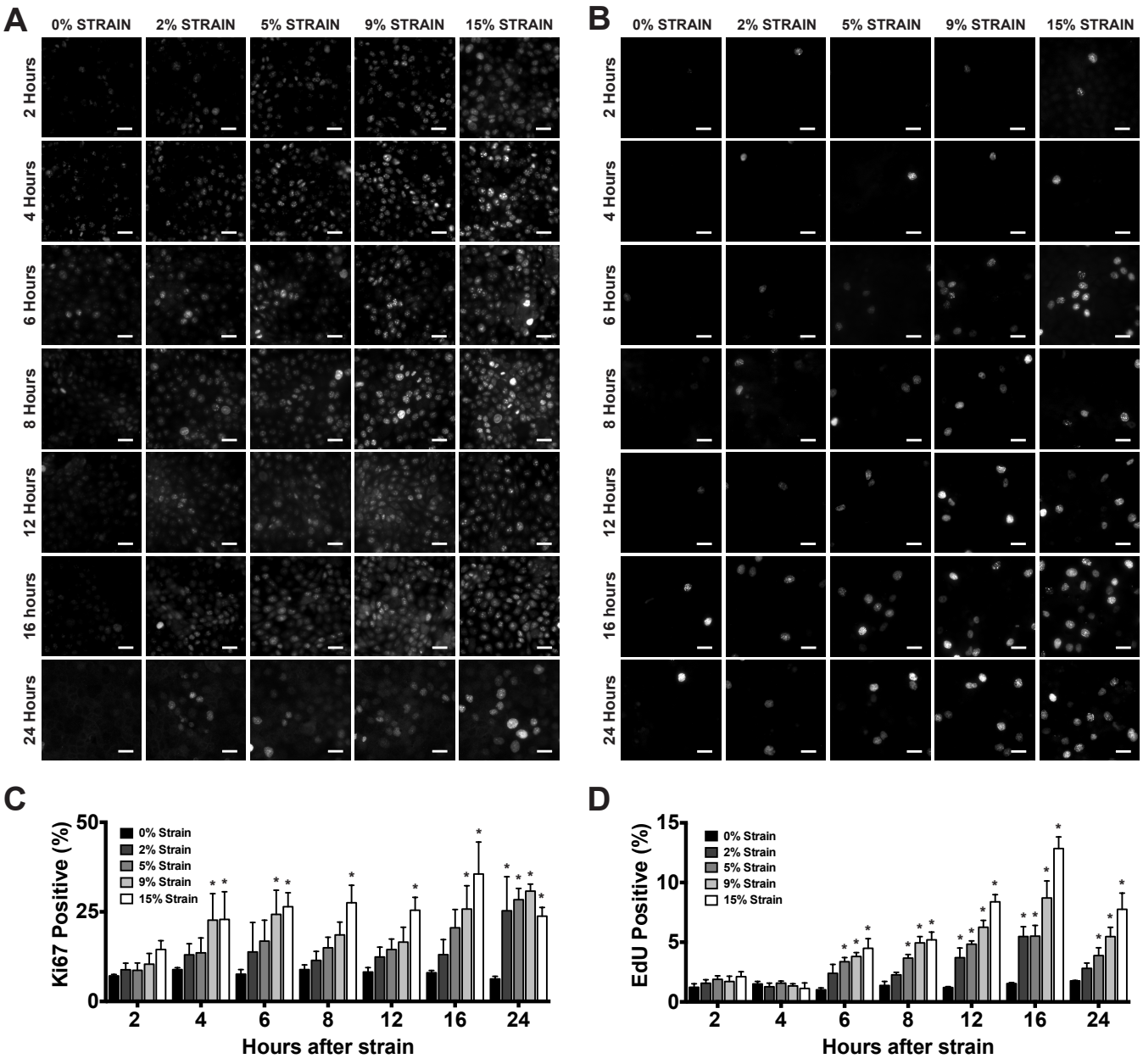

Fig. S2

Strain-induced cell cycle re-entry followed by DNA synthesis in quiescent MDCK monolayers. The G0/G1 transition was assessed by measuring levels of Ki67 immunofluorescence (**A**), while EdU incorporation (**B**) was used as a measure of DNA synthesis (S phase progression). Quiescent MDCK cell monolayers were strained for 2, 4, 6, 8, 12, 16 and 24 hours prior to fixation and staining. Strains applied were 0%, 2%, 5%, 9% or 15%. Quantification of Ki67- (**C**) and EdU-positive cells (**D**) for conditions shown in **A** and **B** are also provided. The quantifications show that increased Ki67 levels preceded EdU incorporation by several hours, consistent with the interpretation that cells first re-entered the cell cycle, then proceeding through the G1/S transition. Scale bars: 25 μm. All quantifications were performed over at least 3 independent experiments with 2 replicate monolayers per condition. Quantifications are mean +/- SEM; with an unpaired t-test p value < 0.05 (*) compared to the no strain treatment at the same time point.

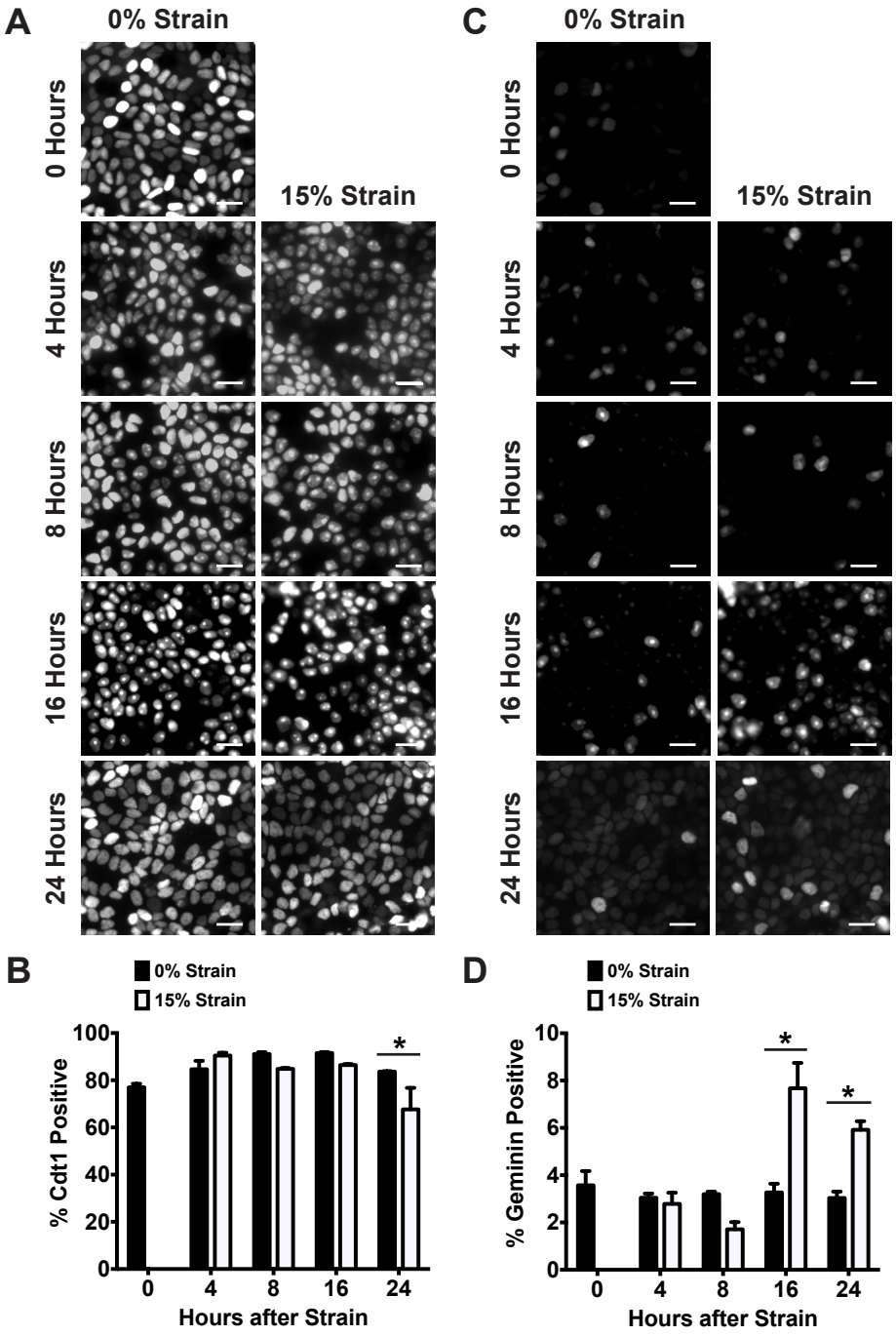

Fig. S3

Strain induced a decrease in Cdt1 reporter expression, and increased Geminin reporter signal in an MDCK cell line stably expressing the Fucci cell cycle marker (2); G0/G1 cells appear red (mKO2-Cdt1) and G2 cells appear green (mAG-Geminin). Cdt1 (**A**) and Geminin (**C**) signal was imaged in quiescent monolayers at 0 hours and at 4, 8, 16, and 24 hours following application of 0% or 15% strain. Quantification of Cdt1 levels (**B**) showed that intensity dropped significantly at 24 hours, indicating a decrease in the number of cells in G0/G1. Quantification of Geminin levels (**D**) showed a strain-induced increase in signal at 16 and 24 hours, consistent with cells progressing through S phase into G2, as also determined by EdU incorporation (Fig. 1, Fig. S2). Scale bars: 25μm; All quantifications were from 3 independent experiments with 2 replicate monolayers per condition. Quantifications are mean +/- SEM; with an unpaired t-test p value < 0.05 (*) compared to the no strain treatment at the same time point.

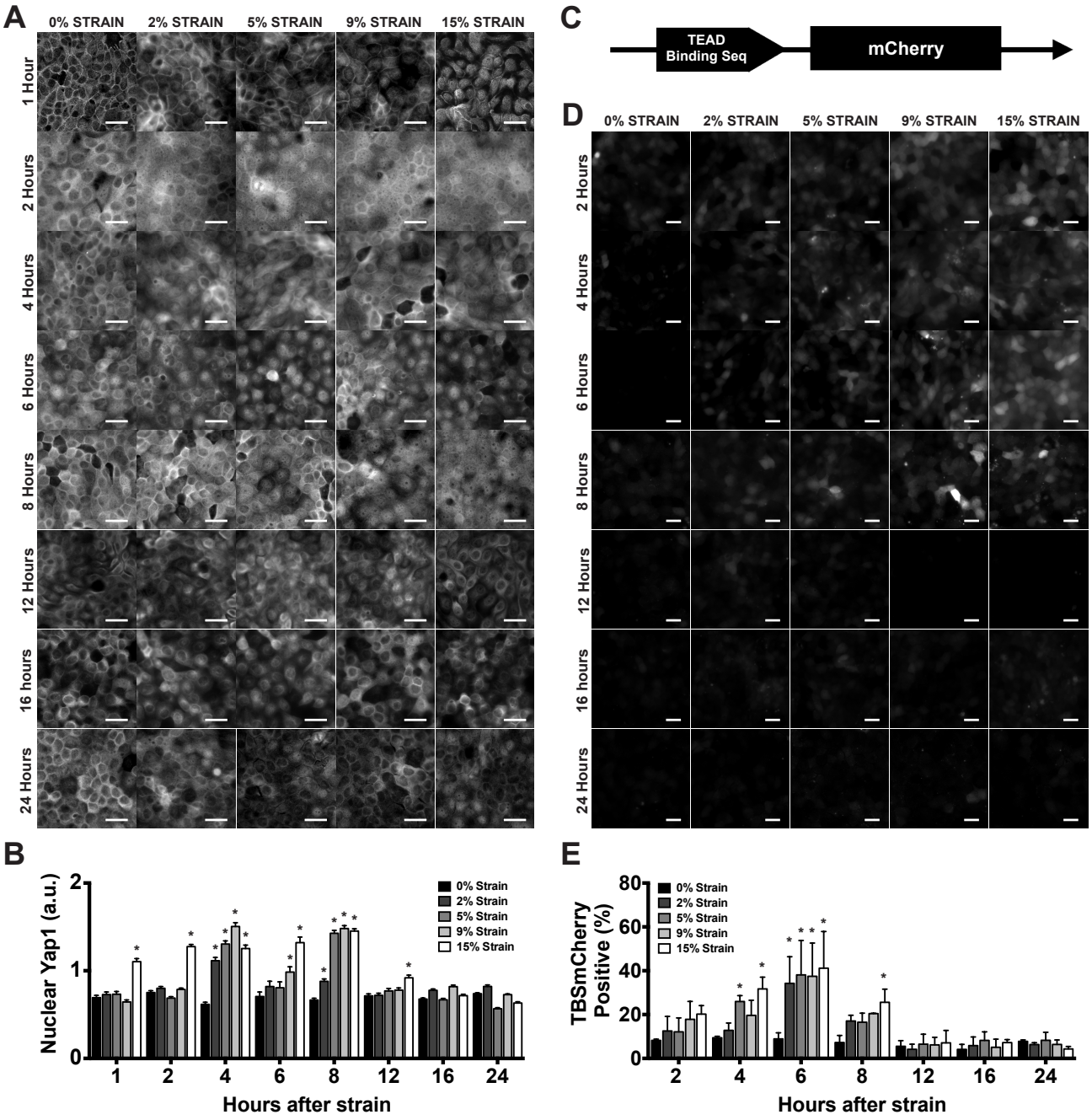

Fig. S4

Strain induced transient nuclear accumulation and transcriptional activity of Yap1. Distributions of Yap1 (**A**) and TBSmCherry transcriptional reporter (**D**) in quiescent MDCK monolayers 1, 2, 4, 6, 8, 12, 16, and 24 hours after application of 0%, 2%, 5%, 9% or 15% strain. Hoescht staining showed that 'dark' regions of monolayers stained for Yap1 are cells with low Yap1 expression, rather than regions without cells. (**C**) Design of the TBSmCherry reporter construct *(13)*. Single cell nuclear Yap1 (n = 346 -1637) (**B**) and mean % cells TBSmCherry positive (**E**) for all conditions shown in A and D. Scale bars: 25μm; All quantifications were performed over at least 2 independent experiments. Quantifications are mean +/- SEM; Yap1 nuclear intensities are Mann-Whitney test p values < 0.05 (*) and TBSmCherry % positive are unpaired t-test p values <0.05 (*) compared to no strain at the same time point.

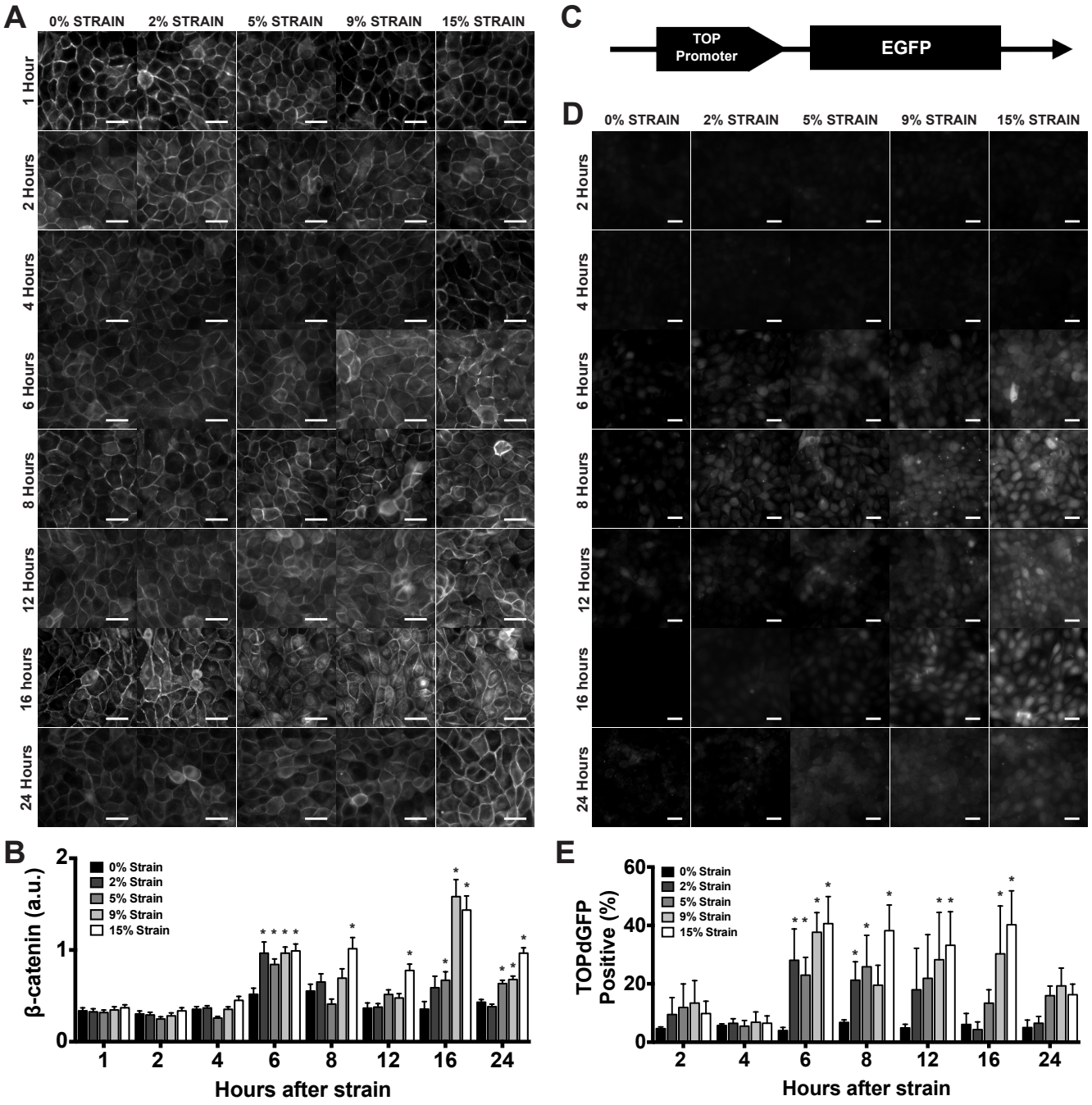

Fig. S5

Strain-induced nuclear accumulation and transcriptional activity of β-catenin. Distribution of β-catenin (**A**) and TOPdGFP transcriptional reporter (**D**) in quiescent MDCK monolayers 2, 4, 6, 8, 12, 16 and 24 hours after application of 0%, 2%, 5%, 9% or 15% strain. Design of TOPdGFP reporter construct (**C**, see also *19)*. Single cell nuclear β-catenin (n = 289-1727) (**B**) and mean % cells TOPdGFP positive (**E**) for all time points and strain levels shown in A and D. Scale bars: 25 μm; All quantifications were performed over at least 2 independent experiments with 2 replicate monolayers per condition. Quantifications are mean +/- SEM; β-catenin nuclear intensities are Mann-Whitney test p values < 0.05 (*) and TOPdGFP % positive are unpaired t-test p values <0.05 (*) compared to no strain at the same time point.

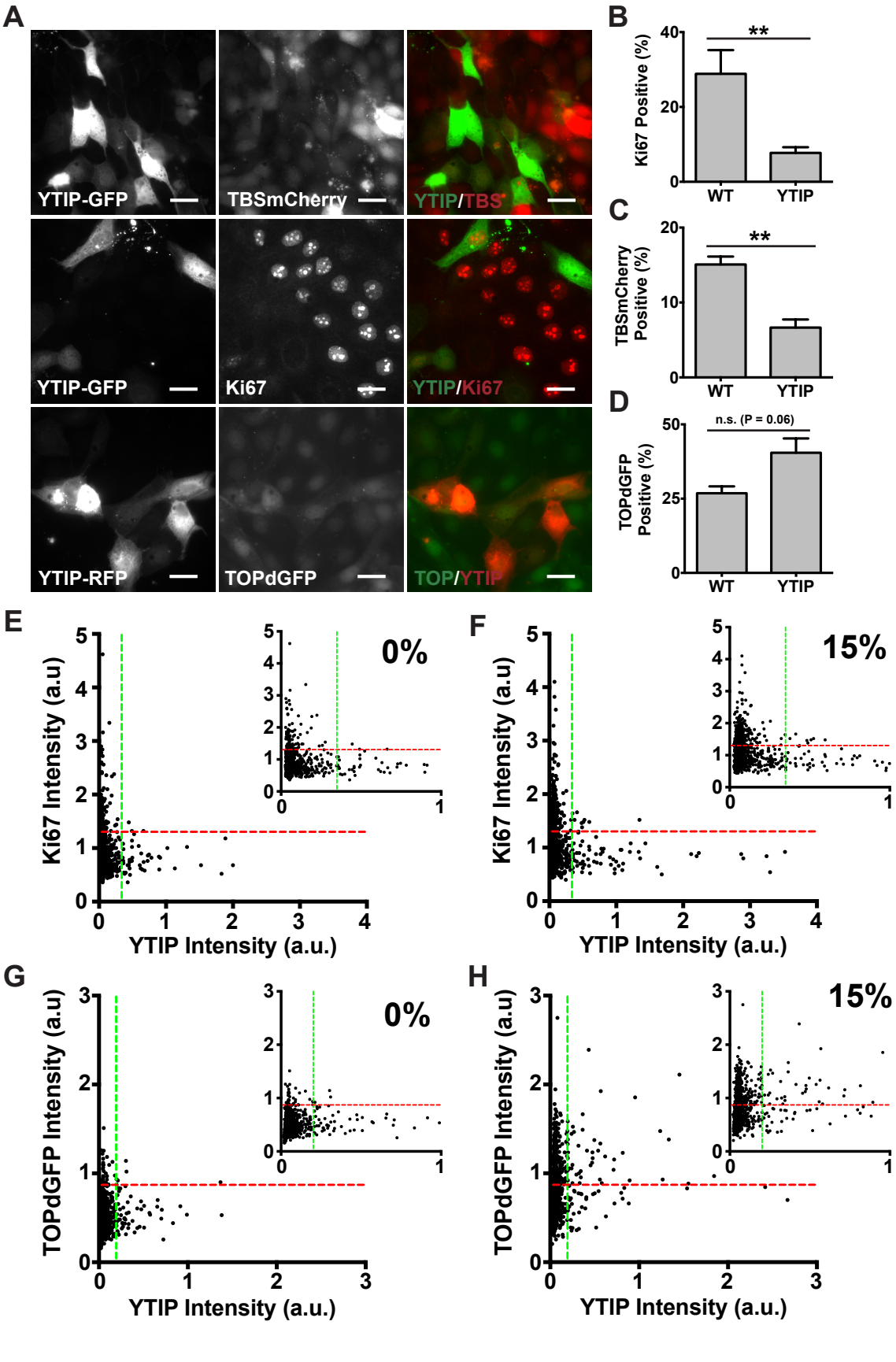
A
YTIP-GFP
TBSmCherry
YTIP/TBS
YTIP-GFP
Ki67
YTIP/Ki67
YTIP-RFP
TOPdGFP
TOP/YTIP
B
Ki67 Positive (%)
**
WT
YTIP
C
TBSmCherry Positive (%)
**
WT
YTIP
D
TOPdGFP Positive (%)
n.s. (P = 0.06)
WT
YTIP
E
0%
Ki67 Intensity (a.u)
YTIP Intensity (a.u.)
F
15%
Ki67 Intensity (a.u)
YTIP Intensity (a.u.)
G
0%
TOPdGFP Intensity (a.u)
YTIP Intensity (a.u.)
H
15%
TOPdGFP Intensity (a.u)
YTIP Intensity (a.u.)

Fig. S6
The Yap-TEAD inhibitory peptide (YTIP) inhibits cell cycle progression, but does not inhibit β-catenin transcriptional activity. (**A**) Distributions of TBSmCherry, Ki67, and TOPdGFP in MDCK cells transiently expressing different levels of YTIP-GFP or YTIP-RFP. Mean % cells positive for TBSmCherry (**B**), Ki67 (**C**), or TOPdGFP (**D**) were quantified in WT and YTIP-expressing MDCK cells using a custom MATLAB image-processing routine (See Materials and Methods). The mechanism underlying the increase in TOPdGFP levels in the presence of YTIP in unstrained cells is not known. It is possible, however, that this result reflects a competition between Yap1 binding to TEAD binding sites or coordinating with β-catenin-mediated transcription activity *(22)*. Thus inhibition of TEAD binding sites by YTIP might increase the availability of nuclear Yap1 to coordinate with β-catenin to increase TOPdGFP expression. In this context, inhibition of Yap1 with Verteporfin did not increase TOPdGFP (see Fig. S8); since Verteporfin binds directly to Yap1, Yap1 binding to either TEAD binding sites or, perhaps, β-catenin targets are inhibited. Example plots of YTIP-GFP vs. Ki67 under 0% strain (n=901) (**E**) and 15% strain (n=1178) (**F**) from a single experiment show how YTIP expression was inversely correlated with Ki67 levels induced by strain. In contrast, increased TOPdGFP signal when comparing 0% strain (n=1084) to 15% strain (n=883) was not affected by the level of YTIP expression (**G** and **H**). Insets show an expanded YTIP intensity axis from 0-1 to demonstrate the range of cells around the threshold intersection point. Thresholds for delineating YTIP positive cells (green dotted line) and biomarker positive cells (red dotted line) were chosen and remained constant in 0% strain and 15% strain conditions, thus allowing the visualization of strain induced changes to the population. Scale bars: 25 μm; Quantifications were performed over at least 3 independent experiments. Quantifications are mean +/- SEM; unpaired t test p values <0.01 (**) compared to no strain at the same time point.

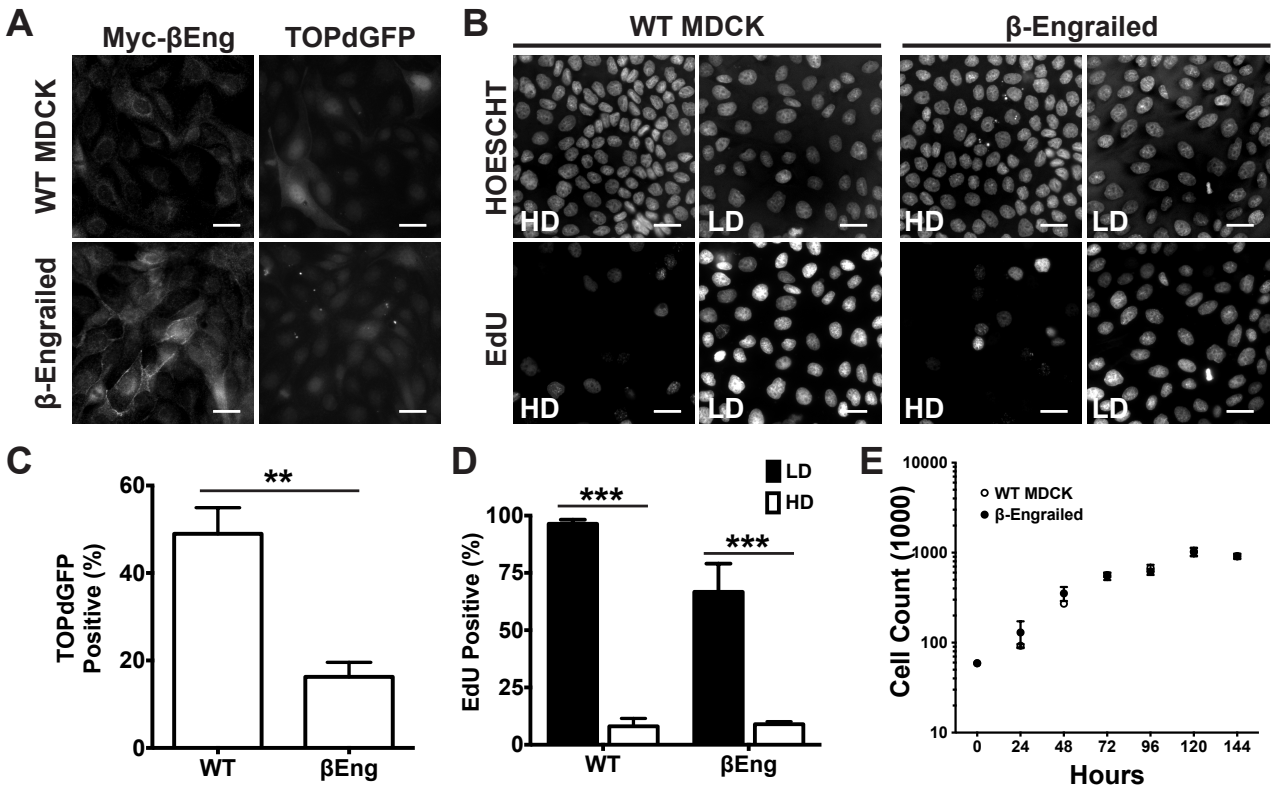

Fig. S7
MDCK cells stably expressing β-catenin-Engrailed (βEng) have low β-catenin transcriptional activity, but grow at rates and become contact inhibited at cell densities comparable to control MDCK cells. (**A**) Distribution of Myc tag and TOPdGFP in control and βEng MDCK cells. (**B**) Distribution of EdU incorporation at high (HD) and low (LD) densities in control and βEng MDCK cells. (**C**) Mean % cells TOPdGFP positive in control (n=4) and βEng (n=4) MDCK cells. (**D**) Mean % cells EdU positive at high and low densities in control (n=3) and βEng (n=3) MDCK cells after 24 hour incubation. (**E**) A growth curve of control and βEng MDCK cells monitored for 6 days after being plated at 25% confluence (n=4) show that βEng cells are contact inhibited (limited division) at high densities. Scale bars: 25 μm; Quantifications were mean +/- SEM; unpaired t-test p values <0.01 (**), p values < 0.001(***).

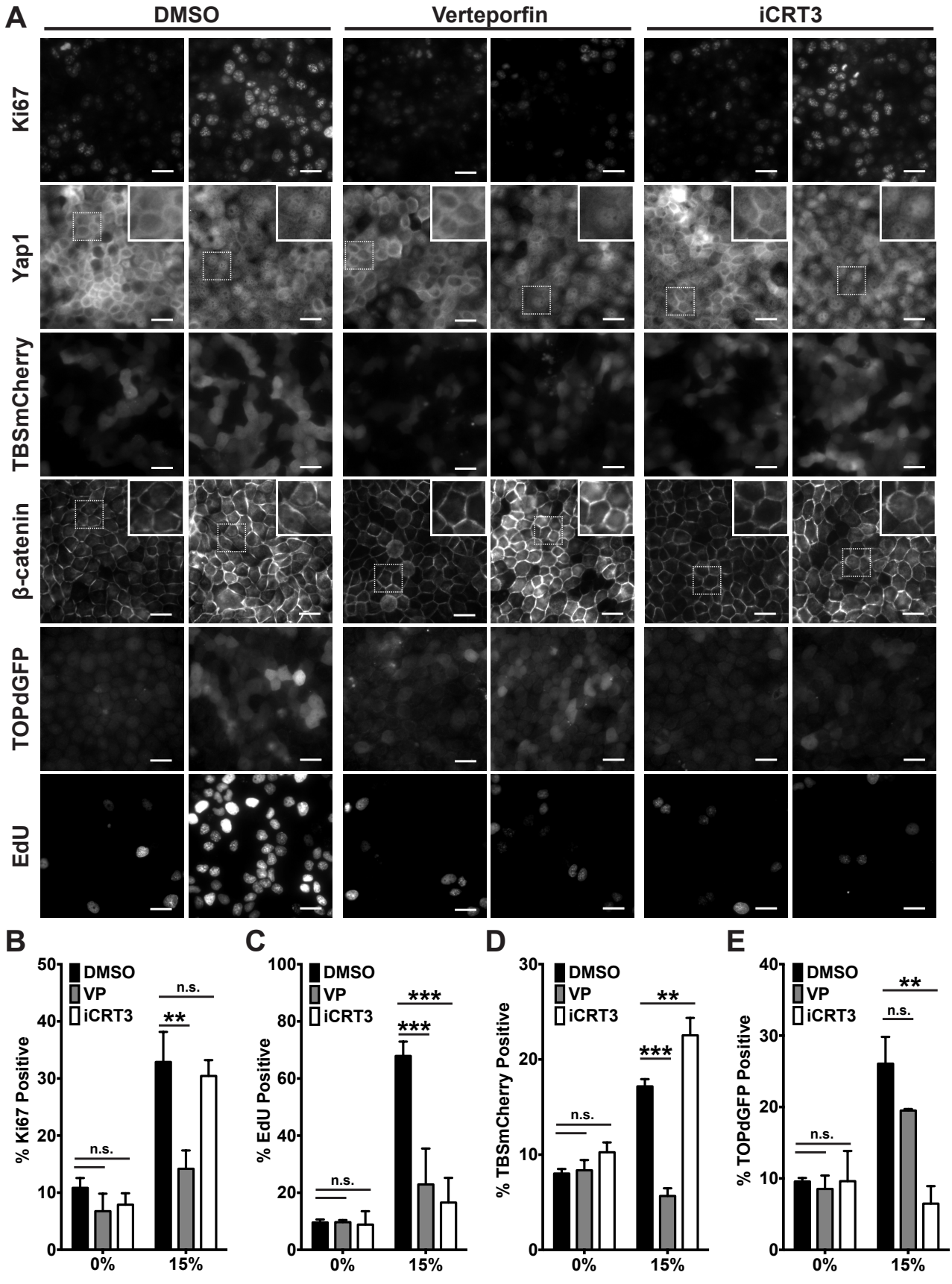
A
DMSO
Verteporfin
iCRT3
Ki67
Yap1
TBSmCherry
β-catenin
TOPdGFP
EdU
B
% Ki67 Positive
DMSO
VP
iCRT3
n.s.
**
n.s.
0%
15%
C
% EdU Positive
***
***
D
% TBSmCherry Positive
**
***
E
% TOPdGFP Positive
**

Fig. S8
Inhibition of Yap1 by Verteporfin blocks strain-induced cell cycle re-entry, but not strain-induced β-catenin signaling; inhibition of β-catenin by iCRT3 blocks progression from G1 into S following strain, but not cell cycle re-entry or Yap1 transcriptional activity. (**A**) Distributions of Ki67 (4hrs), Yap1 (4hrs), TBSmCherry (4hrs), β-catenin (8hrs), TOPdGFP (8hrs) and EdU incorporation (24 hrs) in control MDCK monolayers following application of 0% or 15% strain in the presence of DMSO (vehicle control), Verteporfin (5μM), or iCRT3 (25μM). Dotted lines indicate region shown in higher magnification insets. (**B**) The percentage of cells Ki67-positive 4 hours after strain and percentage of cells EdU-positive 24 hours after strain (**C**) with and without addition of Verteporfin (n=3) or iCRT3 (n=3) showed that Verteporfin blocked cell-cycle re-entry and subsequent progression from G1 into S phase. In the presence of iCRT3, cells re-entered the cell cycle, but did not progress from G1 to S phase. Thus, Yap1-mediated transcription was required for cell-cycle re-entry, while β-catenin-mediated transcription was required for progression from G1 into S phase. The percentage of cells that were TBSmCherry-positive 4 hours after strain (**D**) decreased with Verteporfin (n=3), but not iCRT3 (n=3), confirming that Verteporfin, but not iCRT3 inhibited Yap1/TEAD-mediated gene transcription. Similarly, The percentage of cells that were TOPdGFP-positive 8 hours after strain (**E**) decreased with iCRT3 (n=3), but not Verteporfin (n=3), confirming that iCRT3, but not Verteporfin inhibited β-catenin-mediated gene transcription. Scale bars: 25 μm; Quantifications were performed over 3 independent experiments with 2 replicate monolayers for each condition. Quantifications are mean +/- SEM; unpaired t-test p values <0.01 (**), p values < 0.001 (***).

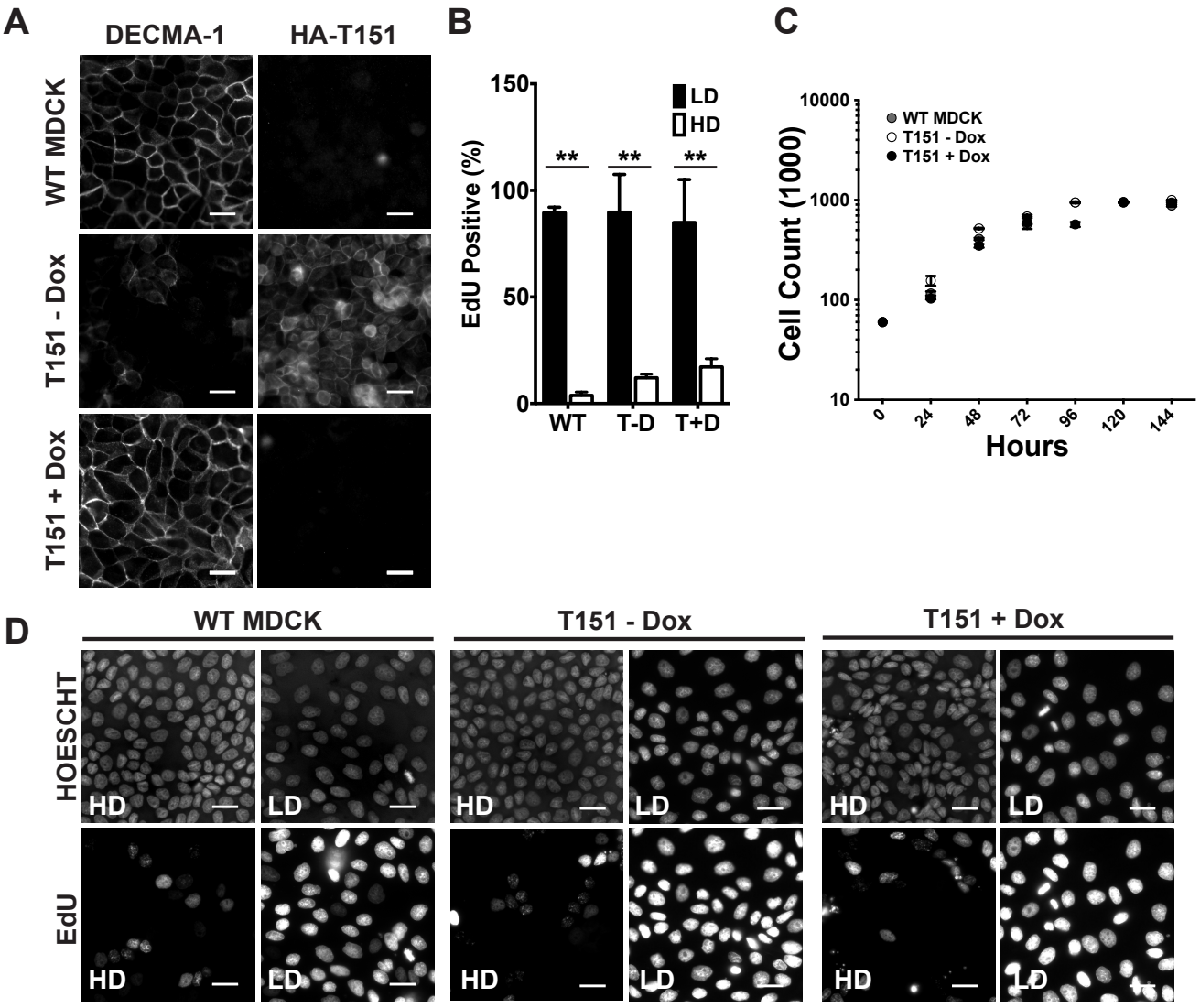

Fig. S9
T151 MDCK cells stably expressing the E-cadherin T151 truncation mutant (-DOX) had decreased expression of endogenous E-cadherin, but grew at rates and became contact inhibited at cell densities comparable to control cells (+DOX). (**A**) Distributions of HA tag (T151 E-cadherin) and endogenous E-cadherin (Decma-1) in control (+DOX) and T151 MDCK cells (-DOX). (**B**) Mean % cells EdU positive at high (HD) and low densities (LD) in control (+DOX) (n=3) and T151-expressing (n=3) MDCK cells (-DOX). (**C**) Growth curve of control (+DOX) and T151-expressing MDCK cells (-DOX) monitored for 6 days after being plated at 25% confluence. (**D**) Distributions of EdU incorporation over 24 hours at high (HD) and low (LD) densities in control (+DOX) and T151-expressing MDCK cells (-DOX). Scale bars: 25 μm; Quantifications were mean +/- SEM; unpaired t-test p values < 0.01 (**).

Table S1. Details on number of experiments and cells analyzed for all presented data.