SUPPLEMENTAL MATERIALS

Mechanisms promoting escape from mitotic-stress induced tumor cell death

Rebecca Sinnott[1], Leah Winters[2], Brittany Larson[3], Daniela Mytsa[1], Patrick Taus[1], Kathryn M. Cappell[4], and Angelique W. Whitehurst[5]

[1]Department of Pharmacology and Lineberger Comprehensive Cancer Center 4093 Genetic Medicine Building CB 7365, Chapel Hill, NC 27599-7365
[2]Department of Anesthesiology, University of Colorado, 12631 E 17th Ave. Aurora, CO 80045
[3]Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157
[4]Deparmtent of Medicine, Stanford University, 300 Pasteur Drive, L154 Stanford, CA
[5]Simmons Comprehensive Cancer Center, UT-Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390

#Address correspondence to: Angelique Whitehurst, PhD. UT-Southwestern Medical Center 6001 Forest Park Drive, Dallas, TX 75390-8807. Phone (214)-645-6066. 214-645-6347 angelique.whitehurst@utsouthwestern.edu.

Inventory:

Supplemental Methods

Supplemental Figures 1-5

Excel file with siRNA screen data

Supplemental Methods:

Pan-genomic screen data analysis: Raw luminescence values were normalized to median values in each row to correct for position effects. The viability ratio for each siRNA pool was then calculated as $mean_{paclitaxel}/mean_{vehicle}$. A z-score for each siRNA was calculated using the following formula: z-score = $(z - \mu)/\sigma$: z = ratio for an individual siRNA, μ = average ratios for all siRNAs/day and σ = standard deviation of all ratios/day. For hit identification, we used criteria of $z < -2.5$ and demanded that the ratio demonstrate a loss of viability > than 15% in the presence of paclitaxel and less than 15% loss in viability in vehicle treated conditions. siRNA pools with a variance of greater than 10% were not considered. siRNA pools that were previously shown to decrease viability in normal immortalized human bronchial epithelial cells (HBEC3KT) were removed (13). A number of genes were subsequently discontinued from the REF-Seq database and were also removed.

EdU Analysis: Cells were seeded in a 96 well plate such that they were 50% confluent 48 hours post plating. Cells were then exposed to 20 μM EdU (5-ethynyl-2'-deoxyuridine) for 1 hour and processed with the Click-iT® (Life Technologies) standard protocol. Cells incorporating EdU were scored by manual inspection.

Protein Purification: Recombinant pGEX4T1-GST-TRIM69A was expressed in E.coli (BL21) in the presence of $ZnCl_2$. Bacteria were lysed with protein buffer (50 mM, Tris pH 7.7, 150 mM KCl, 0.1% Triton X-100, 1 mM DTT) supplemented with lysozyme. GST-TRIM69A was isolated using glutathione-agarose (Sigma) and eluted with protein buffer supplemented with reduced glutathione.

In vitro auto ubiquitinylation assay: The Enzo® Auto-ubiquitinylation kit was used per manufacturer instructions, supplemented with 1 µg GST-TRIM69A, 1 µg Flag-Ubiquitin (Sigma) and 10 µg bovine ubiquitin (Sigma) in the presence and absence of 0.05 µg/mL UbcH5b (E2) to evaluate TRIM69A for auto-ubiquitinylation activity.

Analysis of Gene Expression Data Sets: Evaluation of CASC1 expression in The Cancer Genome Atlas datasets was performed through the CBioPortal. Lung squamous, lung adenocarcinoma, breast and ovarian cases were provisional RNAseq data sets deposited by TCGA. Expression cutoffs were based on z-score threshold of +/- 2.

Cell synchronization: H1299 cells were synchronized by a double thymidine-nocodazole mitotic block. Briefly, cells were treated with 2 mM thymidine for 18 hours, then rinsed 2x with PBS and incubated with fresh medium for 9 hours to release the cells. After release, cells were treated with 2 mM thymidine for 17 hours. After the second block, cells were rinsed and released into fresh medium for 3 hours before treatment with 100 ng/mL nocodazole for 4 hours. Cells were then collected for immunoprecipitation assays.

Supplementary Figure 1:

(A) Indicated cell lines were exposed to paclitaxel for 48 hours. Cells were then fixed and stained with DAPI. Scale bars represent 5 μm. (B) Single-cell lineage tracing of HCC366-GFP-H2B cells starting 72 hours post exposure to 10 nM paclitaxel. 100 micronucleated cells evaluated, each represented as a bar with color indicating cell cycle phase. (C) Colony formation assay in HCC366 cells following exposure to vehicle or 10 nM paclitaxel for 48 hours. Bars represent mean ± SEM for 6 independent experiments. (D) Single cell lineage tracing of H1155 cells exposed to indicated concentrations of paclitaxel. 25 cells were evaluated per condition. Each circle represents a single cell with the color representing mitotic outcome as indicated. (E) Workflow for pan-genomic two-condition screen in HCC366 cells. (F) Z-score distribution of ratios (treated/vehicle) for each siRNA pool.

Supplementary Figure 2:

(A) HCC366 GFP-H2B cells transfected with indicated siRNAs were exposed to 10 nM paclitaxel and imaged for 48 hours. Mitotic transit time was quantitated for 50 cells (30 cells for RBM22) in two independent experiments. Bars represent the mean % increase in mitotic transit time ± range as compared to control transfected and paclitaxel treated cells (B) HCC366 cells were transfected with indicated siRNAs for 48 hours. Cells were then exposed to vehicle or 10 nM paclitaxel for an additional 48 hours. Cleaved-caspase 3/7 activity was assessed by APO-ONE®. Values were normalized to control transfected cells under the same treatment condition (vehicle or paclitaxel). Bars represent mean ± range from 2 independent experiments. (C) HCC366 cells were transfected with indicated siRNAs. 72 hours following transfection, RNA was harvested and target mRNA levels assessed by qRT-PCR. Bars represent mean ± range from 2 independent experiments. (D) HCC366 cells were transfected with indicated siRNA pools and

individual siRNAs for 48 hours followed by exposure to 10 nM paclitaxel for 48 hours. WCLs were immunoblotted with indicated antibodies. (E) Indicated NSCLC cell lines were transfected for 48 hours with indicated siRNAs prior to exposure to vehicle (black bar) or 10 nM paclitaxel (grey bar) for 24 hours. Cells were fixed and stained with anti-phospho-histone H3 (ser10) and DAPI. Mitotic Index (% of total cells in mitosis) was scored by manual inspection. Bars represent mean ± SEM for 3 independent experiments. * P =< 0.05. ** P < 0.01. (unpaired two-tailed student's t-test).

Supplementary Figure 3

(A) Mitotic transit time for HCC366 cells from Figure 2C and D. (B) HCC366 cells were transfected with indicated siRNAs for 48 hours followed by exposure to 10 nM paclitaxel for 48 hours. Whole-cell lysates were immunoblotted with indicated antibodies. (C) Indicated cells were pulsed with EdU and incorporation manually quantitated. Bars represent mean from 6 independent experiments ± SD. (D) HCC366 cells were exposed to indicated doses of proTAME for 24 hours. Caspase 3/7 activity was assessed (APO-ONE®) and normalized to untreated control. Bars represent mean from 3 independent experiments ± SD.

Supplementary Figure 4

(A) Expression and amplification of CASC1 in tumor samples from indicated tumor types as analyzed by the cBio Data Portal. Each rectangle represents a single case. (B) Bars represent mean of abnormal spindles which were manually scored as mitotic cells with <1 or >2 pericentrin positive foci. Error bars represent SEM from a minimum of 3 independent experiments. *** P < 0.001 ** P < 0.01 (two-tailed unpaired student's t-test). (C) H1155-GFP-H2B cells were transfected with indicated siRNAs for 48 hours followed by exposure to 10 nM paclitaxel for 48 hours. Single-cell lineage tracing was performed on 50 cells to measure length

of mitotic transit time and mitotic outcome. (D) Microtubule regrowth assays were performed on indicated cells lines. Cells were immunostained with pericentrin (red) and β-tubulin (green) and DAPI (blue), Scale bar = 10 μm. (E) Indicated cell lines were transfected with indicated siRNAs for 48 hours followed by incubation with vehicle or 10 nM paclitaxel for an additional 48 hours. Whole cell lysates were immunoblotted with indicated antibodies.

Supplementary Figure 5

(A) Domain schematic of human TRIM69 isoforms. (B) An in vitro ubiquitination assay was performed with purified GST-TRIM69A in the presence and absence of E2, UbcH5b. Samples were resolved on SDS-PAGE and blotted with indicated antibodies. (C) Quantitation of micronucleated or multipolar cells from Figure 4B. (D) Cells were transfected with indicated cDNA for 24 hours followed exposure to 11 μM nocodazole for indicated times. In vivo polymerized tubulin assay were then performed and lysates immunoblotted for indicated proteins. (E) Quantitation of Figure 5B. (F) Synchronized H1299-myc-TRIM69A cells were immunoprecipitated with indicated antibodies or control IgG. Immunoprecipitates were resolved by SDS-page and immunoblotted with indicated antibodies. (G) Putative protein interaction network for TRIM69 based on interactions detected by Mitocheck Consortium and Human Interactome Database.

Supplementary Table 1.

Row-median normalized values for each siRNA used in screening. Z-score was calculated based on the mean values for each day of screening. T and U indicate 10nM paclitaxel or vehicle treatment, respectively.

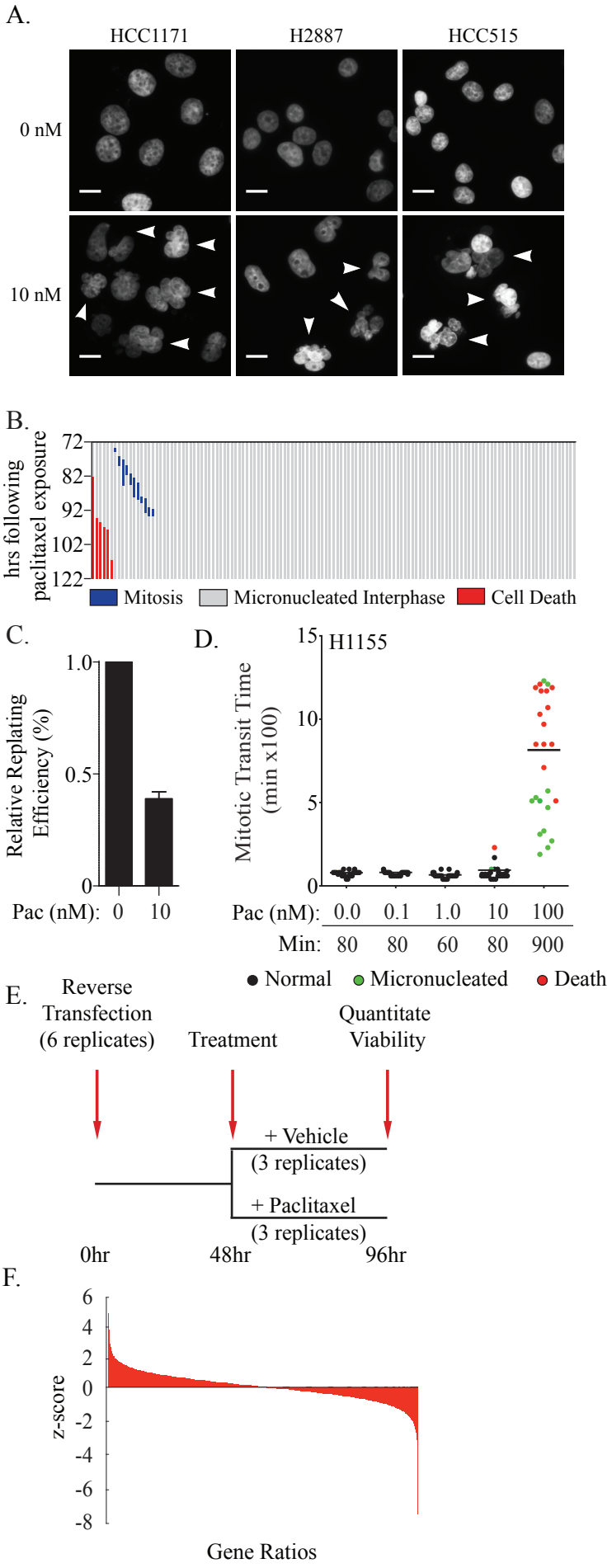
Supplementary Table 2.

High interest chemosensitizers based on siRNAs isolated from pan-genomic screen. See supplemental materials and methods for filter method.

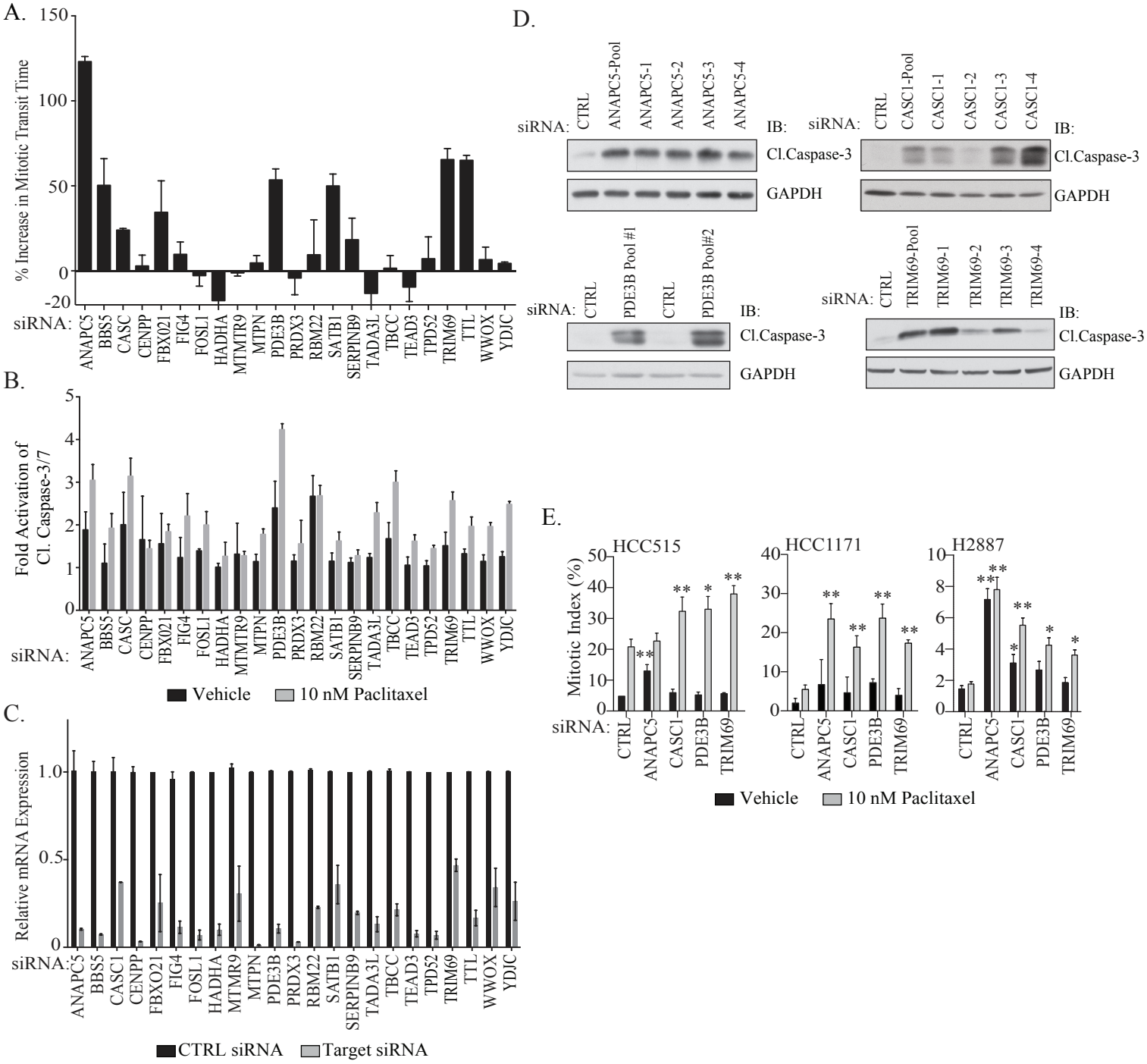
Supplementary Video 1. Micronucleated mitosis

HCC366GFP-H2B cells were exposed to paclitaxel for 48 hours and imaged for a subsequent 48 hours. During imaging, frames were acquired at 20-minute intervals and the movie is presented at 6 frames per second.

Sinnott_ Figure S1

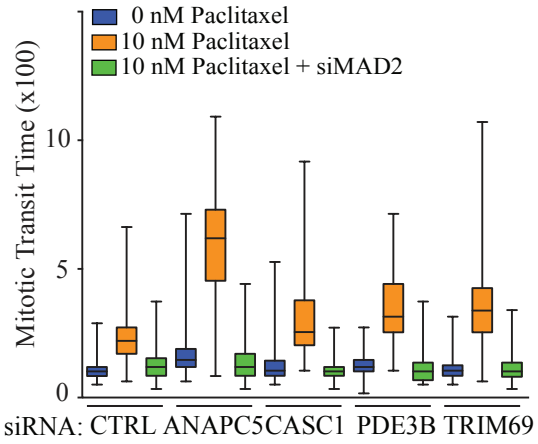


Sinnott Figure S2



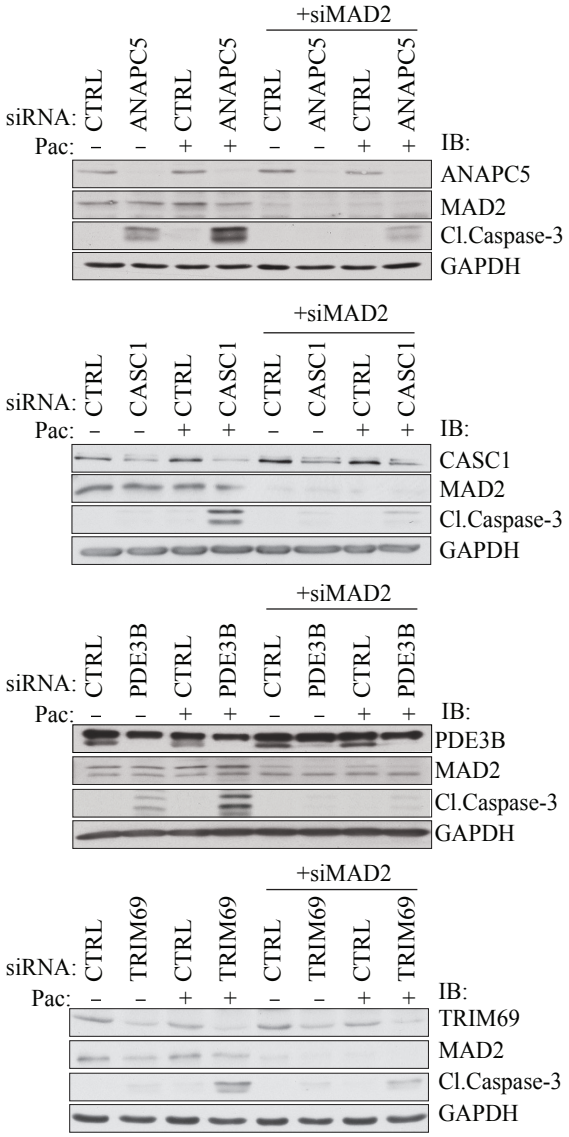
Sinnott Figure S3

A.

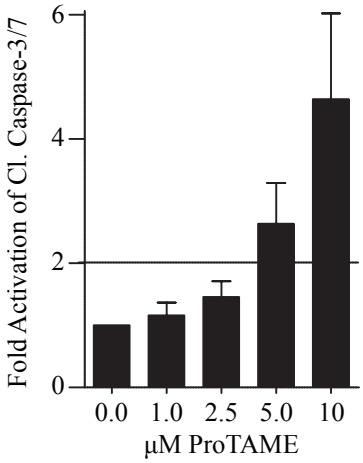


C.

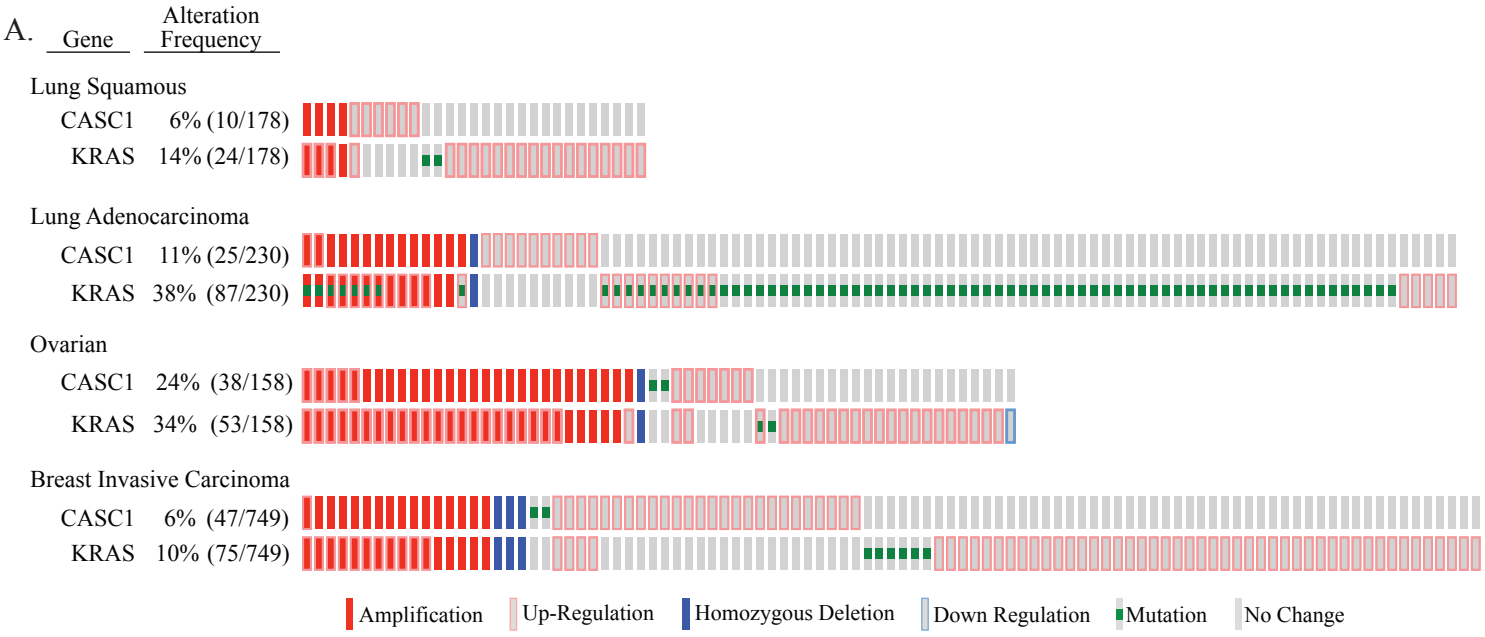
B.

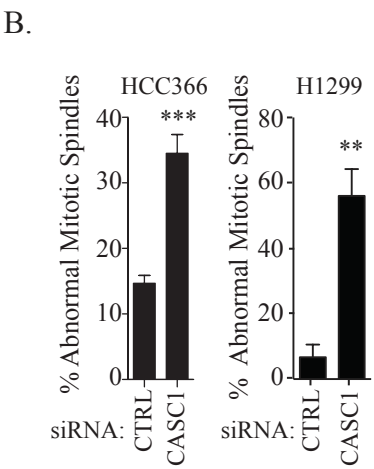


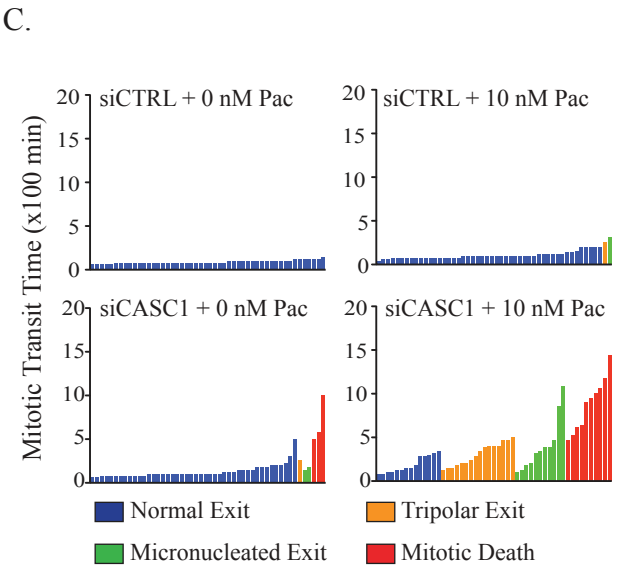
D.

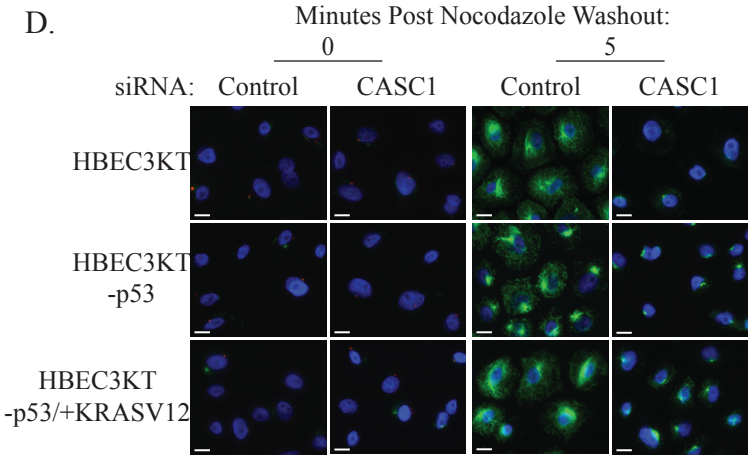


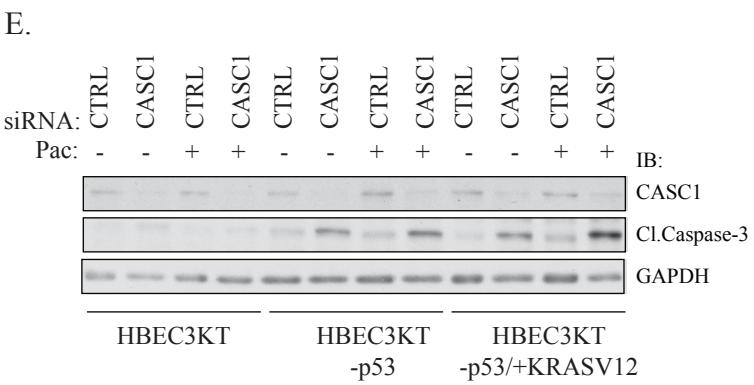
Sinnott Figure S4





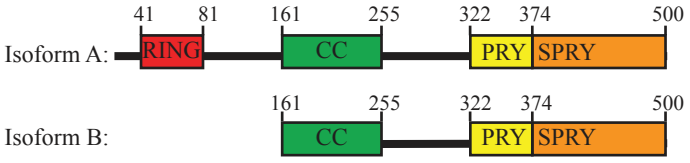






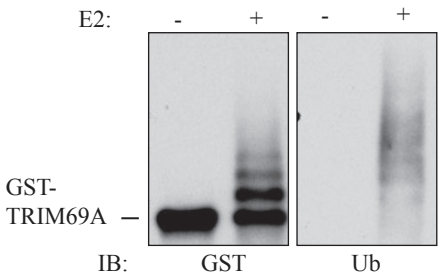
Sinnott Figure S5

A.

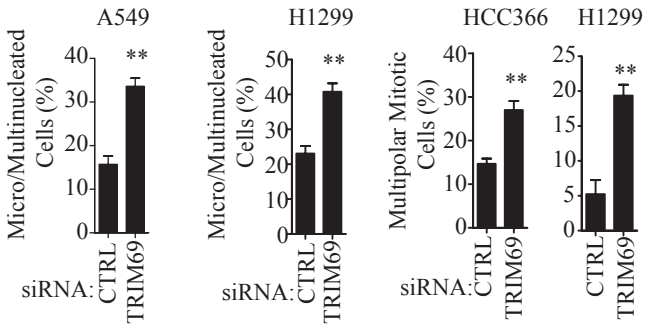


RING = Really Interesting New Gene
CC = Coiled Coil
PRY = PYRin family genes
SPRY = SP1A and in RYanodine receptor subtypes

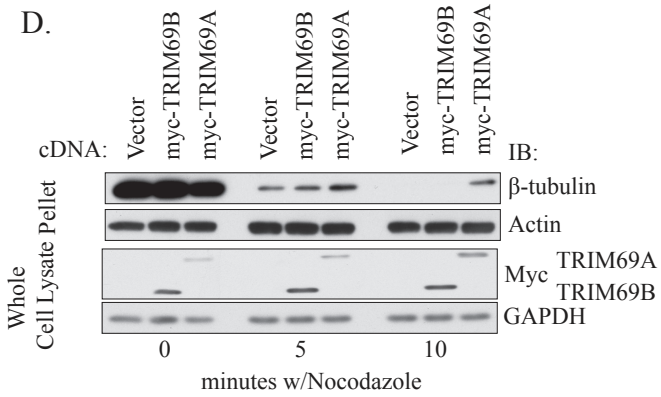
B.



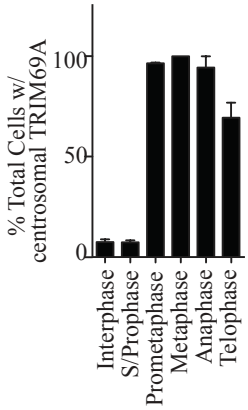
C.



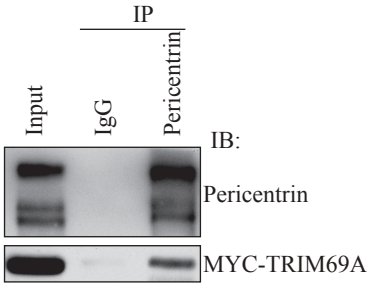
D.



E.



F.



G.