

Supplemental Methods

Antibodies. The following antibodies were used in the supplementary figures: phosphoS473-AKT (44621G) from Biosource Inter. (Camarillo, CA); total AKT (610860) from BD Biosciences (San Jose, CA); vinculin (V4505) from Sigma-Aldrich (St. Louis, MO); cyclin D1 (sc718), B-RAF (sc5284) and p53 (sc126) from Santa Cruz Biotech; α v β 3 (LM609), β 1 (P4C10), α 1 (FB12) and α 2 (P1E6) integrin antibodies from Millipore.

shRNA sequences. The following oligonucleotide sequences were used for shRNAs.

Control: 5'-

CACCGTAGCGACTAAACACATCAATTCAAGAGATTGATGTGTTTAGTCGCTA and 5'-AAAATAGCGACTAAACACATCAATTCTCTTGAATTGATGTGTTTAGTCGCTAC.

Rnd3^{#1}:

5'-CACCGCTACAGTGTTTGAGAATTATTCAAGAGATAATTCTCAAACACTGTAG and 5'-AAAACACTACAGTGTTTGAGAATTATTCTCTTGAATAATTCTCAAACACTGTAGC

Rnd3^{#2}:

5'-CACCGCGGACAGATGTTAGTACATTCAAGAGATGTACTAACATCTGTCCGC and 5'-AAAAGCGGACAGATGTTAGTACATTCTCTTGAATGTACTAACATCTGTCCGC. The

underlined bases signify the hairpin.

Collagen gel adhesion. Cell adhesion was performed using WM793TR Ctl and Rnd3 shRNA cells after being treated +/- dox for 3 days. An equal number of cells were resuspended and plated onto a layer of bovine collagen I gel for increasing times (5, 15, 30, 60 and 90 min). Gels were washed with PBS and fixed with 3.7% paraformaldehyde. The number of cells occupying six different fields viewed by x20 magnification using an Olympus IX-70 inverted

microscope was counted. Experiments were performed in triplicate wells and reproduced in three independent experiments.

Supplemental Figure Legends

Supplementary Figure 1. Endogenous Rnd3 regulates alterations in F-actin organization in melanoma. *A*, Examples of WM793TR melanoma cell actin organization demonstrating reduced, normal or increased actin stress fibers. Bar, 50 μm . *B-C*, Doxycycline-inducible WM115 melanoma cells expressing Rnd3^{#2} shRNA cultured for 72 hours \pm 0.1 $\mu\text{g/ml}$ dox. *B*, Cell lysates were analyzed by western blotting for Rnd3 and β -actin. *C*, F-actin organization visualized using TRITC-phalloidin. Bar, 50 μm . *D*, Serum-containing medium is required for Rnd3 knockdown-induced stress fiber formation. WM793TR-Rnd3^{#2} shRNA cells were plated on glass coverslips for 24 hours in complete medium. Afterwards, the cell culture medium was replaced with either complete medium \pm dox or serum-free medium containing 0.5 % BSA \pm dox for 72 hours. Cells were then fixed and processed to visualize F-actin organization using TRITC-phalloidin. Bar, 50 μm .

Supplementary Figure 2. Depletion of endogenous Rnd3 alters melanoma focal adhesions. *A*, Vinculin localization in the WM793TR Ctl, Rnd3^{#1} and Rnd3^{#2} shRNA melanoma cells. Cells cultured \pm dox for 72 hours were fixed and stained with anti-vinculin antibody. Vinculin staining was visualized using Alexa-Fluor 488 conjugated anti-mouse secondary antibodies. Bar, 50 μm .

Supplementary Figure 3. Rnd3 expression does not alter cell surface integrin expression or adhesion to collagen gel. Melanoma cells were cultured in complete medium \pm 0.1 $\mu\text{g/ml}$ dox for 72 hours to induce expression of Ctl, Rnd3^{#1} or Rnd3^{#2} shRNA. *A*, Cell surface expression of

integrins $\alpha v\beta 3$, $\beta 1$, $\alpha 1$ and $\alpha 2$ was analyzed by flow cytometry. Depicted are representative traces from one of two-three independent experiments. *B*, Time course of cell adhesion to collagen gel. Equal numbers of cells were treated \pm dox for 72 hours and then replated onto bovine type I collagen gel for the indicated times (5, 15, 30, 60, 90 min). Cell layers were washed, fixed and counted. Graphed is the mean \pm SD from one experiment performed in triplicate. Two independent experiments were performed with comparable results.

Supplementary Figure 4. Depletion of endogenous Rnd3 does not alter ERK1/2 and AKT phosphorylation or cyclin D1 expression in melanoma cells. Dox-inducible WM793 melanoma cells expressing either Ctl, Rnd3^{#1} or Rnd3^{#2} shRNA were cultured for 72 hours \pm dox. Cell lysates were generated and analyzed by western blotting for *A*, phospho ERK1/2 and total ERK2 levels; *B*, phospho(pS473) AKT and total AKT levels; and *C*, cyclin D1 and ERK2 levels.

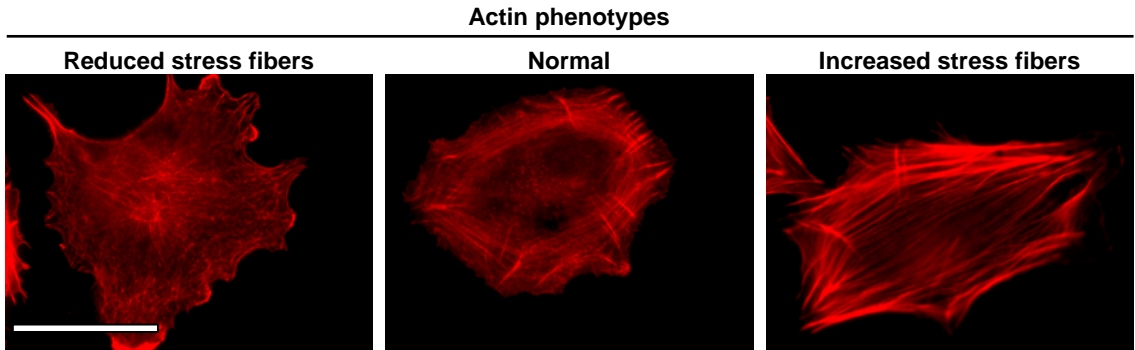
Supplementary Figure 5. Diagram outlining the role of Rnd3 in the regulation of signaling pathways and melanoma cell migration. High Rnd3 expression in invasive melanoma provides a negative feedback loop to constrain RhoA-ROCK signaling via p190RhoGAP, and possibly additional effectors. When expression of Rnd3 is low, elevated RhoA-ROCK signaling supports the phosphorylation of myosin light chain and cofilin, and promotes actin stress fiber formation.

Supplementary Figure 6. Rnd3 expression is p53 independent in WM793 melanoma cells. WM793 cells were transfected with siRNA duplexes targeting Ctl, B-RAF or p53 for 72 hours. Ctl, B-RAF^{#1} and p53 siRNAs have been previously published (1, 2). Cell lysates were subjected to Western blot analysis using antibodies for B-RAF, p53, Rnd3 and ERK2.

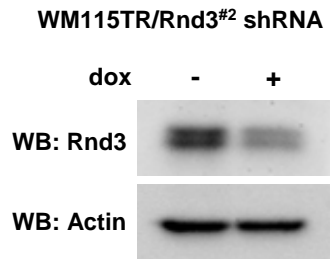
References

1. Klein RM, Spofford LS, Abel EV, Ortiz A, Aplin AE. B-RAF regulation of Rnd3 participates in actin cytoskeletal and focal adhesion organization. *Mol Biol Cell* 2008;19:498-508.
2. Hu R, Aplin AE. Skp2 regulates G2/M progression in a p53-dependent manner. *Mol Biol Cell* 2008;19:4602-10.

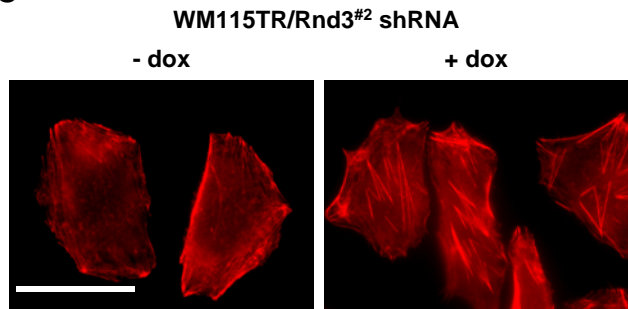
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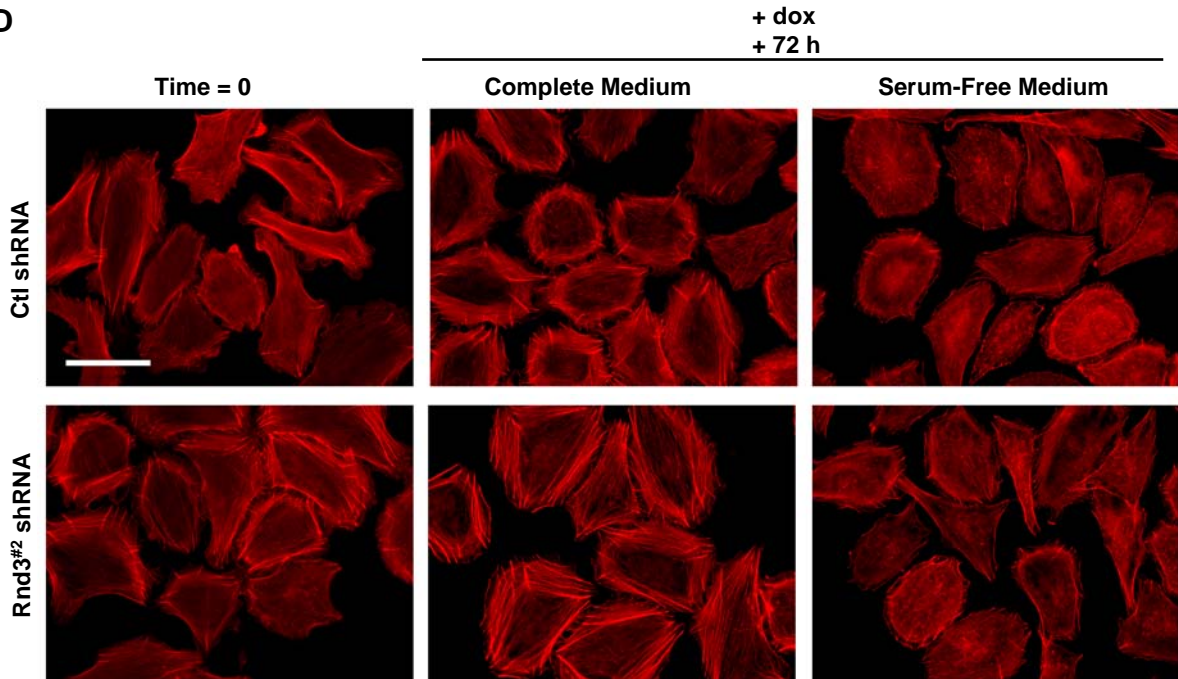
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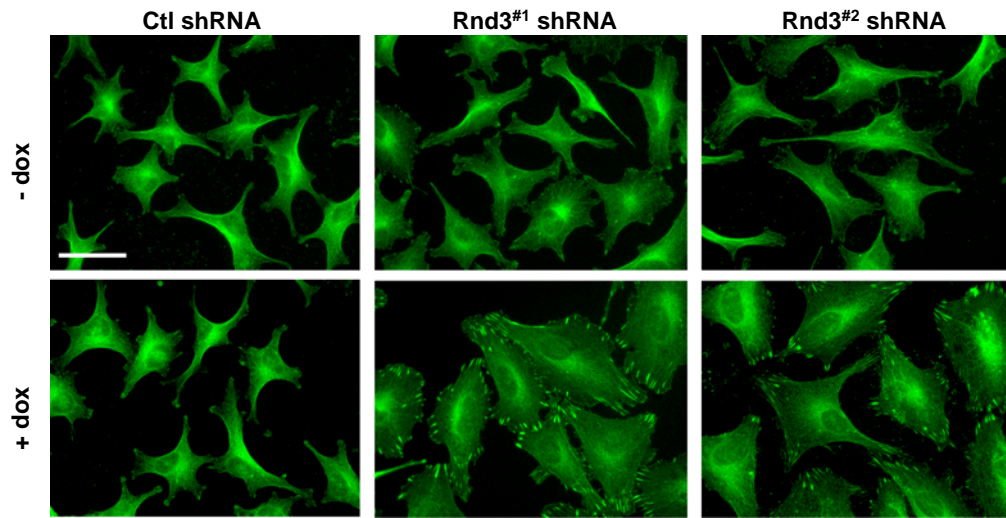


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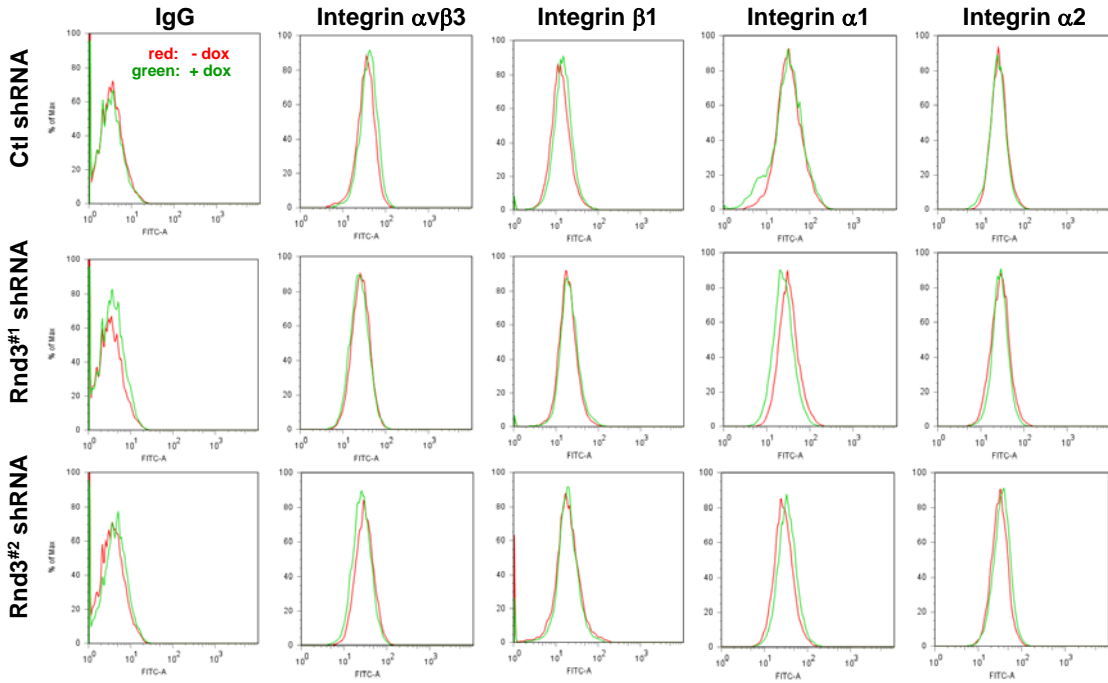


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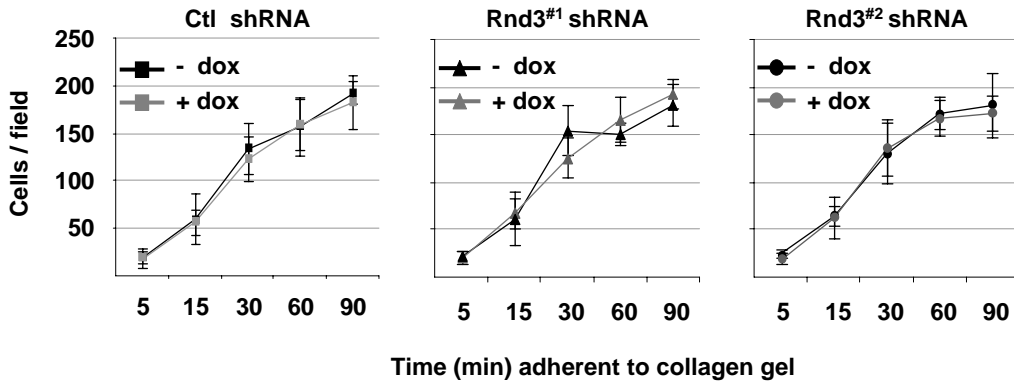


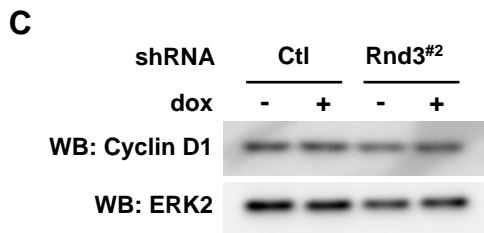
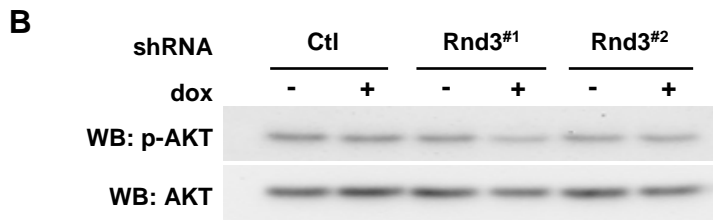
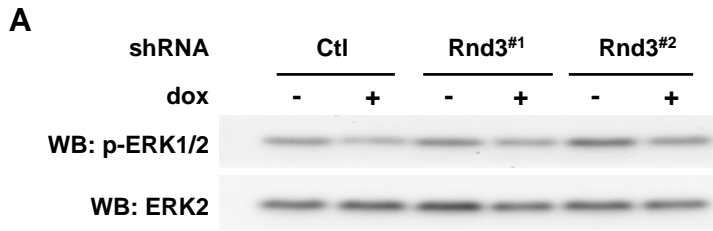


A



B

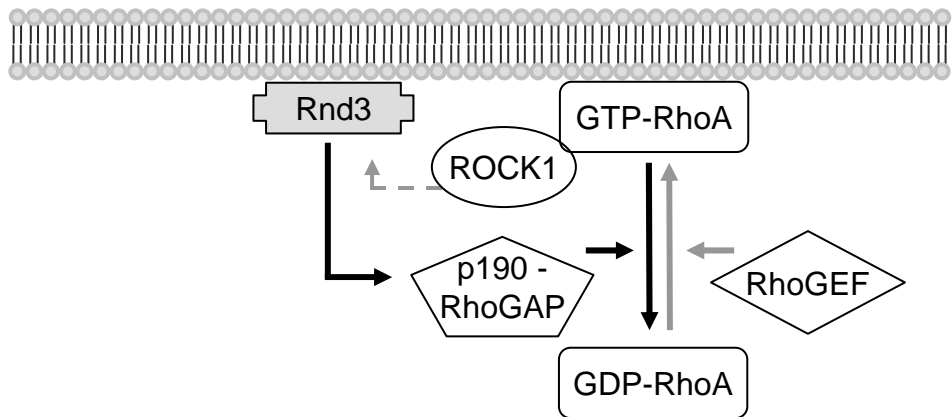




High Rnd3 Expression

Suppressed Actin Stress Fibers – 2D

Efficient Cell Migration – 3D



Low Rnd3 Expression

Increased Actin Stress Fibers – 2D

Reduced Cell Migration – 3D

