Science Signaling
AAAS

www.sciencesignaling.org/cgi/content/full/6/265/ra14/DC1

Supplementary Materials for

The Small GTPase ARF6 Stimulates β-Catenin Transcriptional Activity During WNT5A-Mediated Melanoma Invasion and Metastasis

Allie H. Grossmann, Jae Hyuk Yoo, James Clancy, Lise K. Sorensen, Alanna Sedgwick, Zongzhong Tong, Kirill Ostanin, Aaron Rogers, Kenneth F. Grossmann, Sheryl R. Tripp, Kirk R. Thomas, Crislyn D'Souza-Schorey, Shannon J. Odelberg,* Dean Y. Li*

*To whom correspondence should be addressed. E-mail: dean.li@u2m2.utah.edu (D.Y.L.); sodelber@genetics.utah.edu (S.J.O.)

Published 5 March 2013, *Sci. Signal.* **6**, ra14 (2013)
DOI: 10.1126/scisignal.2003398

The PDF file includes:

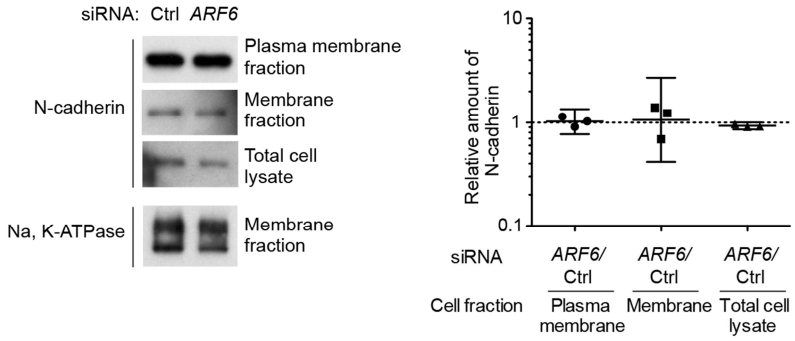
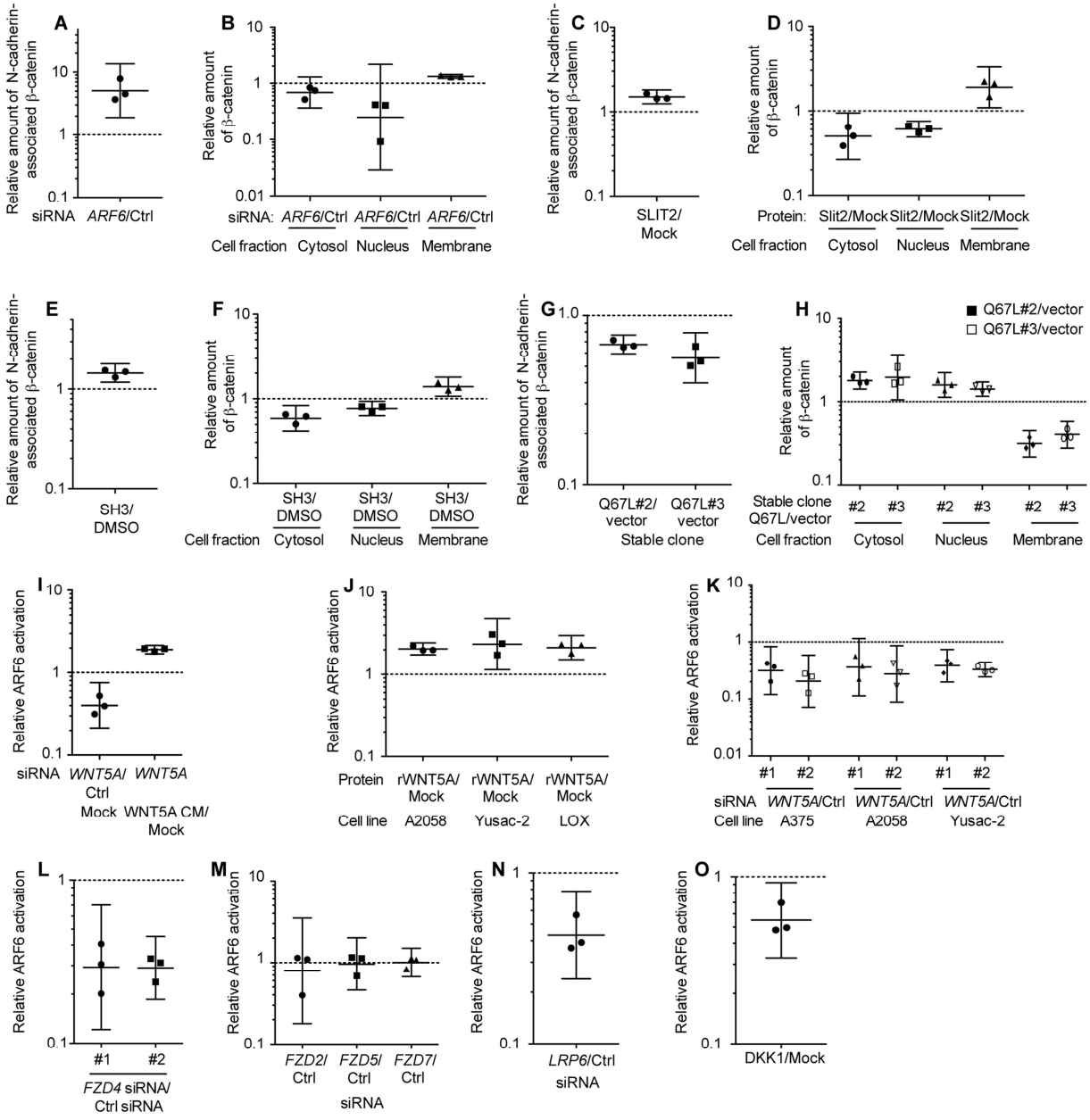


Figure S1: ARF6 knockdown does not alter total N-cadherin protein at the plasma membrane. N-cadherin immunoblots of plasma membrane fractions, membrane fractions, and total cell lysates from LOX cells treated with Control (Ctrl) or *ARF6* siRNAs. Scatter plot shows quantification of immunoblots. Data points = individual experiments (*n=3*). Solid line within data points = geometric mean. Error bars = 95% CI.


A
Relative amount of N-cadherin-associated β-catenin
siRNA ARF6/Ctrl
B
Relative amount of β-catenin
siRNA: ARF6/Ctrl ARF6/Ctrl ARF6/Ctrl
Cell fraction Cytosol Nucleus Membrane
C
Relative amount of N-cadherin-associated β-catenin
SLIT2/ Mock
D
Relative amount of β-catenin
Protein: Slit2/Mock Slit2/Mock Slit2/Mock
Cell fraction Cytosol Nucleus Membrane
E
Relative amount of N-cadherin-associated β-catenin
SH3/ DMSO
F
Relative amount of β-catenin
SH3/ DMSO SH3/ DMSO SH3/ DMSO
Cell fraction Cytosol Nucleus Membrane
G
Relative amount of N-cadherin-associated β-catenin
Q67L#2/ vector Q67L#3 vector
Stable clone
H
Q67L#2/vector
Q67L#3/vector
Relative amount of β-catenin
Stable clone #2 #3 #2 #3 #2 #3
Q67L/vector
Cell fraction Cytosol Nucleus Membrane
I
Relative ARF6 activation
siRNA WNT5A/ Ctrl WNT5A
Mock Mock WNT5A CM/ Mock
J
Relative ARF6 activation
Protein rWNT5A/ Mock rWNT5A/ Mock rWNT5A/ Mock
Cell line A2058 Yusac-2 LOX
K
Relative ARF6 activation
#1 #2 #1 #2 #1 #2
siRNA WNT5A/Ctrl WNT5A/Ctrl WNT5A/Ctrl
Cell line A375 A2058 Yusac-2
L
Relative ARF6 activation
#1 #2
FZD4 siRNA/ Ctrl siRNA
M
Relative ARF6 activation
FZD2/ Ctrl FZD5/ Ctrl FZD7/ Ctrl
siRNA
N
Relative ARF6 activation
LRP6/Ctrl siRNA
O
Relative ARF6 activation
DKK1/Mock

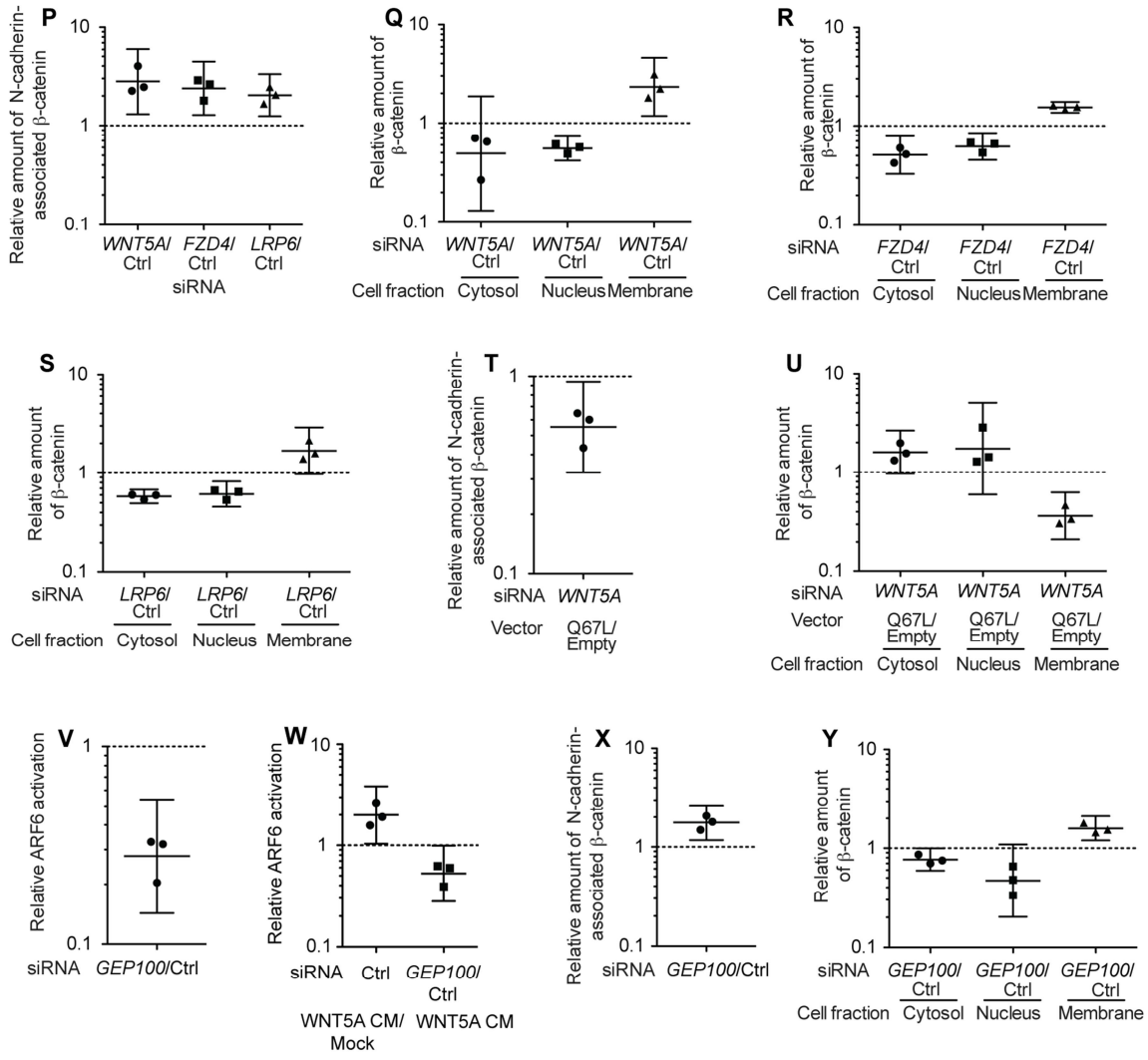


Figure S2: Quantification of immunoblots from main figures. Graphs relate to (A) Fig. 1A, (B) Fig. 1B, (C) Fig. 1C, (D) Fig. 1D, (E) Fig. 1E, (F) Fig. 1F, (G) Fig. 1G, (H) Fig. 1H, (I) Fig. 2A, (J) Fig. 2B, (K) Fig. 2C, (L) Fig. 2D and S9A, (M) Fig. 2E, (N) Fig. 2F, (O) Fig. 2G, (P) Figs. 3A and 3B, (Q) Fig. 3C, (R) Fig. 3D, (S) Fig. 3E, (T) Fig. 4A, (U) Fig. 4B, (V) Fig. 6A, (W) Fig. 6B, (X) Fig. 6C, (Y) Fig. 6D. (A) to (Y) Data points = individual experiments ($n \geq 3$). Solid line within data points = geometric mean. Error bars = 95% CI.

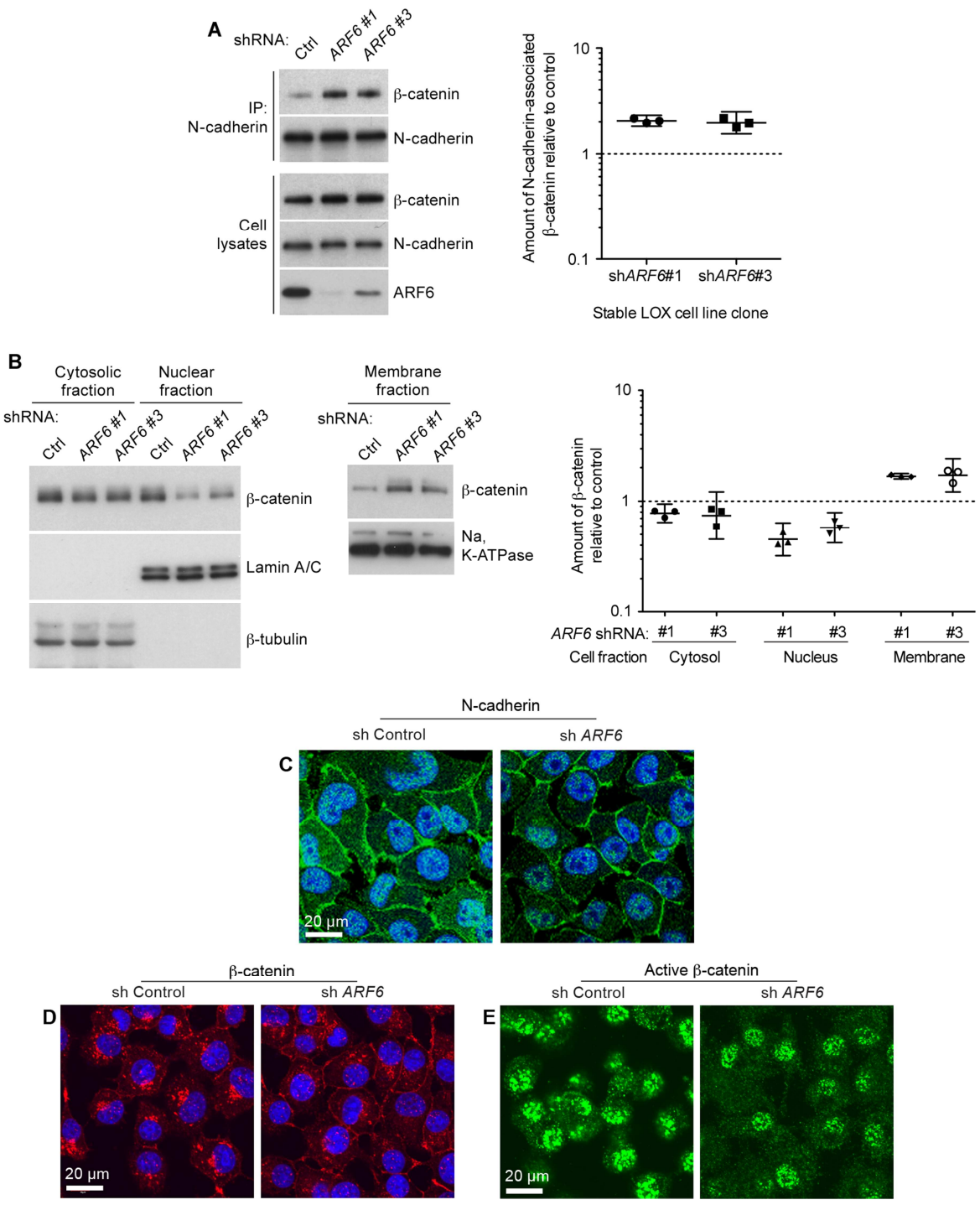

A
shRNA:
Ctrl
ARF6 #1
ARF6 #3
IP:
N-cadherin
β-catenin
N-cadherin
Cell lysates
β-catenin
N-cadherin
ARF6
Amount of N-cadherin-associated β-catenin relative to control
10
1
0.1
shARF6#1
shARF6#3
Stable LOX cell line clone
B
Cytosolic fraction
Nuclear fraction
Membrane fraction
shRNA:
Ctrl
ARF6 #1
ARF6 #3
β-catenin
Lamin A/C
β-tubulin
Na, K-ATPase
Amount of β-catenin relative to control
ARF6 shRNA:
#1
#3
Cell fraction
Cytosol
Nucleus
Membrane
C
N-cadherin
sh Control
sh ARF6
20 μm
D
β-catenin
sh Control
sh ARF6
20 μm
E
Active β-catenin
sh Control
sh ARF6
20 μm

Figure S3

Figure S3: ARF6 knockdown drives β-catenin from the nucleus to N-cadherin.(A) Coimmunoprecipitation of N-cadherin and β-catenin and (B) subcellular fractionation of β-catenin in LOX cells stably expressing Control (Ctrl) or *ARF6* (sh*ARF6* #1 and #3) shRNAs. Scatter plots show quantification of immunoblots. Data points = individual experiments (*n=3*). Solid line within data points = geometric mean. Error bars = 95% CI. (C) to (E) Immunofluorescent staining of (C) N-cadherin, (D) total β-catenin, and (E) active β-catenin in LOX cells stably expressing Ctrl or *ARF6* (sh*ARF6*) shRNAs. ARF6 silencing does not alter N-cadherin localization at the membrane (C) but results in more β-catenin at the cell surface (D) and less in the nucleus (E). (C) to (E) 600X magnification + 2X zoom. For all, *n=3* experiments.

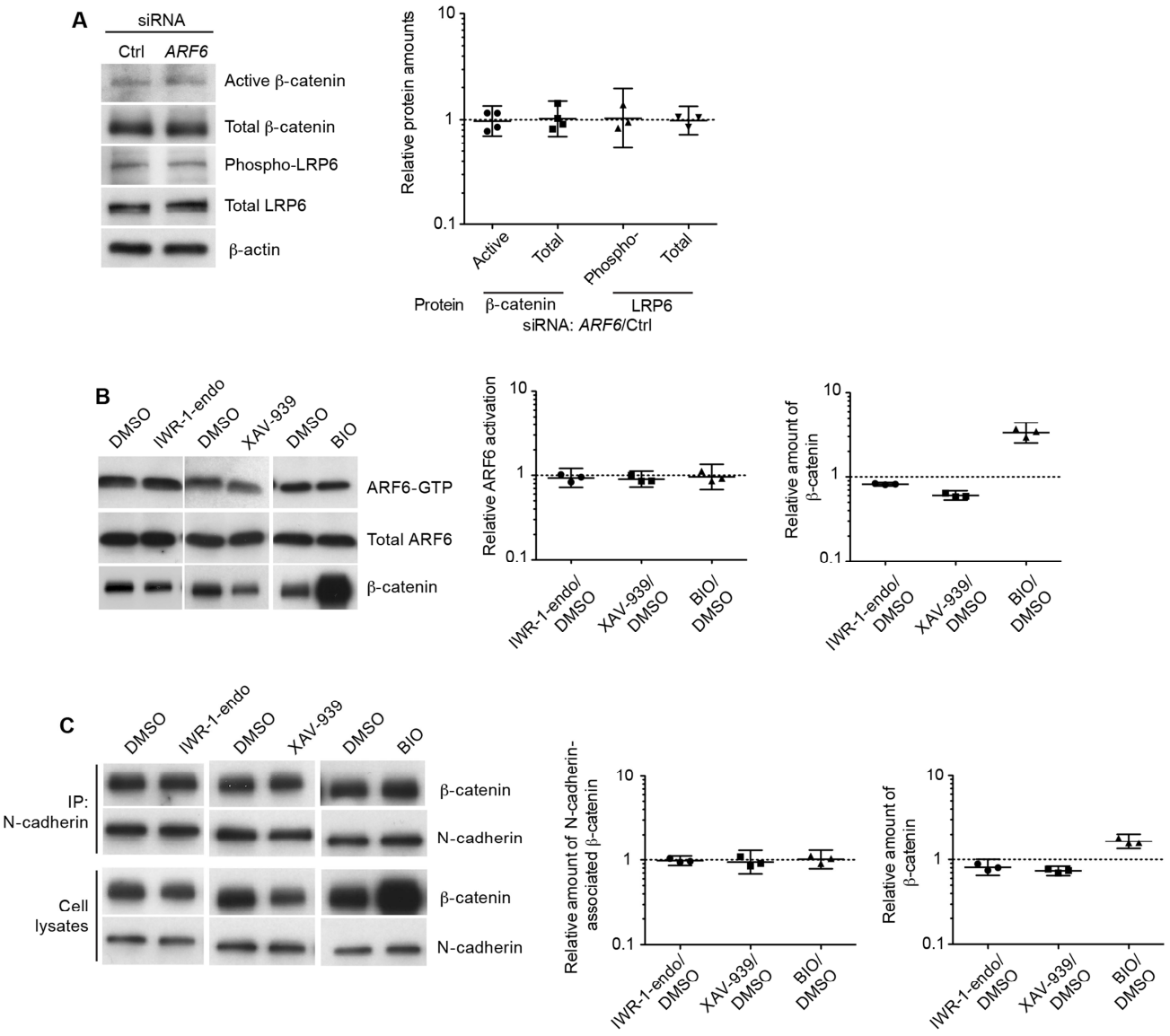


Figure S4: ARF6, junctional β-catenin, and the canonical destruction complex. (A) Immunoblots of active β-catenin, total β-catenin, phosphorylated LRP6, and total LRP6 from LOX cells treated with Control (Ctrl) or *ARF6* siRNAs. (B) GGA3-pulldown of ARF6-GTP in LOX cells treated with IWR-1-*endo*, XAV-939, or BIO. (C) Coimmunoprecipitation of N-cadherin and β-catenin in LOX cells treated with IWR-1-*endo* or XAV-939, or BIO. Scatter plot

shows quantification of immunoblots. Data points = individual experiments (*n=3*). Solid line within data points = geometric mean. Error bars = 95% CI.

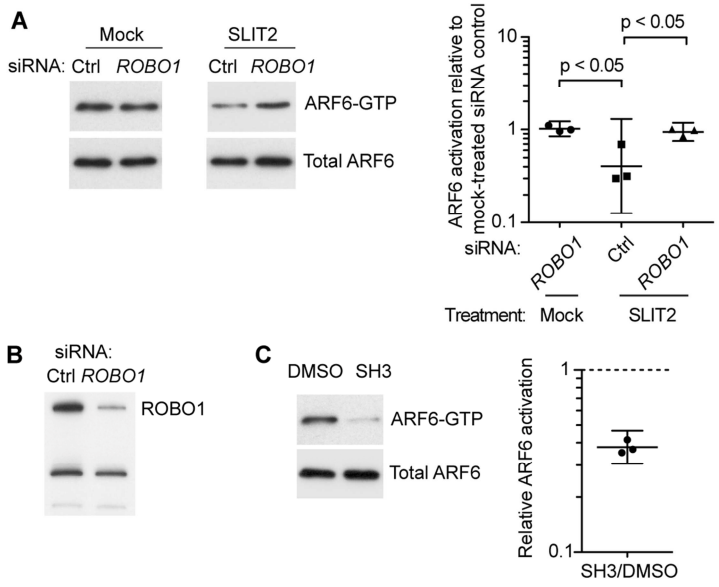


Figure S5: SLIT2-ROBO1 and SecinH3 inhibit ARF6 activation. (A) GGA3-pull down of ARF6-GTP in LOX cells transfected with Control (Ctrl) or *ROBO1* siRNAs and treated with Mock or SLIT2. For plotting purposes, geometric mean and 95% CI were estimated separately for each treatment (two-way ANOVA, Tukey's post hoc test with experiment factor treated as a blocking factor in the statistical analysis). (B) Immunoblot of ROBO1 in LOX cells transfected with Ctrl or *ROBO1* siRNAs. (C) GGA3-pull down of ARF6-GTP in LOX cells treated with DMSO or SecinH3 (SH3). (C) Scatter plot shows quantification of immunoblots. Data points = individual experiments ($n=3$). Solid line within data points = geometric mean. Error bars = 95% CI.

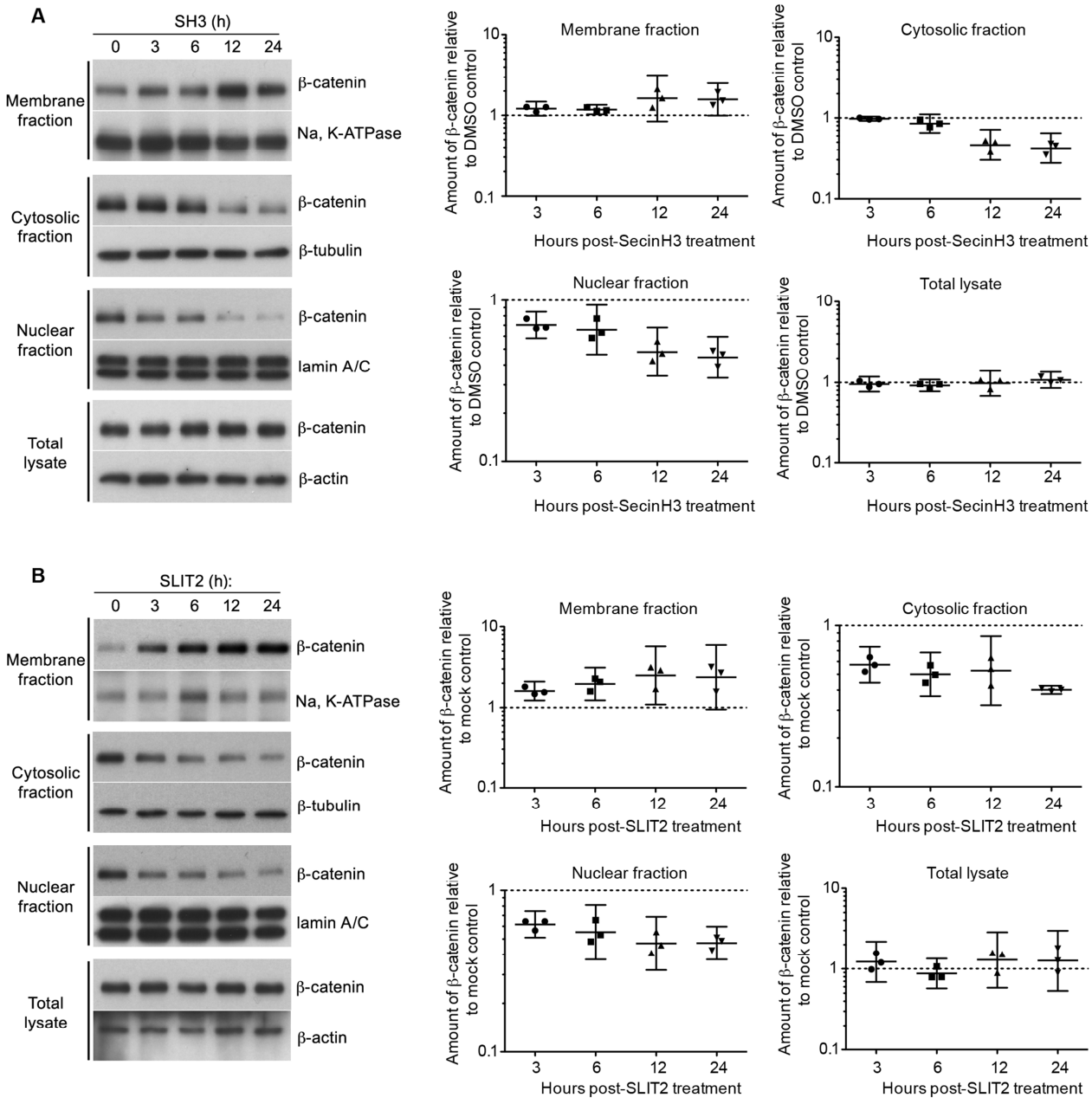


Figure S6: Time course of β-catenin relocalization after ARF6 inhibition. Subcellular fractionation of LOX cells after treatment with (A) SecinH3 (SH3) or (B) SLIT2 at 0, 3, 6, 12, or 24 hours (h). (A) and (B) A shift in β-catenin from the cytosol and nucleus to the membrane fraction was apparent after 3 hours and persisted at each subsequent interval. Scatter plots show

quantification of immunoblots. Data points = individual experiments (*n=3*). Solid line within data points = geometric mean. Error bars = 95% CI.

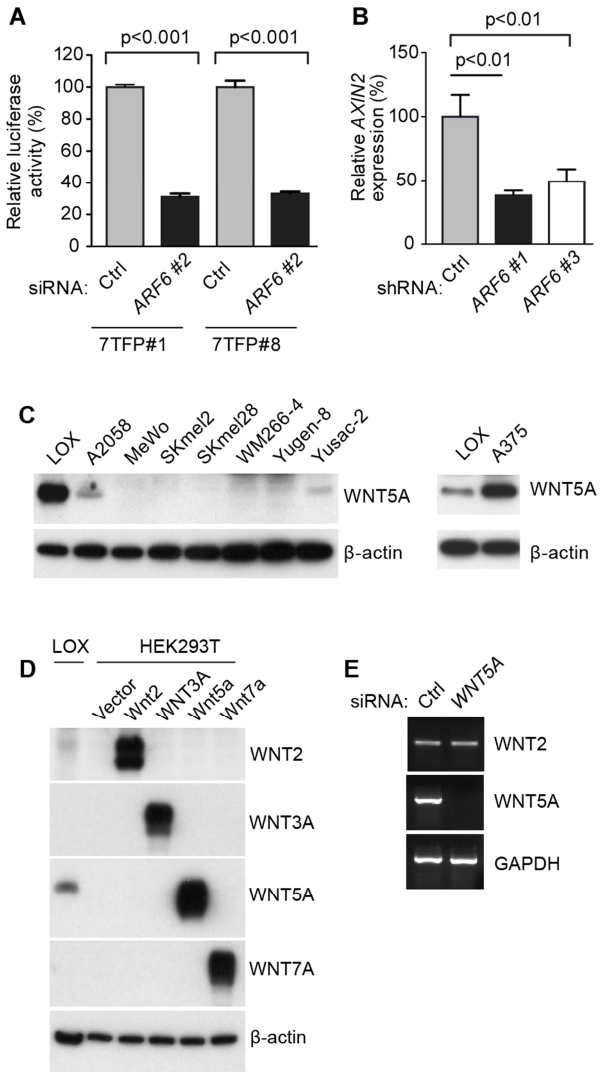


Figure S7: ARF6-dependent β-catenin transactivation and relative WNT production in melanoma cell lines. (A) 7TFP luciferase assay and (B) *Axin2* qRT-PCR in LOX cells stably expressing Control (Ctrl) or *ARF6* (shARF6 #1 and #3) shRNAs (related to Fig. 1I). (C) WNT5A is abundant in LOX and A375 cells and present in A2058 and Yusac2 cells. (D) LOX cells produce low amounts of WNT2, but WNT3A and WNT7A were not detected. (D) HEK293T cells with ectopic overexpression of WNT2, WNT3A, WNT5A, or WNT7A act as immunoblot positive controls. (E) *WNT2* expression is not altered following WNT5A knockdown (see also

Fig. S6A-B). (A) Two-tailed t test. (B) One-way ANOVA with Dunnett's post hoc test test.

For all, error bars = SD, *n=3* experiments.

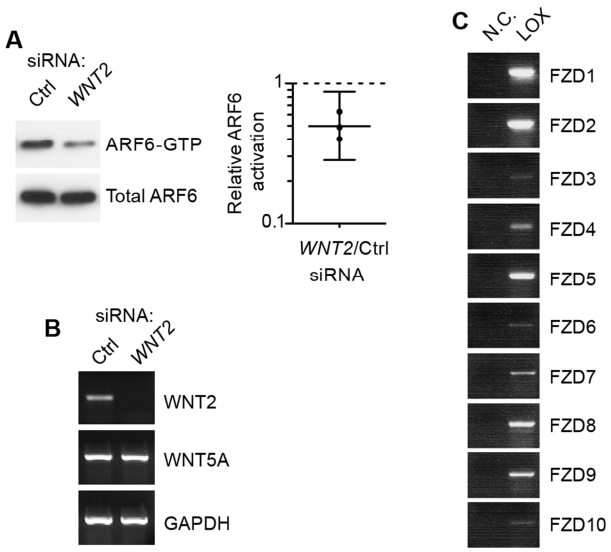


Figure S8: WNT2 knockdown reduces ARF6 activation. (A**)** GGA3 pulldown of ARF6-GTP and (B) confirmation of *WNT2* knockdown with RT-PCR in LOX cells transiently transfected with Control (Ctrl) or *WNT2* siRNAs. (A) Scatter plot shows quantification of immunoblots. Data points = individual experiments (*n=3*). Solid line within data points = geometric mean. Error bars = 95% CI. (B) *WNT5A* mRNA expression, as detected by RT-PCR, is not reduced by siRNA to *WNT2* (related to Fig. S5D). (**C**) Expression of Frizzled (*FZD*) family members in LOX cells, evaluated by RT-PCR. N.C. = no template control. *n=3* experiments.

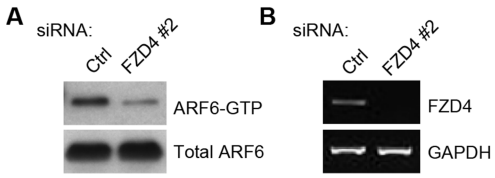


Figure S9: FZD4 knockdown reduces ARF6 activation. (A) GGA3 pulldown of ARF6-GTP and (B) confirmation of knockdown by RT-PCR in LOX cells transiently transfected with Control (Ctrl) or *Frizzled 4* sequence #2 (*FZD4 #2*) siRNAs. See also Fig. 2D and S2L.

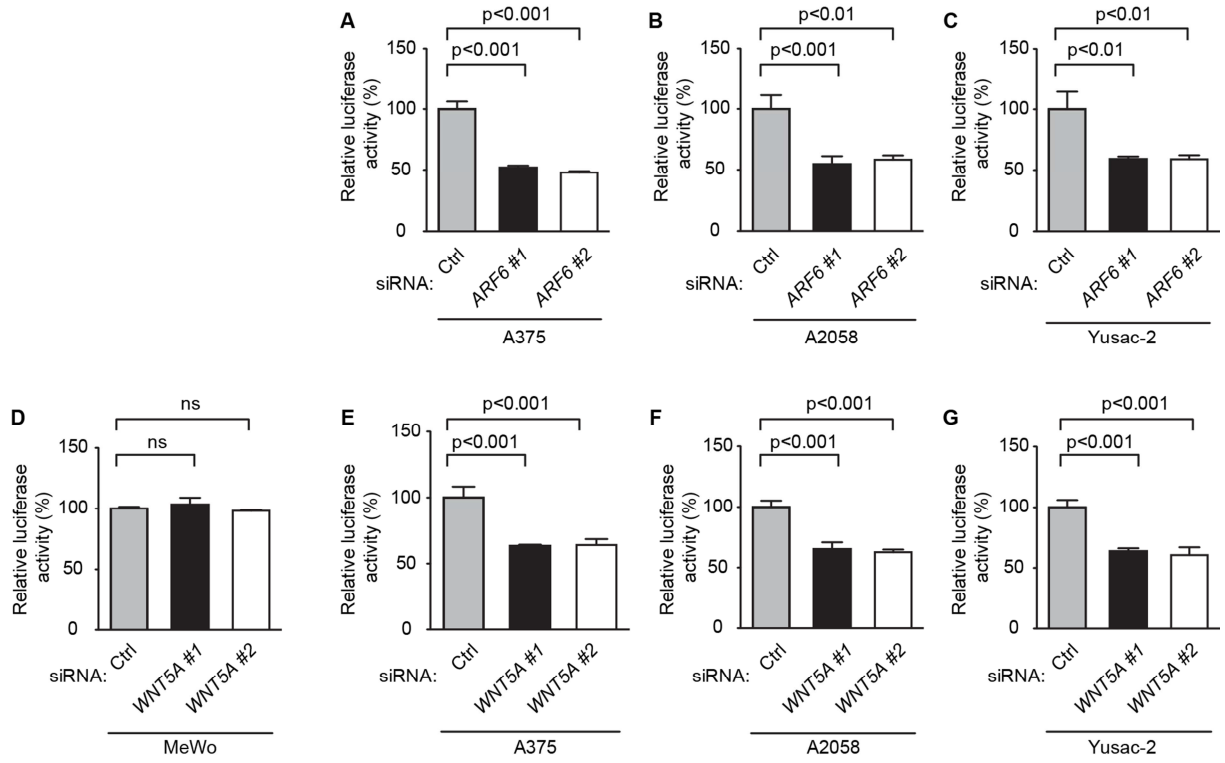


Figure S10: WNT5A, ARF6, and β-catenin signaling in multiple human melanoma cell lines. (A) to (G) 7TFP luciferase assay for β-catenin-mediated transcription in A375, A2058,Yusac-2, and MeWo melanoma cells. (A) to (C) Cells treated with Control (Ctrl) or *ARF6* (ARF6 #1 and #2) siRNAs. (D) to (G) Cells treated with Ctrl or *WNT5A* (WNT5A #1 and #2) siRNAs. (D) MeWo cells, which do not produce WNT5A (see Fig. S7C), were used as a negative control to show the specificity of the *WNT5A* siRNAs. For all, one-way ANOVA followed by Dunnett's post hoc test, error bars = SD, *n=3* experiments.

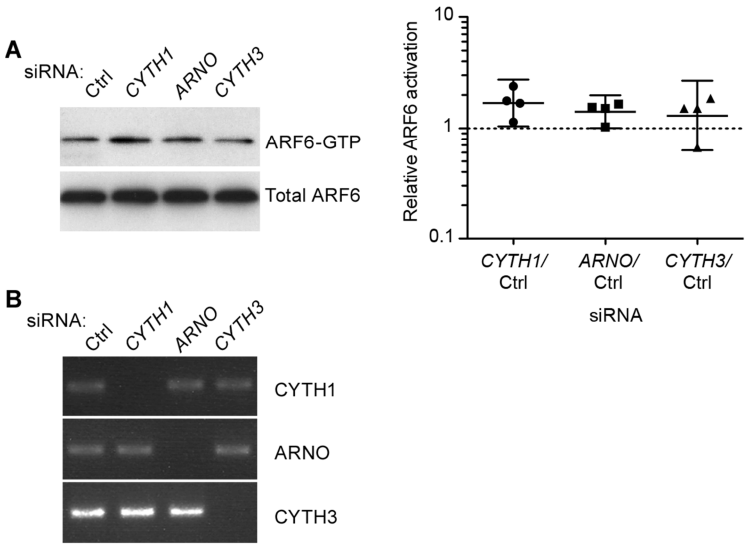


Figure S11: Cytohesin knockdown does not reduce ARF6 activation. (A) GGA3 pulldown of ARF6-GTP and (B) confirmation of knockdown by RT-PCR in LOX cells transiently transfected with Control (Ctrl) or *cytohesion 1* (*CYTH1*), *cytohesin-2* (also called *ARNO*), or *cytohesion 3* (*CYTH3*) siRNAs. (A) Scatter plot shows quantification of immunoblots. Data points = individual experiments (*n=4*). Solid line within data points = geometric mean. Error bars = 95% CI.

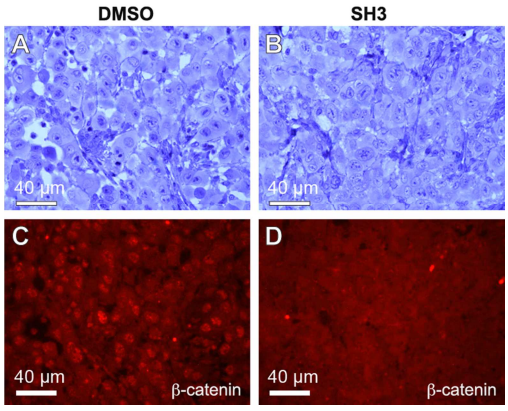


Figure S12: β-Catenin immunostaining of LOX melanoma xenograft tumors. (A) to (B) Representative bright field images of hematoxylin-counterstain and (C) to (D) fluorescent images of β-catenin staining (Alk Phos Red) of LOX melanoma xenograft tumors from mice treated with (A) and (C) DMSO vehicle or (B) and (D) SecinH3 (SH3). 400X magnification. *n=7* primary tumors from each treatment group.

Supplementary Table 1. siRNA and shRNA sequences.

Target Gene Symbol	**siRNA ID**	**Sense Sequence 5'to 3'**	**Vendor**
Control	AllStars Neg. Control siRNA		Qiagen
ARF6 #1	SI02757286	CAACGTGGAGACGGTGACTTA	Qiagen
ARF6 #2	S1565	GUCUCAUCUUCGUAGUGGATT	Ambion
WNT5a #1 (pooled)	J-003939-09	GCCAAGGGCUCCUACGAGA	Thermo
	J-003939-10	GUUCAGAUGUCAGAAGUAU	Thermo
	J-003939-11	CAUCAAAGAAUGCCAGUAU	Thermo
	J-003939-12	GAAACUGUGCCACUUGUAU	Thermo
WNT5a #2	S100051779	CCGGATAACCTTGTAACATAT	Qiagen
FZD4 #1	S15840	CAGUAUGUGCUAUAAUAUUTT	Ambion
FZD4 #2	S100097965	TAGGTGATCGATACTTGTCAA	Qiagen
FZD2	S10275743	CACGGTCTACATGATCAAATA	Qiagen
FZD5	S102757650	TAAGGTTGGCGTTGTAATGAA	Qiagen
FZD7	SI02631237	TCACCTACCTGGTGGACATGC	Qiagen
LRP6 (pooled)	J-003845-12	GCUCAACCGUGAAGUUAUA	Thermo
	J-003845-11	CCACAGAGCGAUCACAUUA	Thermo
	J-003845-10	CAGAUGAACUGGAUUGUUA	Thermo
	J-003845-09	GCAGAUAUCAGACGAAUUU	Thermo
ROBO1	S103055472	CACAAGGGCTCTCAAAGTATA	Qiagen
WNT2	S104271694	CAGGAAGGCTGTAAAGCGGTT	Qiagen
CYTH1	S104217185	CGGGACAGAGGTTCCGGATAA	Qiagen
ARNO	S100061299	CACGCTGTTGGTAATCTTATT	Qiagen
CYTH3	S100061257	CAGCATGTTGTGCTCGGACAA	Qiagen
GEP100	S103019408	CTGAAGGGTAGCAGTAATGAA	Qiagen
Target Gene Symbol	**shRNA**	**Sense Sequence 5'to 3'**	**Vendor**
ARF6_#1	TRCN0000048003	GTCAAGTTCAACGTATGGGAT	SIGMA
ARF6_#3	TRCN0000048005	CTCACATGGTTAACCTCTAACT	SIGMA

Supplementary Table 2. Primer sequences for RT-PCR.

Gene		Sequences (5' to 3')
hFZD1	(F)	gtgagccgaccaaggtgtat
	(R)	cagccggacaagaagatgat
hFZD2	(F)	gcgtcttctccgtgctctac
	(R)	ctgttggtgaggcgagtgta
hFZD3	(F)	tgagtgttcgaagctctatgg
	(R)	atcacgcacatgcagaaaag
hFZD4	(F)	ccaacatggctgttgaaatg
	(R)	tcacccaaccatttcctctc
hFZD5	(F)	tgctaccagccgtccttcagt
	(R)	ccatgccgaagaagtagaccag
hFZD6	(F)	attttggtgtccaaggcatc
	(R)	tattgcaggctgtgctatcg
hFZD7	(F)	gtgcagtgttctcccgaact
	(R)	gaacggtaaagagcgtcgag
hFZD8	(F)	tcttgtcgctcacatggttc
	(R)	tgtagagcacggtgaacagg
hFZD9	(F)	cgctggtcttcctactgctc
	(R)	agaagaccccgatcttgacc
hFZD10	(F)	gcggtgaagaccatcctg
	(R)	gcacggtgtacagcacagag
hGAPDH	(F)	accacagtccatgcatcac
	(R)	tccaccaccctgttgctgt
hAxin2	(F)	ctggctttggtgaactgttg
	(R)	agttgctcacagccaagaca
hWNT2	(F)	actctcaggacatgctggct
	(R)	acgaggtcatttttcgttgg
hWNT5a	(F)	ccacatgcagtacatcggag
	(R)	cactctcgtaggagcccttg
hGEP100	(F)	gcctttagcaacgatgtcatc
	(R)	cacatggtcctcattggtctt
CYTH1	(F)	ctgtgaggaaggttatcgg
	(R)	tccagagtagtccagttagg
ARNO	(F)	tgtggtcttggaggtggagtc
	(R)	ggctgctgctgcttctgg
CYTH3	(F)	ggagaagcagcaggaagg
	(R)	tctaactcagcaccacagc