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Supplemental Information

piggyBac Insertional Mutagenesis Screen Identifies a Role for Nuclear RHOA in Human ES Cell Differentiation

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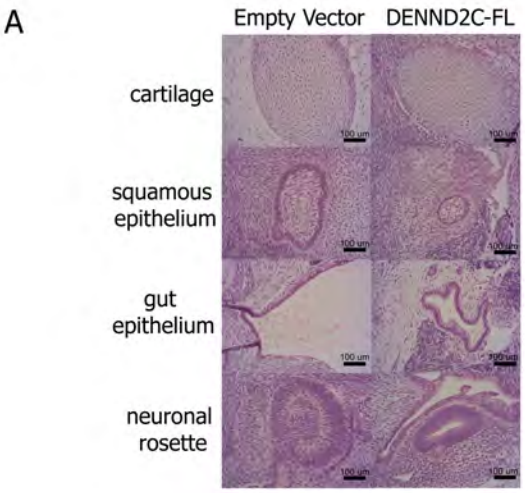
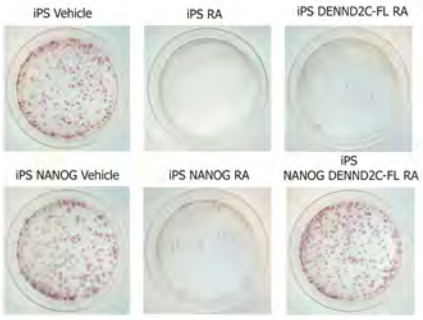
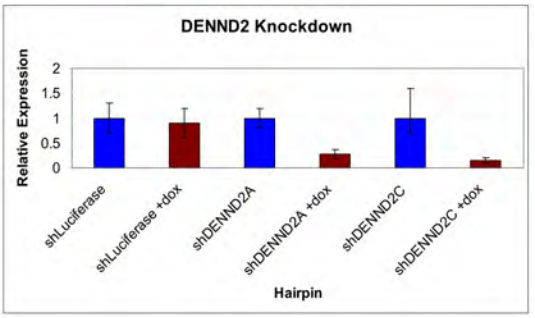
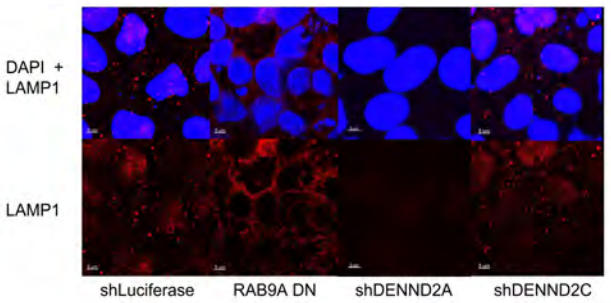
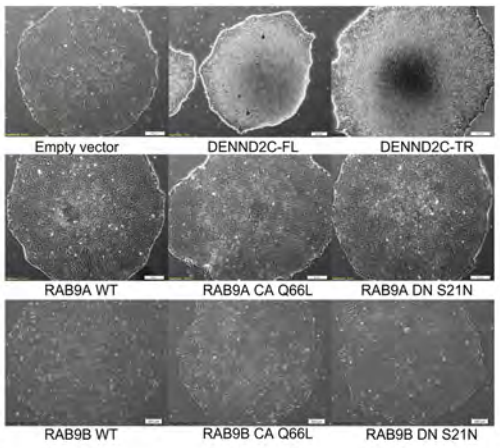
Supplemental Figure 1

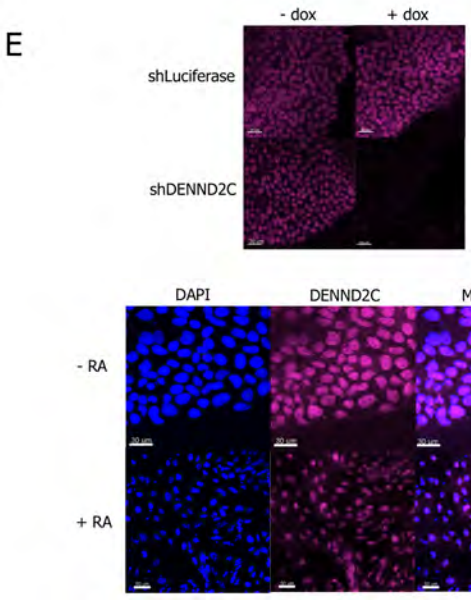


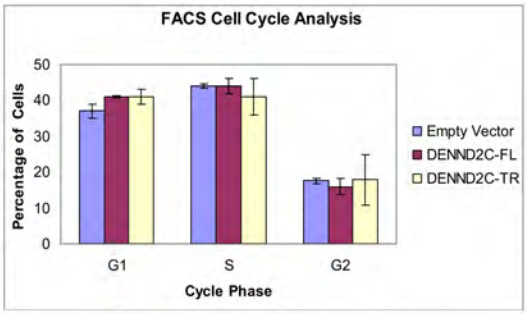


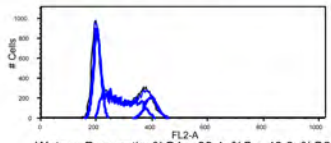












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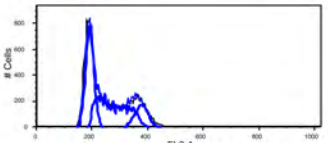
Empty Vector
Event Count: 19181



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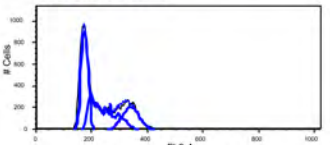
DENND2C-FL
Event Count: 17980



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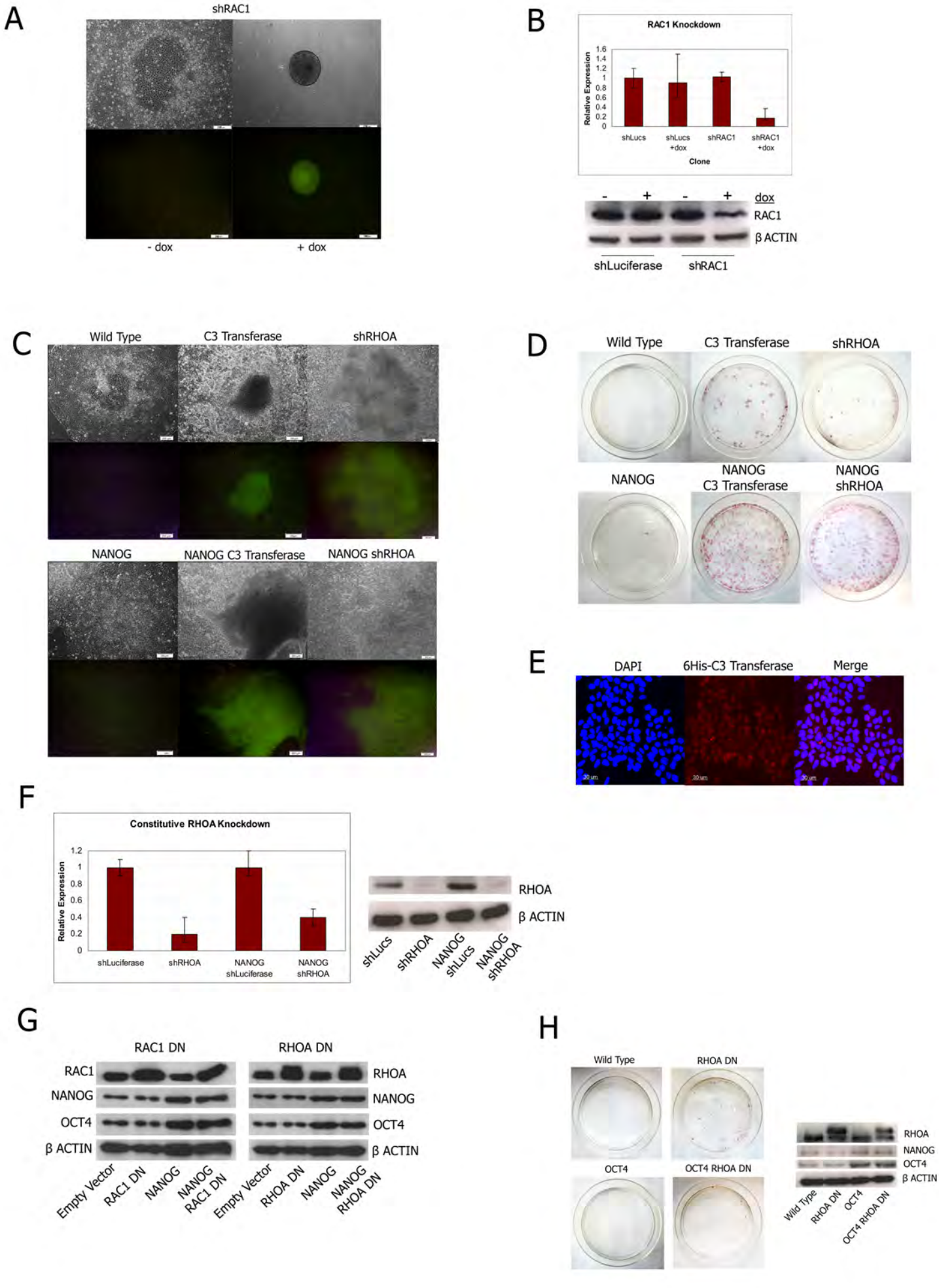
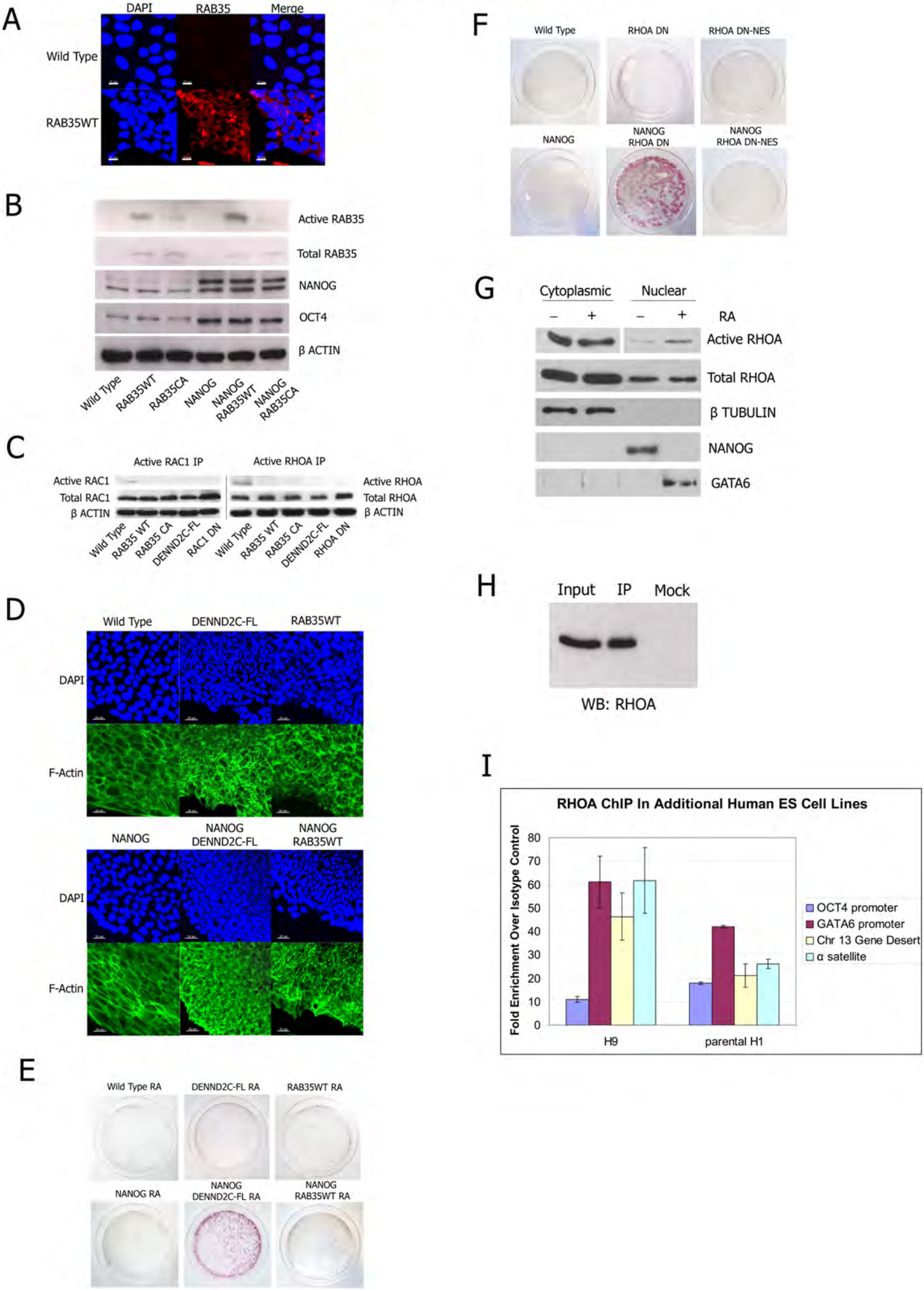
DENND2C-TR
Event Count: 18308

Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure Legends

Supplemental Figure S1, related to Figure 3.

(A) Teratoma formation of empty vector and DENND2C expressing human ES cells in SCID/beige mice. Empty vector tumor formation n=5/6. DENND2C tumor formation n=10/10. Scale bar represents 100 μm.

(B) Plates containing RA differentiated induced pluripotent stem (iPS) cells stained for the stemness marker alkaline phosphatase. Experiments performed in triplicate. A representative plate is shown. Wild type RA-differentiated iPS cells are negative for strong alkaline phosphatase staining, although differentiated cells were present on the plates (not shown). Either NANOG or DENND2C-FL expression produces weak resistance of iPS cells to RA-induced differentiation. NANOG and DENND2C-FL co-expression induces enhanced resistance to RA.

(C) Top: Real time PCR results showing doxycycline inducible knockdown with a negative control hairpin targeting luciferase and hairpins targeting DENND2A and DENND2C. Mean of three technical replicates shown. Error bars represent standard deviation. Bottom: Immunostaining showing LAMP1 positive lysosomes in doxycycline treated human ES cells. Scale bar represents 5 μm.

(D) Morphology of human ES cells overexpressing DENND2C and RAB9 wild type (WT), constitutively active (CA) and dominant negative (DN) constructs within *piggyBac* vectors. All RAB9 mutants have normal colony morphology and do not phenocopy the effects of DENND2C overexpression. Scale bar represents 200 µm.

(E) Top: Immunostaining of DENND2C in wild type human ES cells bearing a doxycycline inducible hairpin targeting either luciferase or DENND2C. Scale bar represents 30 µm. Bottom: Immunostaining of DENND2C in wild type human ES cells treated with or without RA for 8 days. Scale bar represents 30 µm.

(F) Cell cycle analysis of propidum iodide-stained human ES cells containing empty *piggyBac* vector or *piggyBac* containing full length (FL) and truncated (TR) DENND2C. DENND2C overexpression does not alter cell cycle. Graph shows mean of triplicate independent experiments. Error bars represent standard deviation.

Supplemental Figure S2, related to Figure 4.

(A) Brightfield and *OCT4*-EGFP reporter images of the doxycycline inducible RAC1 knockdown human ES cell line. Induced expression of the hairpin causes cells to pile into OCT4 positive, unproliferative balls of cells after two weeks. Scale bar represents 200 μm.

(B) Real time PCR and Western blot validation of RAC1 knockdown with doxycycline inducible hairpins targeted against RAC1 and a luciferase negative control. Mean of two technical replicates shown. Error bars represent standard deviation.

(C) Brightfield and *OCT4*-EGFP reporter images of C3-Transferase treated human ES cells and RHOA knockdown cells expressing a constitutive hairpin targeted against RHOA. Cells tend to pile up into dense, large colonies that resist spontaneous differentiation at the colony center. Scale bar represents 200 μm.

(D) Alkaline phosphatase stained plates post-retinoic acid/G418 treatment. C3 Transferase treatment and RHOA knockdown cooperate with NANOG overexpression for enhanced resistance to RA-induced differentiation. Experiments performed in triplicate. A representative plate is shown.

(E) Immunostaining of human ES cells with anti-6His tag antibody to detect localization of His-tagged C3-Transferase after one week of treatment. After one week C3 Transferase is detected in the cytoplasm and is particularly enriched in the nucleus. Scale bar represents 30 μm.

(F) Real Time PCR and Western blot validating RHOA knockdown in cells constitutively expressing a hairpin targeted against RHOA. Mean of two technical replicates shown. Error bars represent standard deviation.

(G) Western blots validating overexpression of RAC1 DN and RHOA DN from stable transfection of *piggyBac* constructs. Mutant cells maintain stemness marker expression at levels compared to the parental cell lines.

(H) Left: Alkaline phosphatase stained plates post-retinoic acid/G418 treatment. OCT4 overexpression alone in human ES cells does not induce retinoic acid resistance nor does it enhance resistance of RHOA DN expressing cells. Experiments performed in triplicate. A representative plate is shown.
Right: Western blot confirming overexpression of OCT4 and RHOA DN in mutant cells. OCT4 overexpression does not induce significant NANOG overexpression.

Supplemental Figure S3, related to Figure 5.

(A) Immunostaining showing ectopic expression of cytoplasmic Rho inhibitor RAB35 in human ES cells. Wild type human ES cells do not express RAB35 by immunofluoresence. Scale bar represents 15 µm.

(B) Immunoprecipitation of active RAB35 from RAB35 expressing human ES cells. Wild type human ES cells do not appear to express RAB35. Ectopic expression of wild type RAB35 (WT) and constitutively active RAB35 Q67L (CA) results in RAB35 activation. Ectopic expression of RABWT results in greater activation of RAB35 than RAB35 CA.

(C) Ectopic expression of wild type (WT) and constitutively active (CA) RAB35 in human ES cells results in reduction of active RAC1 and RHOA in whole cell lysate similar to the effects of DENND2C overexpression.

(D) RAB35 results in small nuclear and disorganized cortical actin phenotypes similar to the effects caused by DENND2C overexpression. Scale bar represents 20 µm.

(E) Comparison of the effects of DENND2C and RAB35 on the differentiation of human ES cells. Plates stained for stemness marker alkaline phosphatase post-

retinoic acid/G418 treatment. Experiments performed in triplicate. A representative plate is shown. Unlike DENND2C, RAB35 does not cooperate with NANOG expression for enhanced resistance to retinoic acid.

(F) Comparison of the effects of RHOA DN and RHOA DN tagged with a nuclear exclusion signal (RHOA DN-NES) on the differentiation of human ES cells. Plates stained for stemness marker alkaline phosphatase post-retinoic acid/G418 treatment. Experiments performed in triplicate. A representative plate is shown. Unlike RHOA DN, RHOA DN-NES does not induce cooperation with NANOG.

(G) Immunoprecipitation of active RHOA from cytoplasmic and nuclear lysates of untreated and RA treated wild type human ES cells.

(H) Immunoprecipitation of total RHOA from whole cell lysate using a RHOA monoclonal antibody subsequently used for chIP in this study.

(I) RHOA chromatin immunoprecipitation in H9 human ES cells and the parental H1 human ES cell line from which the *OCT4*- EGFP cells were derived. RHOA is enriched on the four tested loci. Error bars represent the mean percent fold enrichment over IgG control ± the standard errors of the mean for experiments performed in triplicate.

Clone	Candidate Gene	BLAT Coordinates of PB Insertion	Function
2	*DENND2C*	chr1:115,163,248-115,163,295	Unknown
	MRE11	chr11:94,184,355-94,184,506	DNA double strand break repair
1-8	*KIAA0564*	chr13:42,217,579-42,217,700	Unknown
	CPNE8	chr12:39,337,027-39,337,404	Calcium dependent membrane binding protein
	C1S	chr12:7,147,086-7,147,235	Serine protease
	PDE4D	chr5:58316488-58316532	cAMP degradation
22	*ADARB2*	chr10:1,434,460-1,434,879	RNA editing
	ZNF429	chr19:21,519,302-21,760,301	Uncharacterized zinc finger transcription factor
25	*NINJ2*	chr12:743,116-743,227	Adhesion protein involved in nerve regeneration
	ADCY8	chr8:131,962,309-131,962,528	cAMP formation
	NPBF22P	chr5:84,954,194-85,620,860	Neuroblastoma breakpoint family, member 22, pseudogene, noncoding RNA
5	*PRDM16*	chr1:3,232,335-3,232,916	Zinc finger transcription factor, brown fat differentiation
	CASZ1	chr1:10857031-10857056	Zinc finger transcription factor, tumor suppressor involved in differentiation
8	*GABBR2*	chr9:101,113,456-101,113,544	GABA-B receptor
	HEMOGEN	chr9:101,113,456-101,113,544	Hematopoietic differentiation
	MGC70870	chr17_gl000205_random:1-171,000	C-terminal binding protein 2 pseudogene, non-coding RNA
	LINC00536	chr8:113,149,745-121,149,744	Long intergenic non-coding RNA
	GNA14	chr9:80,254,047-80,254,301	Guanine nucleotide binding protein
	GCNT2	chr6:10,528,649-10,528,776	Blood group I antigen formation
	CSF1	chr1:110,435,360-110,457,247	Macrophage differentiation

Supplemental Table S1. Candidate genes isolated from the screen.

Provided are the list of candidate genes mutagenized by PB. Insertion coordinates were provided by a BLAT search of mapped sequences adjacent to transposon ends (http://genome.ucsc.edu/).

Supplementary Experimental Procedures

Western Blotting

Whole cell extracts were prepared using lysis buffer included in NewEast Biosciences GTPase activity kits (Cat# 30303). 10-20ug of protein was used per lane in a reducing gel. Protein was transferred to nitrocellulose at 4C for 1hr at 100V and the membrane blocked with 3% BSA for 1hr at room temperature followed by overnight incubation with the appropriate antibody at 4C.

Immunofluorescence

Cells on Matrigel coated 10mm plates or isolated nuclei suspended in hypotonic solution were fixed in 4% PFA for 10 minutes, permeabilized with 0.1% Triton-X for five minutes and blocked in 0.2% goat serum for 30 minutes at room temperature. Cells were incubated with primary antibody overnight at 4C, washed and incubated with a secondary antibody for one hour at room temperature at a 1:400 dilution ratio. Stained samples were mounted with Vectashield (Vector Laboratories Cat# H-1200). Confocal images were taken in a Zeiss LSM510 Meta confocal microscope.

Antibodies

DENND2C (Abcam Cat# ab87164) 1:300 (IF)/ 1:1000 (WB)
NANOG **(**Cell Signaling Cat # 4903) 1:2000 (IF)/ 1:1000 (WB)
OCT4 (Cell Signaling Cat# 2750) 1:200 (IF) / 1:2000 (WB)
GATA6 (Cell Signaling Cat# 5851) 1:1600 (IF)/ 1:1000 (WB)
RHOA (Abcam Cat# ab54835) 1:50 (IF)/ 5:1000 (WB)

RAC1 (Cell Biolabs Cat# 240106) 1:400 (IF)/ 1:10,000 (WB)
LAMP1 (Cell Signaling Cat# 3243) 1:400 (IF)
RAB35 (NewEast Biosciences Cat# 21078) 1:200 (IF) / 1:500 (WB)
β ACTIN (Cell Signaling Cat# 3700) 1:10,000 (WB)
β TUBULIN (Sigma Cat# T3952) 1:10,000 (WB)
anti-His tag (Millipore Cat# 05-949) 1:500 (IF)
Secondary-conjugated phalloidin Invitrogen (Cat#s A22281, A12379, A12381, A22284) 1:400 (IF)

C3 Transferase (Rho Inhibitor I) and Retinoic Acid Treatment

Cells were treated with 0.1 ng/mL C3 Transferase (Cytoskeleton Inc. Cat# CT04) for one week prior to beginning retinoic acid treatment as previously described. Cells were maintained on C3 Transferase for the duration of retinoic acid treatment.

ROCK inhibitor (Y-27632) and Retinoic Acid Treatment

Cells were treated with 10uM ROCK inhibitor (SelleckBio Cat# S1049) for one week prior to beginning retinoic acid treatment as previously described. Cells were maintained on ROCK inhibitor for the duration of retinoic acid treatment.

Immmunoprecipitation

For immunoprecipitation of total RHOA, anti-RHOA beads were prepared by coupling 15ug RHOA antibody to amine reactive resin (Abcam anti-RHOA Cat# ab54835, Pierce Co-Immunoprecipitation Kit Cat# 26149). Precleared whole cell lysate containing 1 mg of protein was then incubated overnight at 4C with either 50uL RHOA conjugated resin or 50uL unconjugated resin for mock immunoprecipitation. Co-IP product was washed and eluted from resin using the Pierce kit.

Immunoprecipitation of active RAC1 from 500ug whole cell lysate was performed with the Cell Biolabs RAC1 Activation Assay (Cat# STA-401-1).
Immunoprecipitation of active RAB35 from 750ug whole cell lysate was performed with the NewEast Biosciences RAB35 Activation Assay (Cat# 82801).

Cell Culture

Induced pluripotent stem cells were derived in the laboratory of Dr. In-Hyun Park from retroviral reprogramming of human neonatal foreskin fibroblasts using OCT4, SOX2, KLF4 and C-MYC. Cells were maintained on Matrigel (BD Biosciences Cat# 354277) in a 37C incubator at 5% CO2 and passaged with manual dissociation with a 5mL pipette after collagenase IV treatment (Gibco Cat# 17104019). Cells were grown in high FGF H1 human embryonic stem cell media as prepared by the staff of Dr. Caihong Qiu at the Yale Stem Cell Core Facility. For RA treatment, cells in 6 well plates at 20% confluency were

differentiated with 1uM RA for 12 days, split 2 wells : 1 10cm plate and allowed to grow for another 8 days in normal high FGF media without RA.

The H9 and H1 human ES cell lines were obtained from the Yale Stem Cell Core from the WiCell Research Institute under the appropriate MTAs and maintained on Matrigel (BD Biosciences Cat# 354277) in a 37C incubator at 5% CO2. Cells were passaged with manual dissociation with a 5mL pipette after collagenase IV treatment (Gibco Cat# 17104019). H9 human ES cells were maintained in mTESR media (Stem Cell Technologies Cat# 05850) and H1 human ES cells were maintained in high FGF H1 human embryonic stem cell media as prepared by the staff of Dr. Caihong Qiu at the Yale Stem Cell Core Facility.

Teratoma Formation

Human ES cells were harvested by Accutase treatment (Millipore Cat# SCR005), and the pellets suspended in high FGF media with 10 uM ROCK inhibitor (Calbiochem Cat# 688000). Before injection, Matrigel (BD) was added to resuspended cells to a final concentration of 30%. 150uL of 10^7/mL cells were intramuscularly injected to the hindlimbs of a SCID/Beige mouse (Charles River). Eight weeks after injection, tumors were dissected and fixed with 10% formalin. Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin. The use of human embryonic stem cells for teratoma assays was approved by Yale Embryonic Stem Cell Research Oversight (ESCRO) under protocol number E-07-019. All animal procedures were approved by the Yale Animal

Resources Center and the Institutional Animal Care and Use Committee under protocol numbers 10230.

FACS Analysis

For transfection efficiency, live cells were analyzed 48h post-transfection on a Stratedigm analyzer. For cell cycle analysis cells were fixed in cold 70% ethanol for at least 30 minutes, treated with 100uL 100ug/mL ribonuclease (Sigma Cat# R4875) and 400uL 50ug/mL propidium iodide (Sigma Cat# P4864) and data acquired on FACSCalibur. All data was analyzed using the Watson Pragmatic model on FlowJo software (Tree Star, Inc.).

Knockdown

Knockdown was performed by subcloning annealed hairpin oligos into either the Tet-pLKO-puro construct (Addgene Plasmid 21915) for doxycycline inducible expression of RAC1 or the pLKO.1-blast construct (Addgene Plasmid 26655) for constitutive repression of RHOA. Constructs were transfected into 293T cells for packaging, and virus supernatant harvested. Human ES cells were pulse infected with 1mL virus supernatant and 8ug/mL polybrene (Sigma Cat# H9268) for one hour and drug selected either with 1ug/mL puromycin or 0.1ug/mL blasticidin for two weeks 48h post-infection. Surviving Tet-pLKO-puro clones were expanded and treated with 0.5ug/mL doxycycline for 5 days to induce hairpin expression.

Hairpin sequences	
Target Gene	**Sequence**
shLuciferase	5’CCGGCGCTGAGTACTTCGAAATGTCCTCGAGGACATTT CGAAGTACTCAGCGTTTTT 3’
shDENND2A	5’CCGGCTGAAGGGACTAGGCAATAAACTCGAGTTTATTG CCTAGTCCCTTCAGTTTT 3’
shDENND2C	5’ CGGGCCTTGTTGTTGTTACATTTACTCGAGTAAATGTAA CAACAACAAGGCTTTT 3’
shRHOA	5’CCGGGAAGGATCTTCGGAATGATTCGAATCATTCCGAAG ATCCTTCTTTTTG 3’
shRAC1	5’CCGGCGCAAACAGATGTGTTCTTAACTCGAGTTAAGAAC ACATCTGTTTGCGTTTTT 3’

Primers used in this work.

Quantitative PCR Primers	
Target Gene	**Sequence**
GAPDH	F 5’AAGGTGAAGGTCGGAGTCAA 3’
	R 5’ AATGAAGGGGTCATTGATGG 3’
OCT4	F 5’ GGGTTTTTGGGATTAAGTTCTTCA 3’
	R 5’ GCCCCCACCCTTTGTGTT 3’
NANOG	F 5’ GATTTGTGGGCCTGAAGAAA 3’
	R 5’ ATGGAGGAGGGAAGAGGAGA 3’
Endogenous NANOG	F 5’ CAGTCTGGACACTGGCTGAA 3’
	R 5’ CTCGCTGATTAGGCTCCAAC 3’
REX1	F 5’ TCACAGTCCAGCAGGTGTTTG 3’
	R 5’ TCTTGTCTTTGCCCGTTTCT 3’
SOX2	F 5’ CAAAAATGGCCATGCAGGTT 3’
	R 5’ AGTTGGGATCGAACAAAAGCTATT 3’
GBX2	F 5’ GTAACTTCGACAAGGCGGAG 3’
	R 5’ TCAGATTGTCATCCGAGCTG 3’
NESTIN	F 5’ CTCAAGATGTCCCTCAGCCT 3’
	R 5’ GGTCCTAGGGAATTGCAGC 3’
TUBB3	F 5’ CCCAGTATGAGGGAGATCGT 3’
	R 5’ CGATGCCATGCTCATCAC 3’
OLIG1	F 5’ CCAGTGTTTTGTCGCAGAGA 3’

	R 5’ GCGGTTGGTTTTCGTTTTTA 3’
HUC	F 5’ GGCACACAAGAATGGTCACT 3’
	R 5’ CGTCAGTGGCTCCATTTGTA 3’
N-CADHERIN	F 5’ CCCACACCCTGGAGACATTG 3’
	R 5’ GCCGCTTTAAGGCCCTCA 3’
GFAP	F 5’ ATCGAGATCGCCACCTACAG 3’
	R 5’ CACCACGATGTTCCTCTTGA 3’
PAX6	F 5’ ACCCATTATCCAGATGTGTTTGCCC GAG 3’
	R 5’ ATGGTGAAGCTGGGCATAGGCGGC AG 3’
NKX2.2	F 5’ TGCCTCTCCTTCTGAACCTTG 3’
	R 5’ GCGAAATCTGCCACCAGTTG 3’
NKX6.1	F 5’ GTTCCTCCTCCTCCTCTTCCTC 3’
	R 5’ AAGATCTGCTGTCCGGAAAAAG 3’
GATA6	F 5’ GCCAACTGTCACACCACAAC 3’
	R 5’ TGGGGGAAGTATTTTTGCTG 3’
BRACHYURY	F 5’ GCGGGAAAGAGCCTCGAGTA 3’
	R 5’ TTCCCCGTTCACGTACTTCC 3’
AMPD1	F 5’ GGATTTCAGCAACAATGCCT 3’
	R 5’ TCCTGACGACCTCCTTCATC 3’
NRAS	F 5’ GCTTCCTCTGTGTATTTGCCA 3’
	R 5’ GCACCATAGGTACATCATCCG
TRIM33	F 5’ CTCCTTCTGCCTGCGCT 3’
	R 5’ ACTGGGCACCGTATTACACC 3’
BCAS2	F 5’ TGGAACTAATGTCACAGCATGG 3’
	R 5’ TGAAGTTCCTTCTGTGCGTG 3’
DENND2C C-term	F 5’ GCGGAGTCTTGGAAGCAAAATG 3’
	R 5’ GAATACTTCATGGGATCCTGACCC 3’
DENND2C N-term	F 5’ GGGAACATGGATGTTGGTTT 3’
	R 5’ TTGCAGTGGCTTCTTGACAG 3’
DENND2A	F 5’ TTCCATCCCTGACACACTCA 3’
	R 5’ CCCGACTCAGCTTCCTAGTG 3’
RAB9A	F 5’ TACCATGCAGATTTGGGACA 3’
	R 5’ TAAGCAGGCAGCAGTCAGAA 3’
RAB9B	F 5’ TGAAGGACCCTGAGCATTTC 3’
	R 5’ TTCCTCTACAGCCAGCACCT 3’
RHOA	F 5’ AAGGACCAGTTCCCAGAGGT 3’
	R 5’ GCTTTCCATCCACCTCGATA 3’
RAC1	F 5’ AACCAATGCATTTCCTGGAG 3’
	R 5’ TCCCATAAGCCCAGATTCAC 3’

Determination of *piggyBac* copy number by quantitative PCR

PiggyBac signal from genomic DNA was normalized to *β ACTIN* signal. Copy number was estimated relative to normalized signal from a control of known copy number previously established by Southern blot.

Determination of *piggyBac* Copy Number by Quantitative PCR		
Purpose	**Primer Name**	**Sequence**
Copy #	PBLRT2	F 5' TCACGCGGTCGTTATAGTTCAA 3'
		R 5' CCGTGAGGCGTGCTTGTC 3'
Control	BACTIN	F 5'TCACCCACACTGTGCCCATCTACGA 3'
		R 5' CAGCGGAACCGCTCATTGCCAATGG 3'

ChIP Primers

OCT4 promoter primers were sourced from Cell Signaling Cat# 4641S. α satellite repeat primers were sourced from Cell Signaling Cat# 4486S.

Chromosome 13 gene desert primers were chosen in a region with no coding genes within 500kb of either side of the sequence. GATA6 promoter primers were chosen from an area within a NANOG-binding region in human ES cells published by Boyer et al. 2005 that gave the highest signal in ChIPs performed with an antibody against NANOG.

ChIP Quantitative PCR Primers	
Ch 13 gene desert	F 5' GATTTTTGGCCACATGCTTC 3'
	R 5' TGGGCAAAGAACACAGACAG 3'
GATA6 promoter	F 5' TACGTGCAGAGGAAACAACG 3'
	R 5' GAGTTGAACTGGGGTGGAGA 3'

DENND2C Cloning

Full length and truncated DENND2C were amplified from human ES cell cDNA using primers containing MluI restriction sites and cloned into a *piggyBac* construct for stable overexpression.

DENND2C Overexpression Construct Primers		
Product	**Primer Name**	**Sequence**
Full Length	DENND2C-FL MLU F	5'ATTACGCGTGCCGCCACCATGGATGTTGGTTTTTCTCGTACTAC 3'
Truncated	DENND2C-TR MLU F	5'ATTACGCGTGCCGCCACCATGCTTGTATTGAAAATAGATGACATATTTGAATC 3'
_____	DENND2C MLU R	5' ATTACGCGTTCATTTCTTTTGCAGAAATTTCATTTTGC 3'

Mutant Constructs

To create mutant constructs, two mutant PCR products were amplified with phosphorylated primers from the wild type template: WT F/CA1 R and CA2 F/WT R. These PCR products were digested with MluI restriction enzyme and triple ligated into a *piggyBac* overexpression construct carrying a β Actin promoter, poly A tail and drug selection cassette. Abbreviations: wild type (WT), constitutively active (CA) and dominant negative (DN).

Mutant Primers			
Target	**Mutation**	**Primer Name**	**Sequence**
RAB9A	Wild Type	RAB9A WT F	5’ATTACGCGTGCCGCCACCATGGCAGG AAAATCATCAC TTTTTAAAGT 3’
		RAB9A WT R	5’ATTACGCGTTCAACAGCAAGATGAGC TAGGC 3’
	Q66L	RAB9A CA1 R	5’ AAGACCTGCCGTGTCCCAAATCTG 3’
		RAB9A CA2 F	5’ GAGCGATTCCGAAGCCTGAGG 3’
RAB9B	Wild Type	RAB9B WT F	5’ATTACGCGTGCCGCCACCATGAGTGGG AAATCCCTGCTCTTAAAG 3’
		RAB9B WT R	5’ATTACGCGTTTAACAGCACGAAGACCC TGCTTTG 3’
	Q66L	RAB9B CA1 R	5’ AAGCCCTGCAGTGTCCCAGATC 3’
		RAB9B CA2 F	5’GAACGTTTCAAGAGCCTTAGGACACC 3’
RHOA	Wild Type	RHOA WT F	5’ATTACGCGTGCCGCCACCATGGCTGCC ATCCGGAAGAAACTGG 3’
		RHOA WT R	5’ATTACGCGTTCACAAGACAAGGCACC CAGATTTTTTCTTCC 3’
	T19N	RHOA DN1 R	5’GTTCTTTCCACAGGCTCCATCACCAAC 3’
		RHOA DN2 F	5’TGCTTGCTCATAGTCTTCAGCAAGGAC C 3’
	NES	RHOA NES R	5’ ATTACGCGTTCAAAGACCAAGAGTAC GAAAAGCATCACGAAGCAAGACAAGGC ACCCAGATTTTTTCTTCC 3’
RAC1	Wild Type	RAC1 WT F	5’ATTACGCGTGCCGCCACCATGCAGGCC AT CAAGTGTGTGGTGG 3’
			5’ATTACGCGTTTACAACAGCAGGCATTT TCTCTTCCTCTTC 3’
	T17N	RAC1 DN1 R	5’ ATTTTTACCTACAGCTCCGTCTCCCA 3’
		RAC1 DN2 F	5’TGCCTACTGATCAGTTACACAACCAAT GC ATT 3’
RAB35	Wild Type	RAB35 WT F	5’ATTACGCGTGCCGCCACCATGGCCCGG GACTACGACCACCTCTTC 3’
		RAB35 WT R	5’ATTACGCGTTTAGCAGCAGCGTTTCTT TCGT TTACTGTTCTTCGTGAGC 3’
	Q67L	RAB35 CA1 R	5’ CAGCCCCGCTGTGTCCCAGATCTGC 3’
		RAB35 CA2 F	5’GAGCGCTTCCGCACCATCACCTCCAC 3’