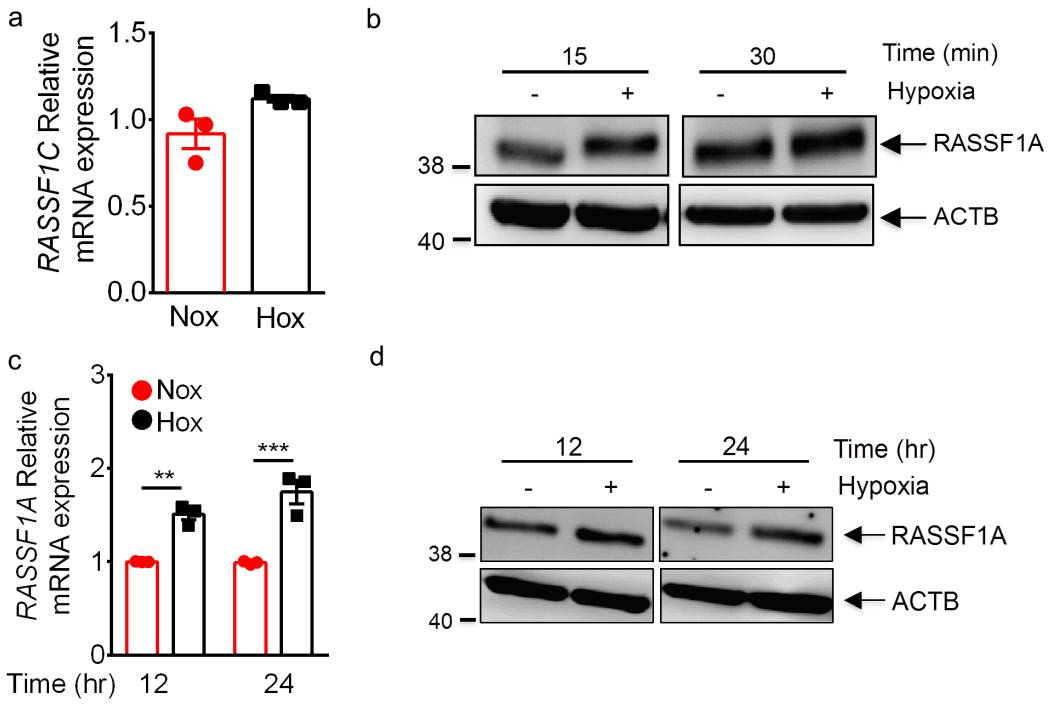
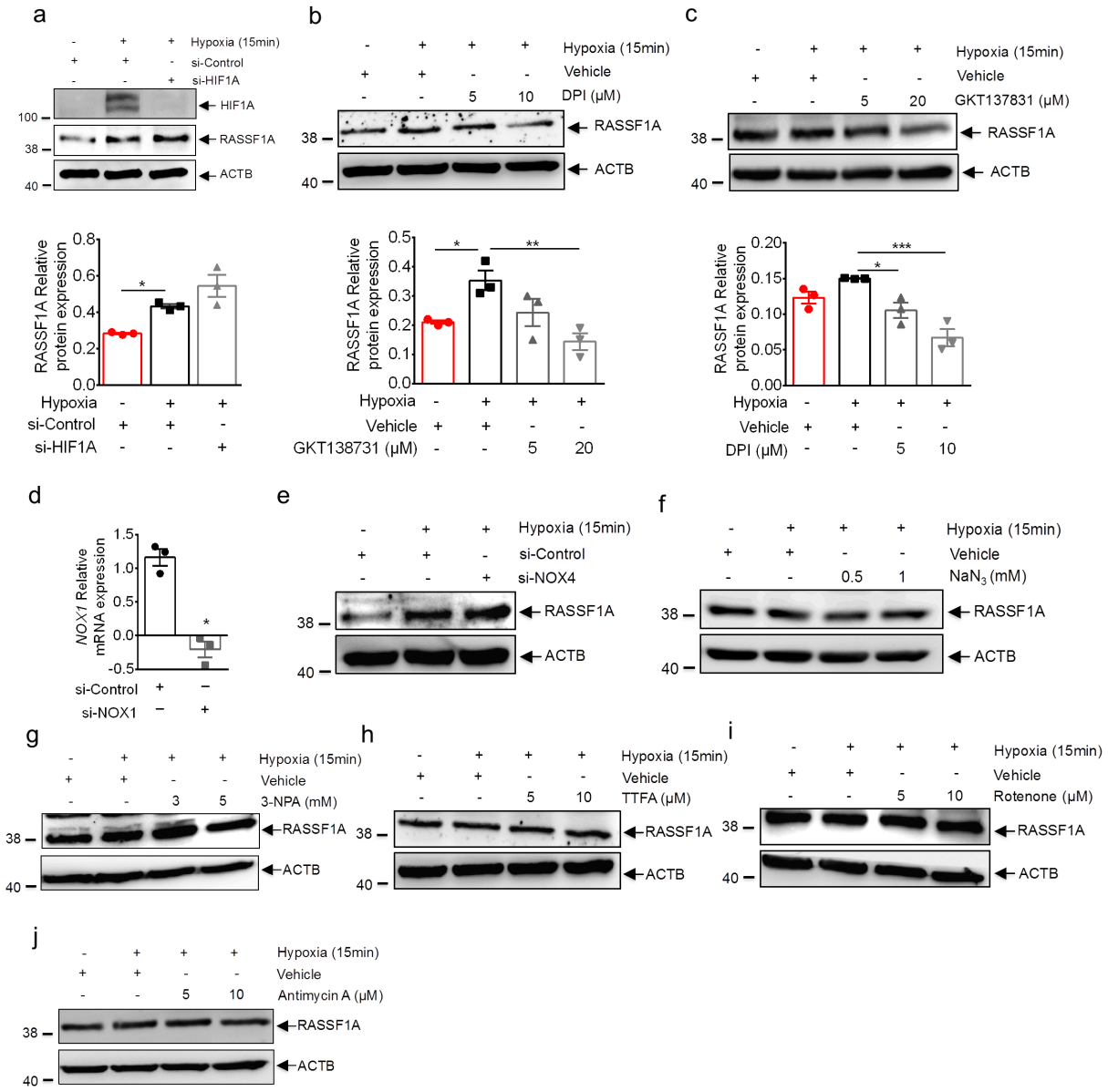
Supplementary Information

A RASSF1A-HIF1α loop driving Warburg effect in cancer and Pulmonary hypertension

Dabral et al.

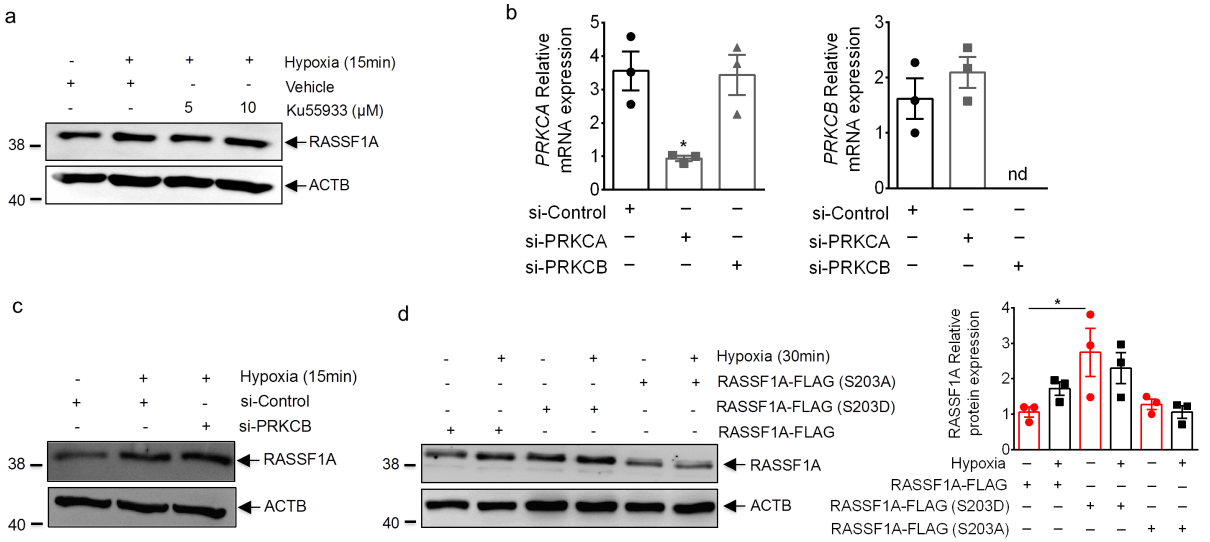


Supplementary Fig. 1: RASSF1A is upregulated in hypoxia exposed pulmonary artery adventitial fibroblasts. (a) Human PASMCs were exposed to 20% (Normoxia) or 1% O_2 (Hypoxia) for 24 hr. Cell lysates were subjected to real time PCR for RASSF1C. **(b–d)** Human PAAFs were exposed to 20% (Normoxia) or 1% O_2 (Hypoxia) for indicated intervals. Cell lysates from each time point were subjected to **(b, d)** western blotting for RASSF1A and ACTB and **(c)** real time PCR for *RASSF1A*. $*P < 0.05$, $***P < 0.001$ compared to Normoxia (Nox), unpaired Student's *t*-test. n = 2 independent experiments from 2 biological replicates**.**

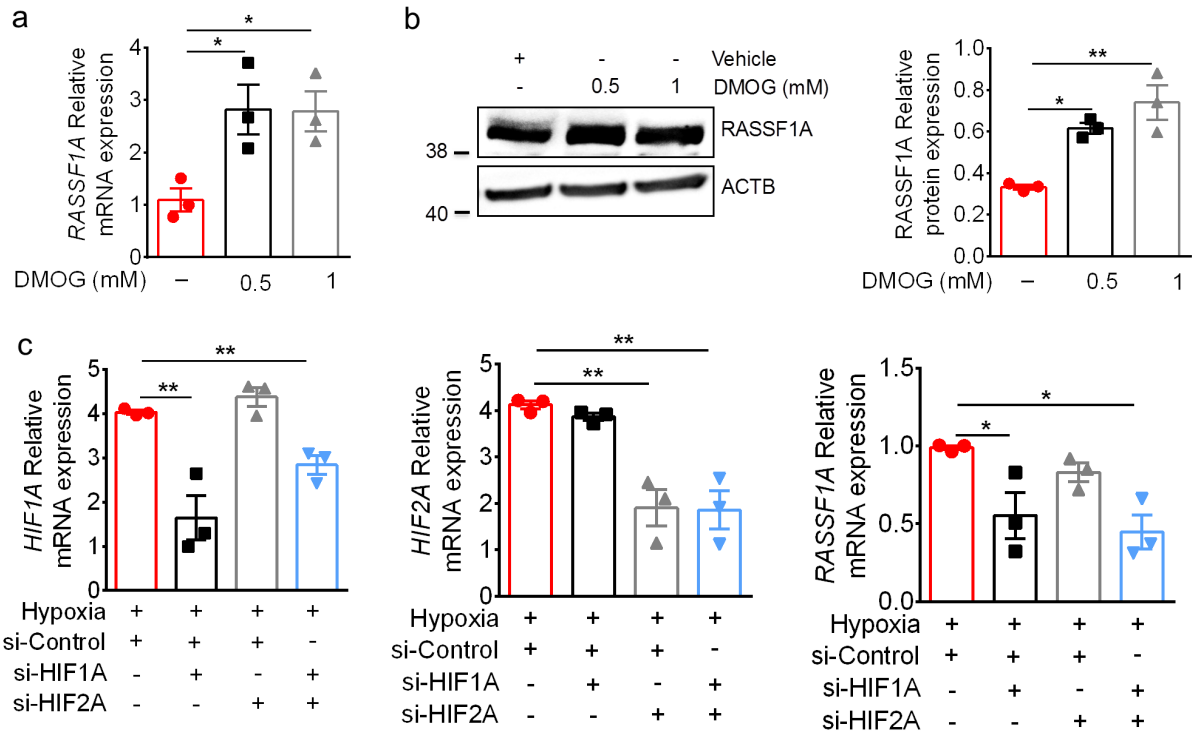


Supplementary Fig. 2: Influence of HIF1A, ROS, NOX and electron transport chain on RASSF1A protein expression. **(a)** Human PASMCs were transfected HIF1A siRNA (si-HIF1A) or control siRNA (si-Control). 24hr later, cells were exposed to hypoxia for 15min, followed by cell lysis and **(a, upper)** western blotting for HIF1A, RASSF1A and ACTB and **(a, lower)** densitometric quantification of relative RASSF1A expression. **(b, c)** Human PASMCs were pre-treated with **(b)** DPI (ROS inhibitor) or **(c)** GTK137831 (NOX inhibitor) at indicated concentrations for 1 h, followed by 15min Hypoxia exposure and cell lysates representing each dose were subjected to **(b–c, upper)** western blotting for RASSF1A and ACTB. **(b–c, lower)** Densitometric quantification of relative RASSF1A expression (RASSF1A/ACTB). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to si-Control (Hypoxia) or vehicle (Hypoxia), one-way ANOVA followed by SNK multiple comparison test. n = 3 independent experiments. **(d)** Human PASMCs were transfected with NOX1 siRNA (si-NOX1). 24hr later, RNA was isolated and real time PCRs were performed. $**P < 0.01$ compared to si-Control, unpaired Student's *t*-test. **(e)** Human PASMCs were transfected with NOX4 siRNA (si-NOX4) or control siRNA (si-Control) for 48hr, followed by 15min Hypoxia exposure. Cell lysates were subjected to western blotting for RASSF1A and ACTB. **(f–k)**

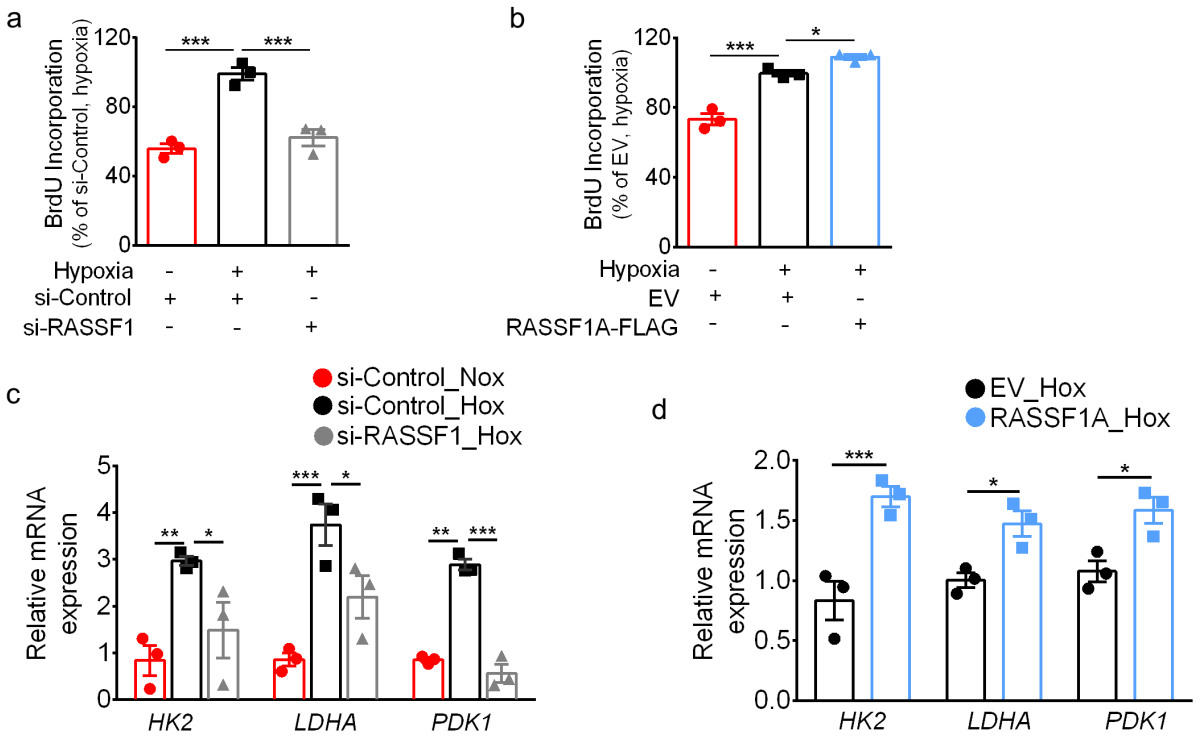
Human PASMCs were pre-treated with different inhibitors of electron chain; **(f)** sodium azide (NaN_3), **(g)** 3-sodium propionic acid (3-NPA), **(h)** thenoyltrifluoroacetone (TTFA)**,** (**i)** rotenone and **(j)** antimycin A at indicated concentrations for 1 h, followed by 15min Hypoxia exposure and cell lysates representing each dose were subjected to western blotting for RASSF1A and ACTB. Data represent mean ± s.e.m all through the figure. n = 3 independent experiments from 3 biological replicates**.**



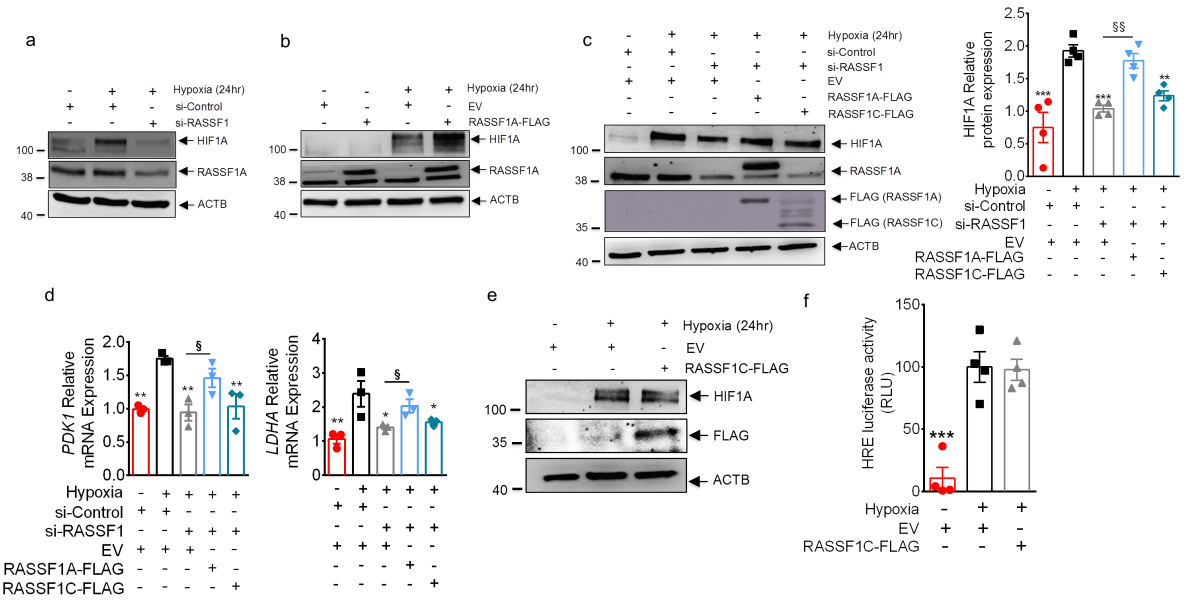
Supplementary Fig. 3: Influence of phosphorylation on RASSF1A expression. (a) Human PASMCs were pre-treated ATM kinase inhibitor (KU55933) at indicated concentrations for 1 h, followed by 15min Hypoxia exposure and cell lysates representing each dose were subjected to western blotting for RASSF1A and ACTB. **(b)** Human PASMCs were transfected with PKCα siRNA (si-PRKCA), PKCβ siRNA (si-PRKCB) or control siRNA (si-Control). 24hr later, RNA was isolated and real time PCRs were performed. $^{**}P < 0.01$ compared to si-Control, unpaired Student's *t*-test. **(c)** Human PASMCs were transfected with si-PRKCB or si-Control for 48hr, followed by 15min Hypoxia exposure. Cell lysates were subjected to western blotting for RASSF1A and ACTB. **(d)** HEK 293 cells were transfected with plasmids indicated in top lane, and exposed to Hypoxia for 30 min. **(d, left)** Cells were lysed and subjected to western blotting for RASSF1A and ACTB, followed by **(d, right)** densitometric quantification of relative RASSF1A expression.* $P < 0.05$, $^{**}P < 0.01$ compared to **(b)** si-Control or **(d)** RASSF1A-FLAG (Normoxia). Data represent mean ± s.e.m all through the figure. n = 3 independent experiments from 3 biological replicates for human PASMCs and n=3 independent experiments for HEK cell lines.



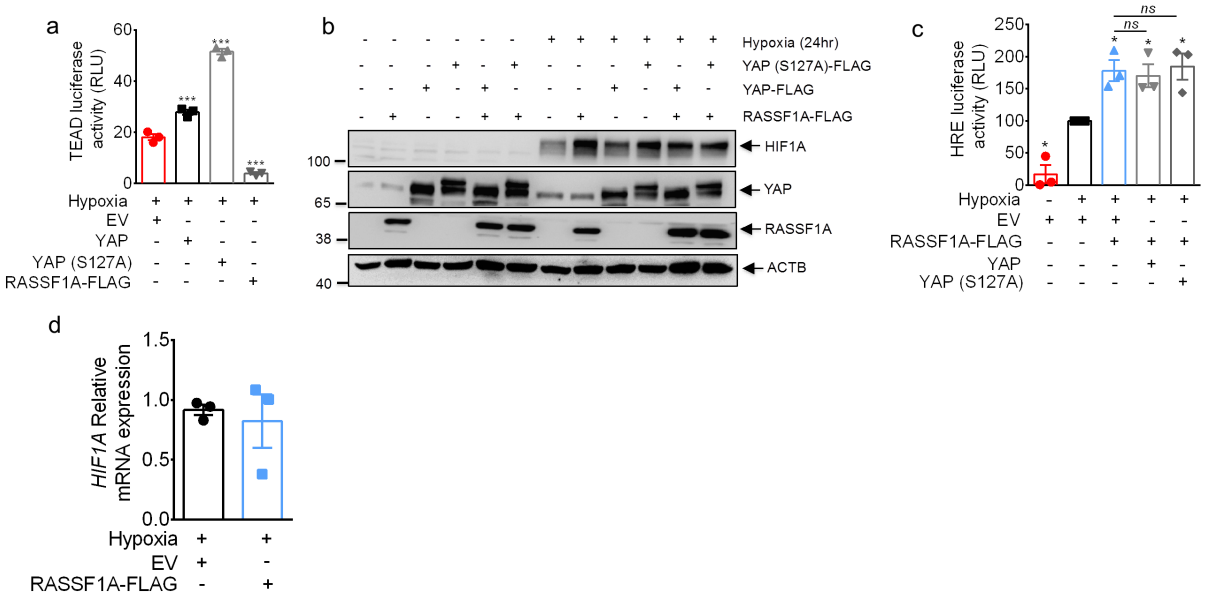
Supplementary Fig. 4: *RASSF1* is a HIF-1 target gene. (a, b) Human PASMCs were treated with indicated concentrations of prolyl hypdroxylase inhibitor, DMOG (hypoxia mimetic), followed by **(a)** real time PCR and **(b, left)** western blotting for RASSF1A and ACTB. **(b, right)** Densitometric quantification of relative RASSF1A expression. **P* < 0.05, ***P* < 0.01 compared to control, one-way ANOVA followed by SNK multiple comparison test. **(c)** Human PASMCs were transfected with HIF-1α siRNA (si-HIF1A) or HIF-2α siRNA (si-HIF2A) or both together for 24hr, with additional 24hr hypoxia exposure. Cell lysates were subjected to real time PCRs for *HIF1A*, *HIF2A* and *RASSF1A*. ***P* < 0.01, ****P* < 0.001 compared to si-Control (Hypoxia), one-way ANOVA followed by SNK multiple comparison test. Data represent mean ± s.e.m all through the figure. n = 3 independent experiments from 3 biological replicates**.**



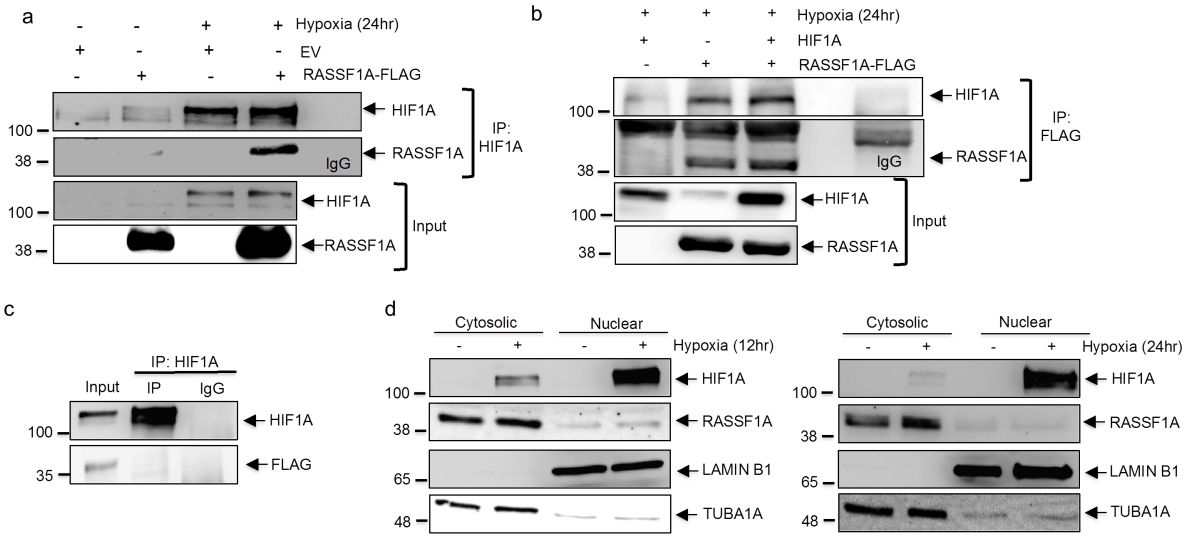
Supplementary Fig. 5: RASSF1A regulates proliferation and glycolytic metabolism of human PAAFs under hypoxia. Human PAAFs were transfected with **(a, c)** RASSF1 siRNA (si-RASSF1) and control siRNA (si-Control) or **(b, d)** RASSF1A-FLAG and empty vector (EV). **(a, b)** 24hr after transfection, cells were exposed to Normoxia or Hypoxia for 48hr and proliferation was measured by BrdU incorporation assay. **(c, d)** 24hr after transfection, cells were exposed to Normoxia or Hypoxia for 24hr. Quantitative real time RT PCRs for *HK2*, *LDHA* and *PDK1* were performed. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to **(a, c)** si-Control (Hypoxia) or **(b, d)** EV (Hypoxia), one-way ANOVA followed by SNK multiple comparison test. n = 2 independent experiments, Data represent mean ± s.e.m all through the figure. n = 3 independent experiments from 3 biological replicates**.**



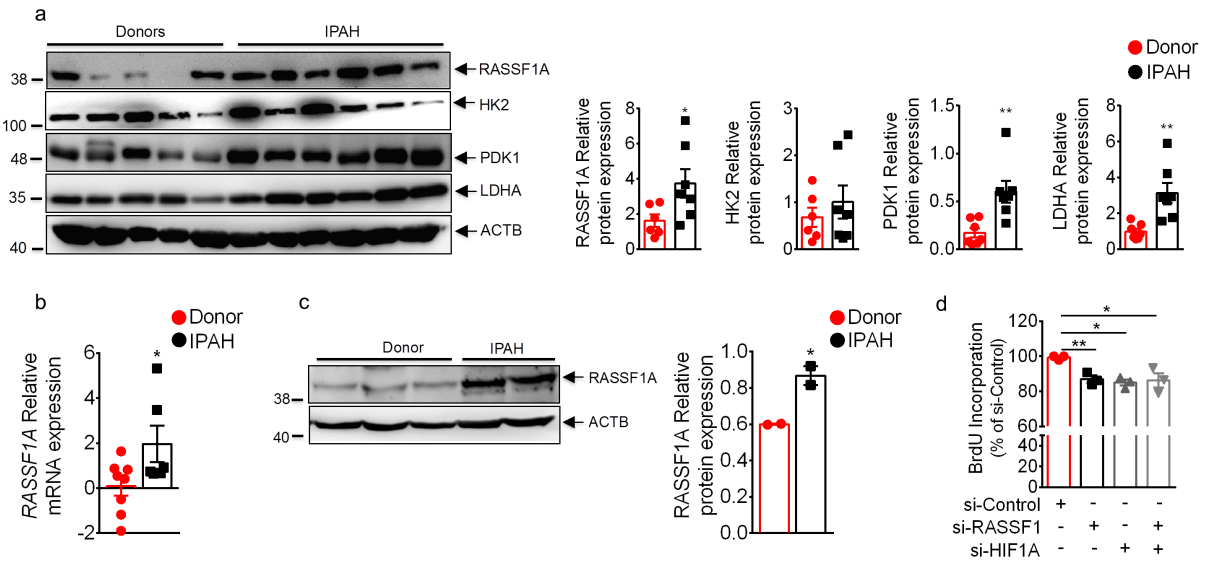
Supplementary Fig. 6: RASSF1A, not RASSF1C regulates HIF1α. (a, b) Human PAAFs were transfected with **(a)** RASSF1 siRNA (si-RASSF1) and control siRNA (si-Control) or **(b)** RASSF1A-FLAG and empty vector (EV). 24hr after transfection, cells were exposed to Normoxia or Hypoxia for 24hr. Cell lysates were subjected to western blotting for HIF1A, RASSF1A and ACTB. **(c, d)** Human PASMCs were transfected with RASSF1 siRNA (si-RASSF1) and control siRNA (si-Control). 24 hr later, cells were further transfected with EV, RASSF1A-FLAG or RASSF1C-FLAG as mentioned in the lanes above the blots. 6 hr later, cells were exposed to Hypoxia or Normoxia for further 24 hr. **(c, left)** Cell lysates were subjected to western blotting for HIF1A, RASSF1A, FLAG, and ACTB. **(c, right)** Densitometrical quantified data of HIF1A/ACTB expression ratio. **(d)** Real time PCRs for PDK1 and LDHA were performed. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to si-Control (Hypoxia), $^{\S\S}P < 0.01$ compared to si-RASSF1+EV (Hypoxia) one-way ANOVA followed by SNK multiple comparison test. **(e)** Human PASMCs were transfected with EV or RASSF1C-FLAG and 24 hr later, cells were exposed to hypoxia for another 24 hr. Cell lysates were subjected to western blotting for HIF1A, FLAG and ACTB. **(f)** A luciferase reporter under control of multiple HIF1α binding sites (HRE) was transfected into cells with EV or RASSF1C-FLAG. 6 hr after transfection, cells were exposed to Hypoxia for 24 hr. Cells were lysed and luciferase activity was measured and normalized to co-transfected Renilla luciferase internal control. $^{***}P < 0.001$ compared to EV (Hypoxia), one-way ANOVA followed by SNK multiple comparison test. Data represent mean ± s.e.m. n = 3 independent experiments.



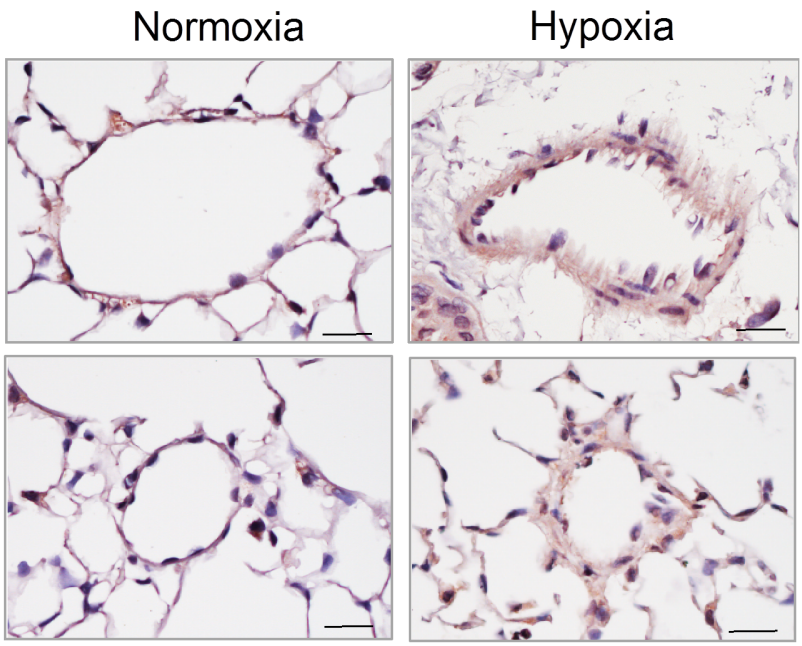
Supplementary Fig. 7: RASSF1A regulates HIF1α, independent of hippo signaling. A luciferase reporter under control of **(a)** TEAD binding sites or **(c)** of multiple HIF1 α binding sites (HRE) was transfected into HeLa cells with EV, YAP, YAP (S127A) and RASSF1A-FLAG. 6 hr after transfection, cells were exposed to Hypoxia for 24 hr. Cells were lysed and luciferase activity was measured and normalized to co-transfected Renilla luciferase internal control. ***$P < 0.001$ compared to EV (hypoxia), one-way ANOVA followed by SNK multiple comparison test. **(b)** HEK 293 cells were transfected with plasmids indicated on top of lanes and exposed to hypoxia for 24 hr, followed by western blotting for HIF1A, YAP, RASSF1A and ACTB. **(d)** Effect of RASSF1A overexpression on mRNA expression of HIF1A in HEK293 cells exposed to 24 hr Hypoxia. Data represent mean ± s.e.m. n = 3 independent experiments.



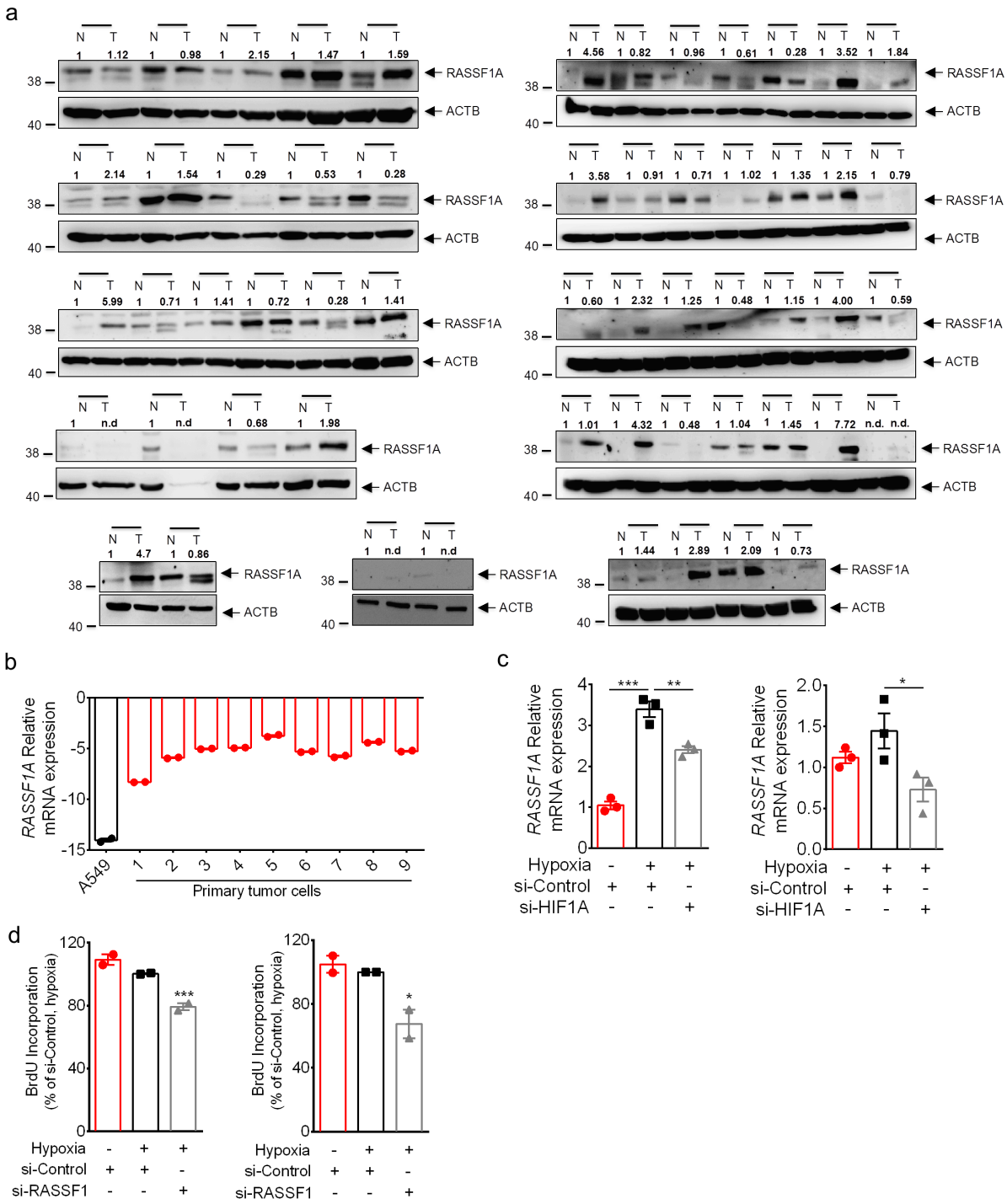
Supplementary Fig. 8: RASSF1A interacts with HIF1α. (a, b) HEK293 cells were transfected with plasmids indicated on top of lanes and exposed to Hypoxia for 24hr. **(a)** HIF1A or **(b)** FLAG was immunoprecipitated (IP) and co- immunprecipitated (co-IP) RASSF1A or HIF1A were detected by western blotting. **(c)** HEK 293 cells were transfected with RASSF1C-FLAG plasmid and exposed to hypoxia for 24 hr, followed by HIF1A IP and western blotting for HIF1A and FLAG. **(d)** Human PASMCs were exposed to Hypoxia or Normoxia for 12 hr and 24 hr, followed by sub cellular fractionation. Cytoplasmic and nuclear lysates were subjected to western blotting for HIF1A, RASSF1A, Lamin B1 and TUBA1A. Lamin B1 and alpha-tubulin (TUBA1A) were used as nuclear and cytoplasmic markers respectively. n = 2 independent experiments. n = 2 independent experiments**.**



Supplementary Fig. 9: Protein expression of RASSF1A and glycolytic enzymes is upregulated in IPAH patients. (a) Protein was isolated from frozen pulmonary arteries of IPAH patients (n=6) and donors (n=5) and subjected to **(a, left)** western blotting for RASSF1A, HK2, PDK1, LDHA and ACTB, followed by **(a, right)** densitometric quantification of RASSF1A, HK2, PDK1 and LDHA to ACTB expression ratio. **(b, c)** Expression of RASSF1A in IPAH- vs donor- PAAFs (n= 2-5) as analyzed using **(b)** real time PCRs and **(c)** western blotting for RASSF1A and ACTB followed by densitometric quantification of relative RASSF1A expression. $*P < 0.05$, $**P < 0.01$ compared to donor, unpaired Student's *t*-test. **(d)** IPAH PASMCs were transfected with RASSF1 siRNA (si-RASSF1), HIF-1α siRNA (si-HIF1A) or in combination. 6 hr after transfection, cells were placed in medium with growth factors for 48 hr. Proliferation was measured by BrdU incorporation assay. $**P < 0.01$, $***P < 0.001$ compared to si-Control, one-way ANOVA followed by SNK multiple comparison test. Data represent mean ± s.e.m. n = 3 independent experiments from 3 biological replicates for IPAH PASMCs.

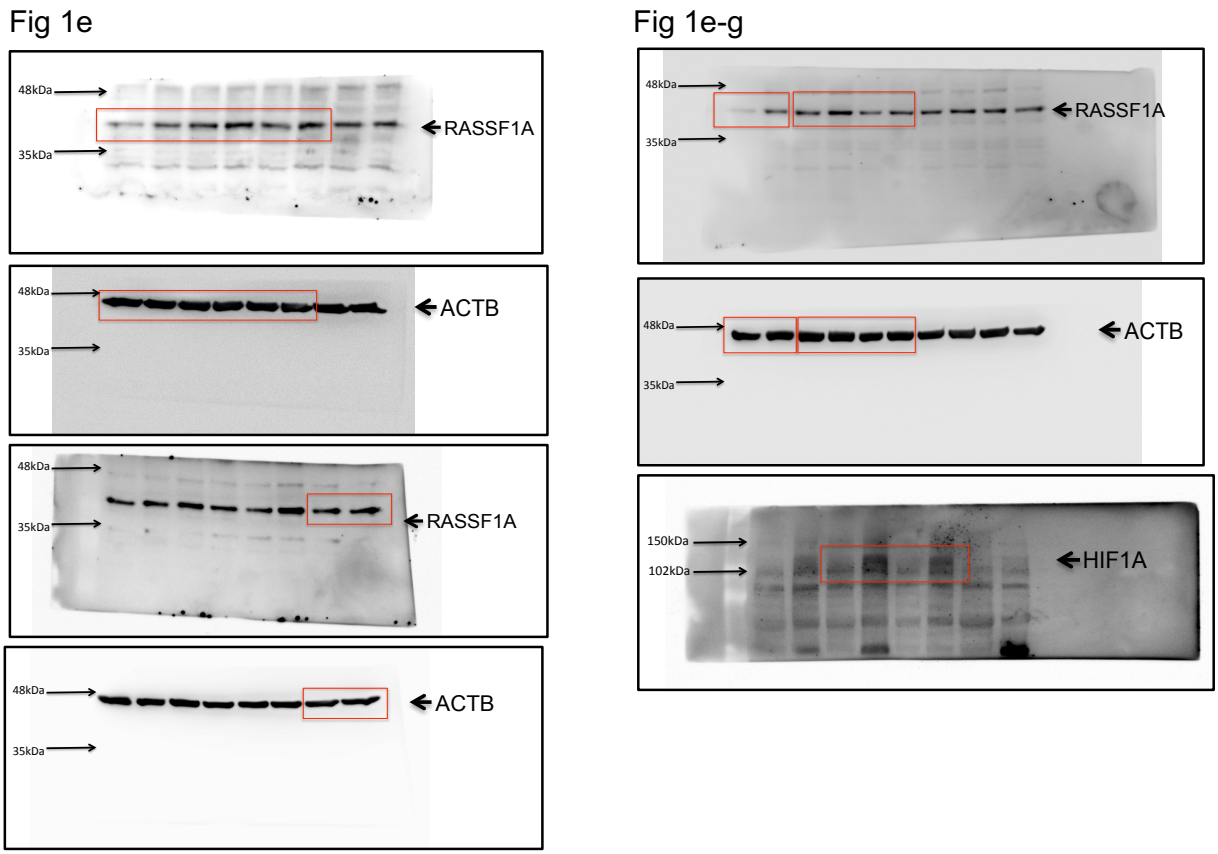


Supplementary Fig. 10: RASSF1A is upregulated in hypoxic mice lungs. Representative immunostaining microphotographs of mice lung sections from normoxic (n=3) and 3-week hypoxic mice (n=3), stained for RASSF1A (brown color). Scale bar: 20 µm.



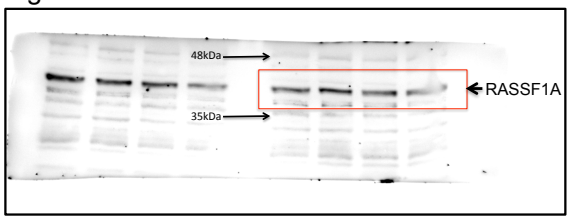
Supplementary Fig. 11: RASSF1A- HIF-1α axis in primary lung tumor cells. (a) Proteins were isolated from tumor (T) and non-tumor (N) areas of human non-small cell lung cancer lungs (n= 57) followed by western blotting for RASSF1A, followed by densitometric analysis. Non-tumor sample values were taken as 1 and expression of RASSF1A in corresponding tumor was calculated as a fold change. **(b)** RASSF1A mRNA expression in different primary tumor cells as analyzed by real time PCRs. **(c)** two different primary tumor cells were transfected with siHIF1α (si-HIF1A) or control siRNA (si-Control). 24hr after transfection, cells were exposed to Normoxia or Hypoxia for 24hr, followed by expression analysis of RASSF1A by real time PCRs. **(d)** Primary lung cancer cells were transfected with RASSF1 siRNA (si-RASSF1) or control siRNA (si-control). 24 hr after transfection, cells were placed in hypoxia for 48 hr. Proliferation was measured by BrdU incorporation assay. $^{**}P < 0.01$, $^{***}P < 0.001$ compared

to si-Control (Hypoxia), one-way ANOVA followed by SNK multiple comparison test. Data represent mean ± s.e.m all through the figure. For primary tumor cells, n=2 independent experiments from 2 biological replicates (represented as separate).



Supplementary Fig. 12: Uncropped scans of western blots for Fig. 1.

Fig 2a



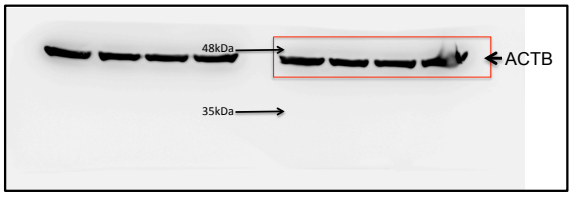


Fig 2b

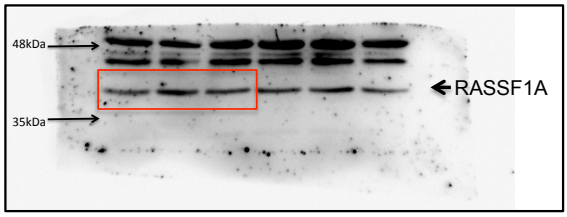
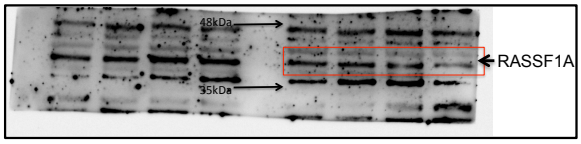


Fig 2c



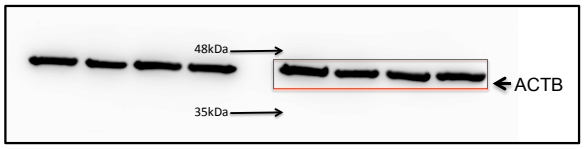


Fig 2d

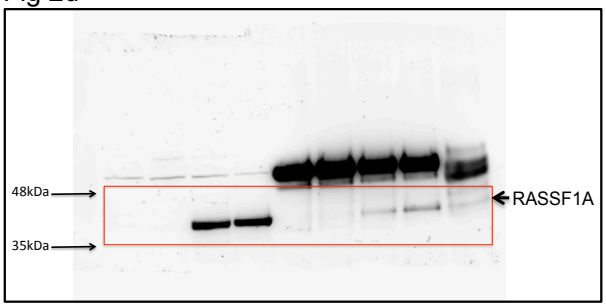


Fig 2e

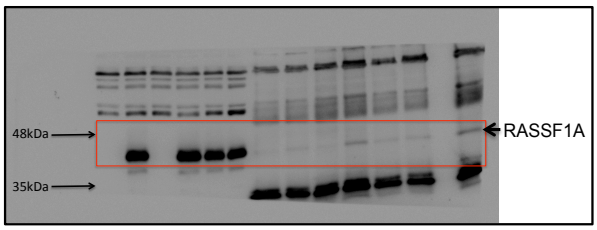
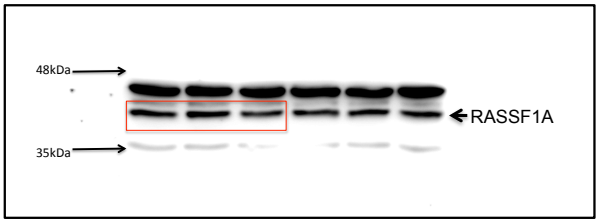
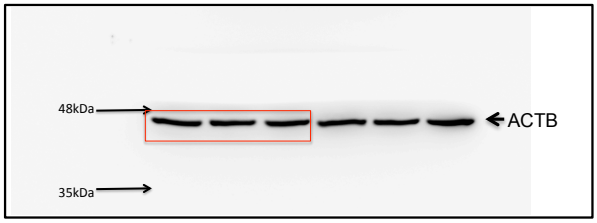
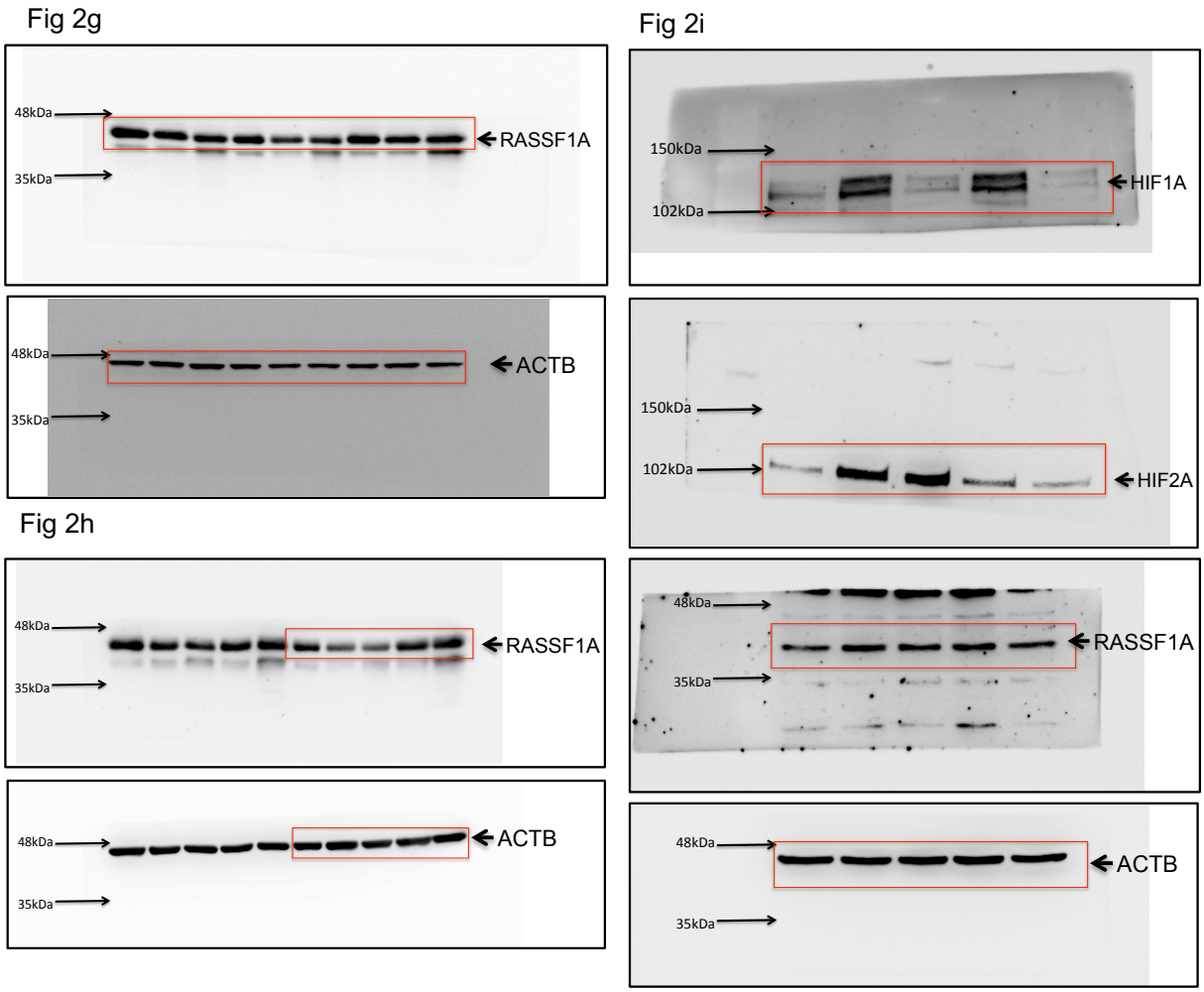


Fig 2f

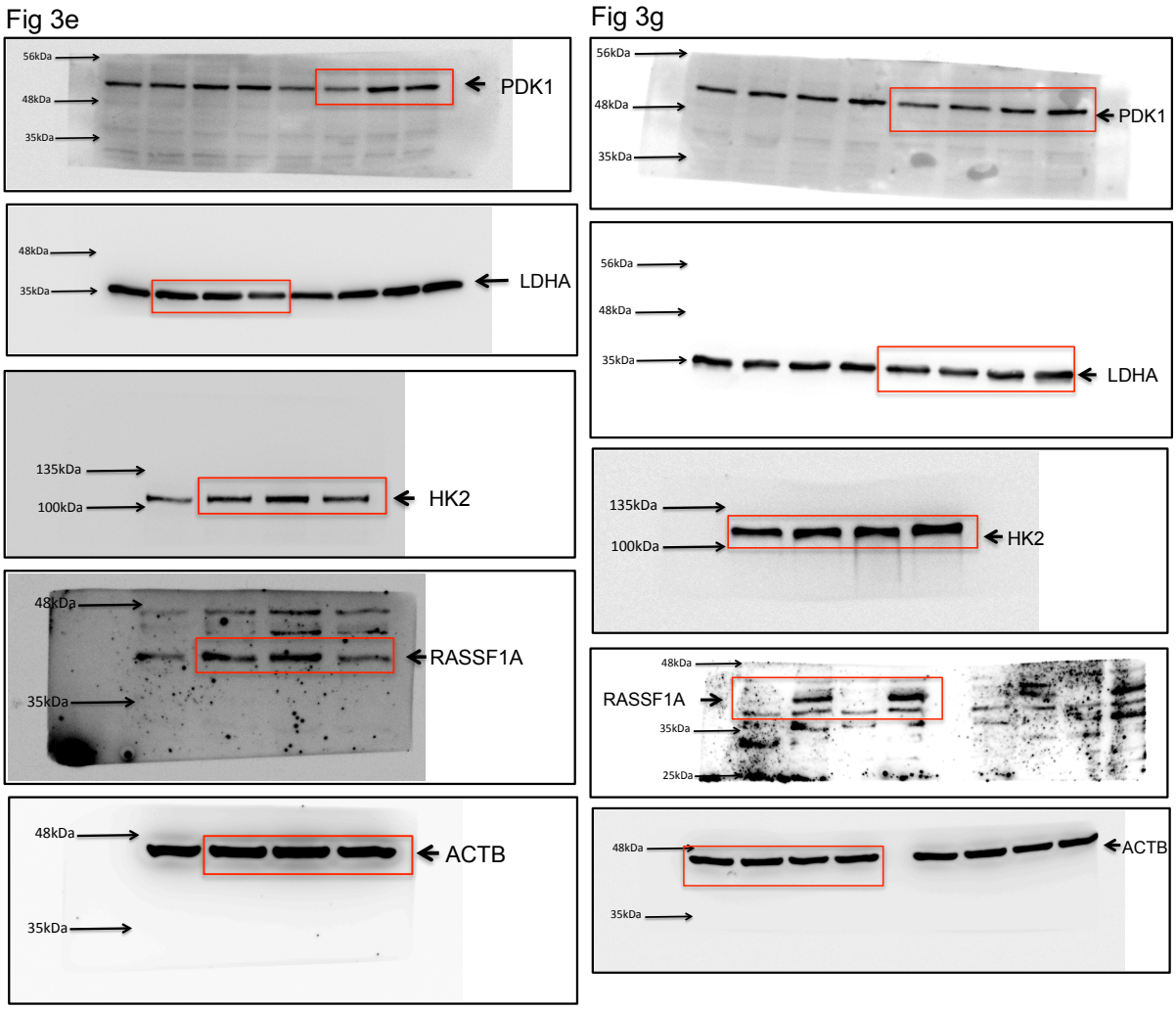




Supplementary Fig. 13: Uncropped scans of western blots for Fig. 2a – 2f.



Supplementary Fig. 14: Uncropped scans of western blots for Fig. 2g – 2i.



Supplementary Fig. 15: Uncropped scans of western blots for Fig. 3e and 3g.

Fig 4a

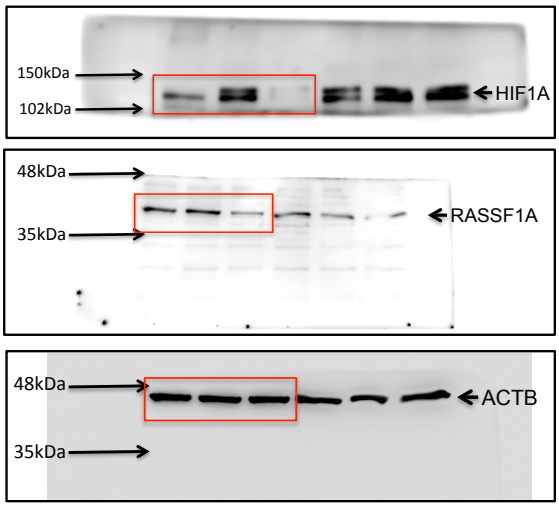


Fig 4e

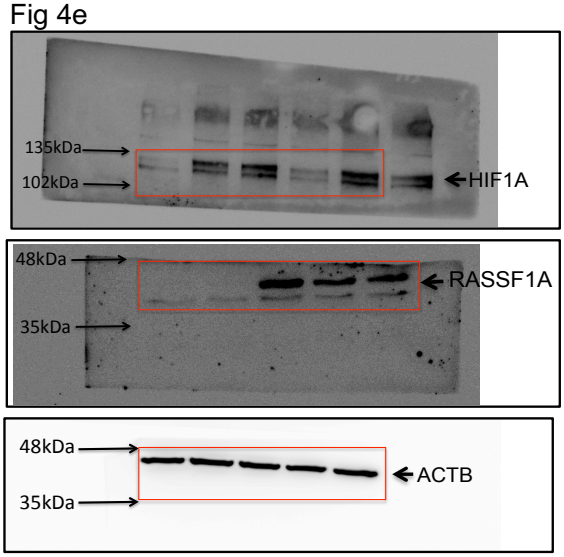
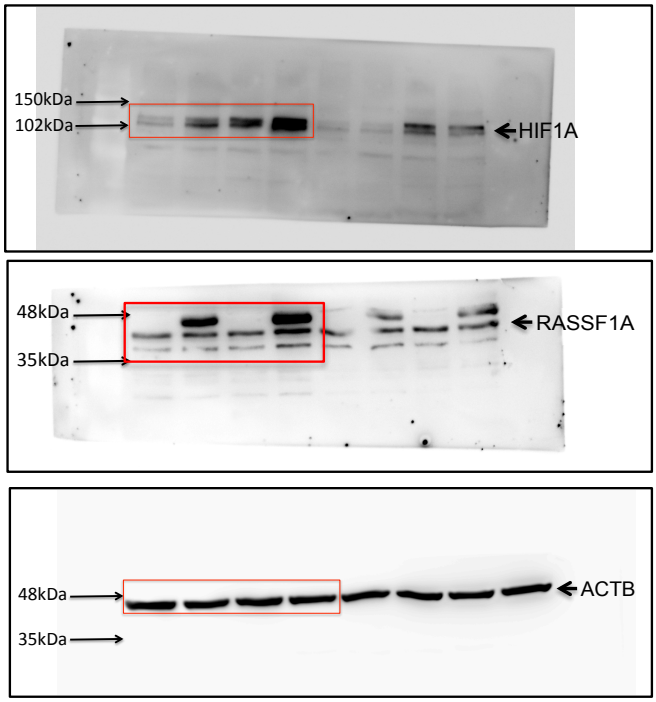
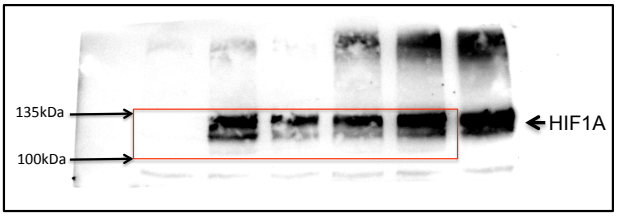


Fig 4b



Supplementary Fig. 16: Uncropped scans of western blots for Fig. 4a, 4b and 4e.

Fig 5a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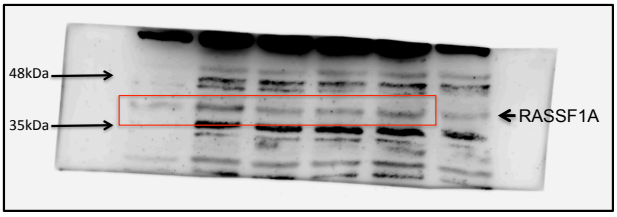
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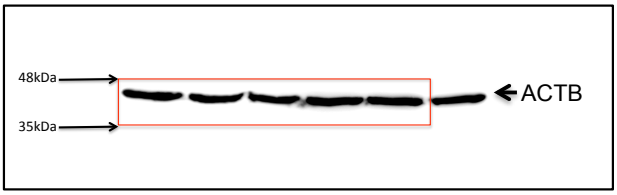
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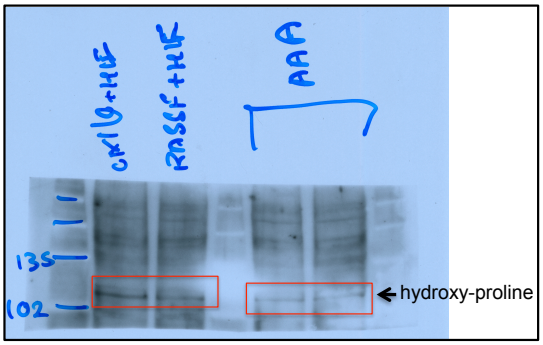
35kDa
ACTB

Fig 5b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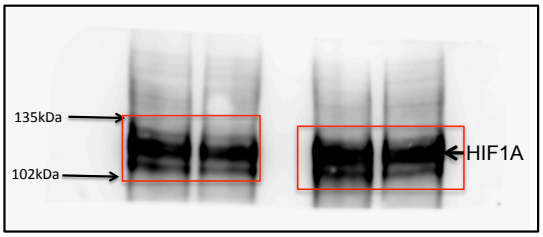
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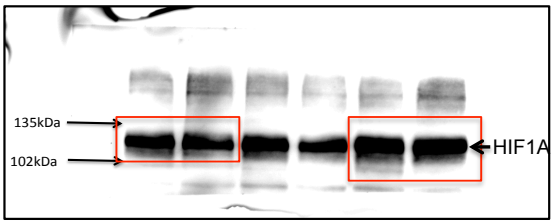
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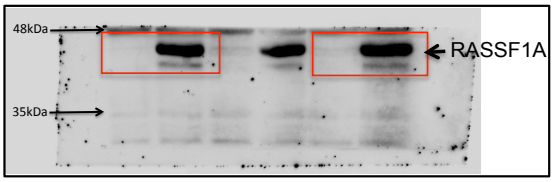
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Fig 5c

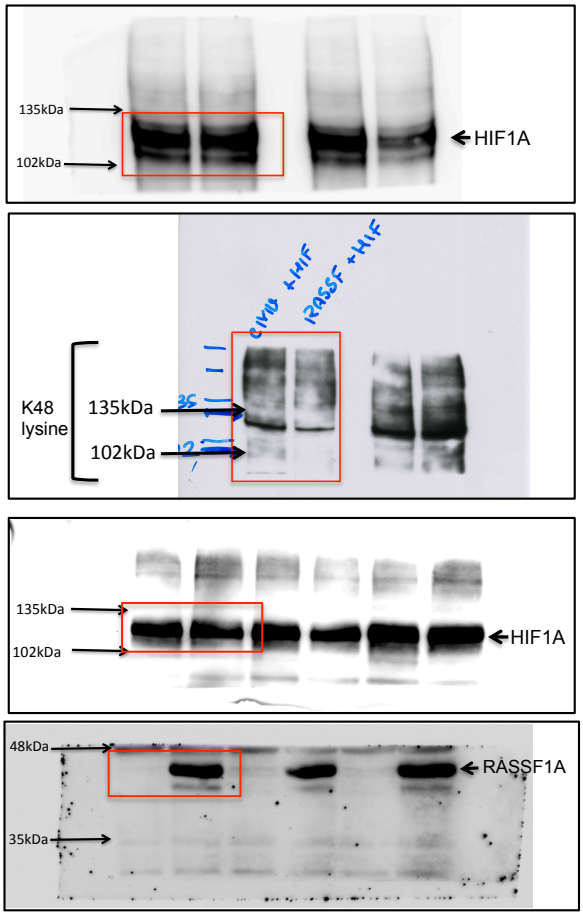


Fig 5d

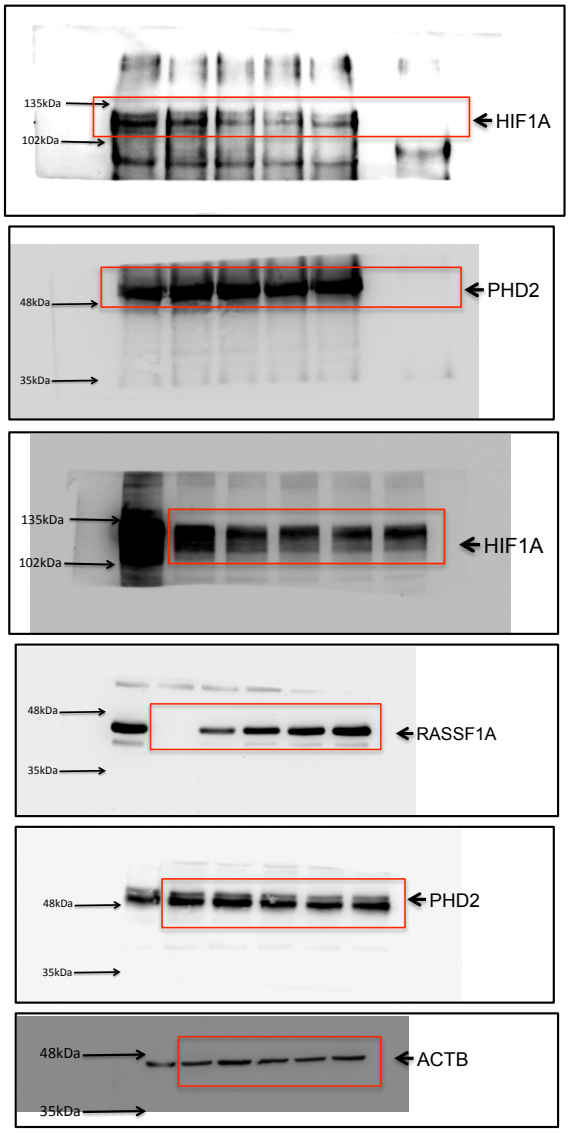


Fig 5e

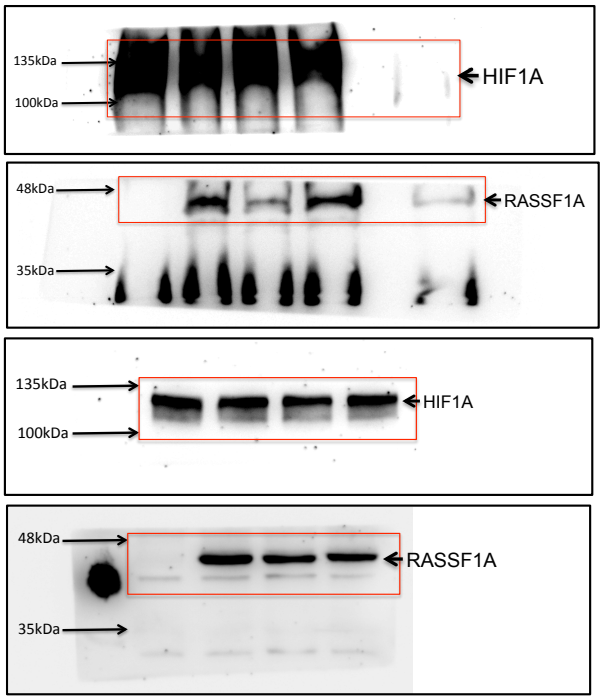
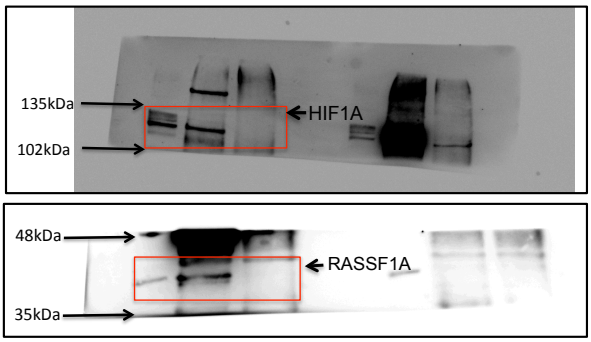
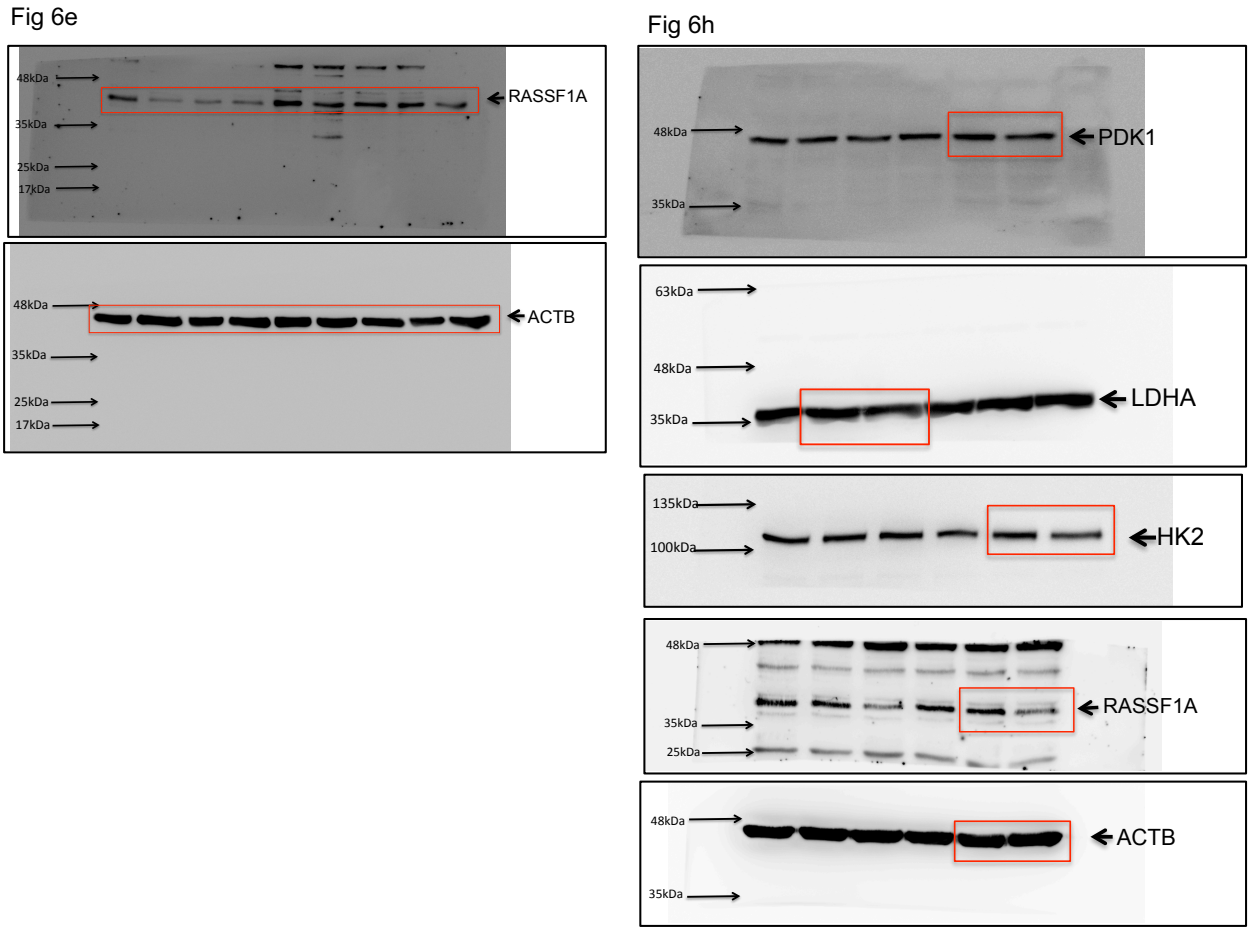


Fig 5f

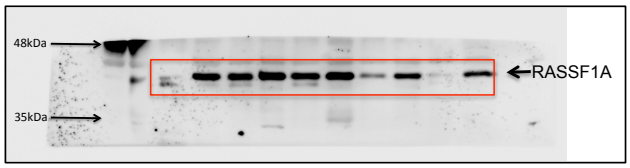


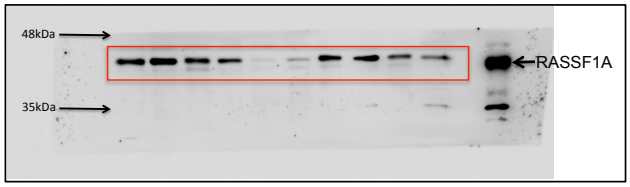
Supplementary Fig. 17: Uncropped scans of western blots for Fig. 5a – 5f.

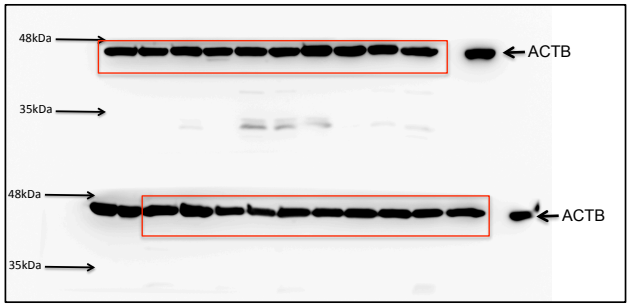


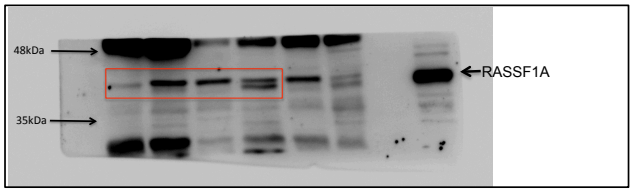
Supplementary Fig. 18: Uncropped scans of western blots for Fig. 6e and 6h.

Fig 8a









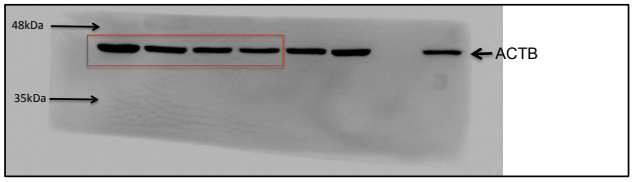
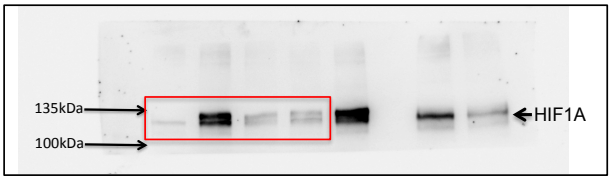
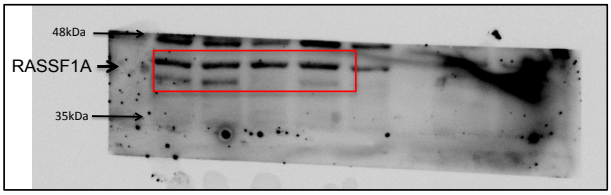


Fig 8c





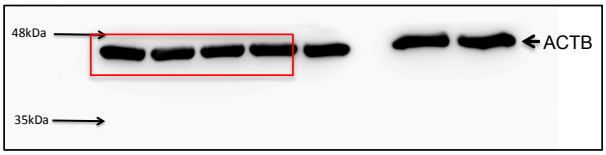
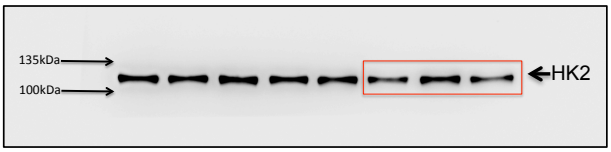
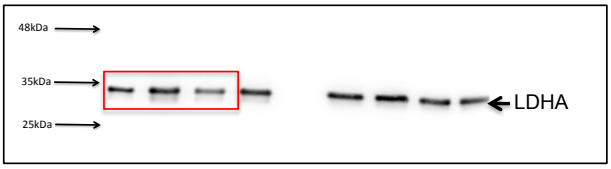
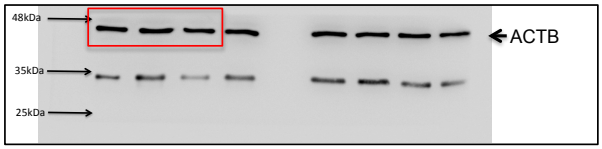
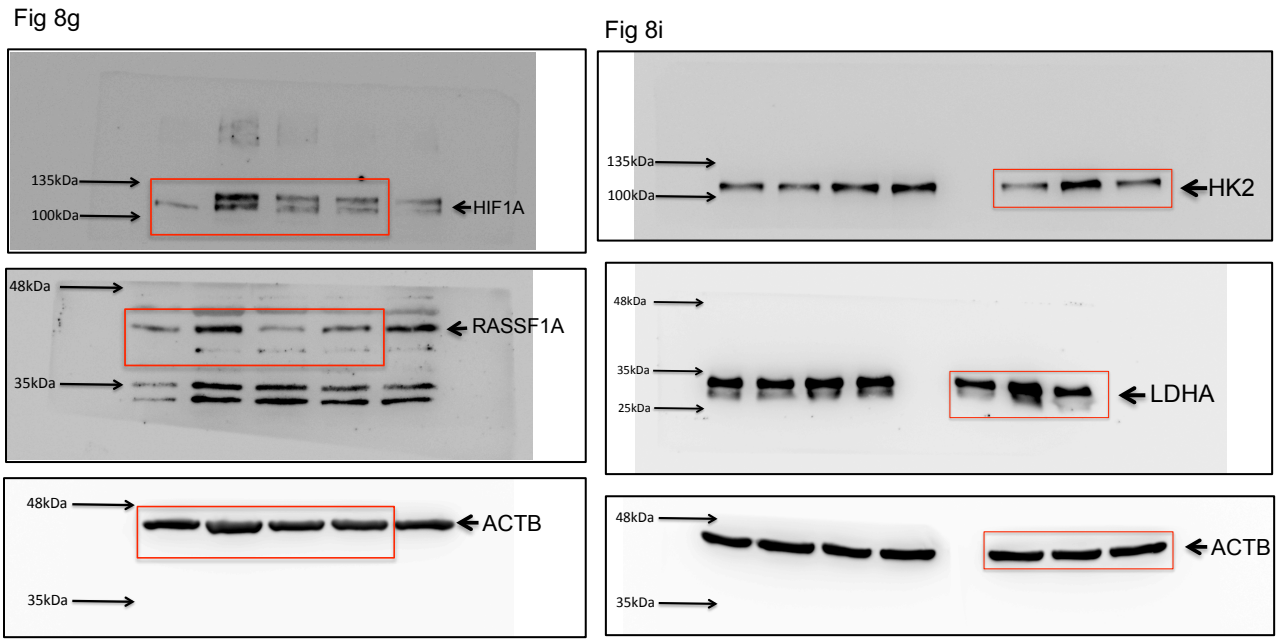


Fig 8e

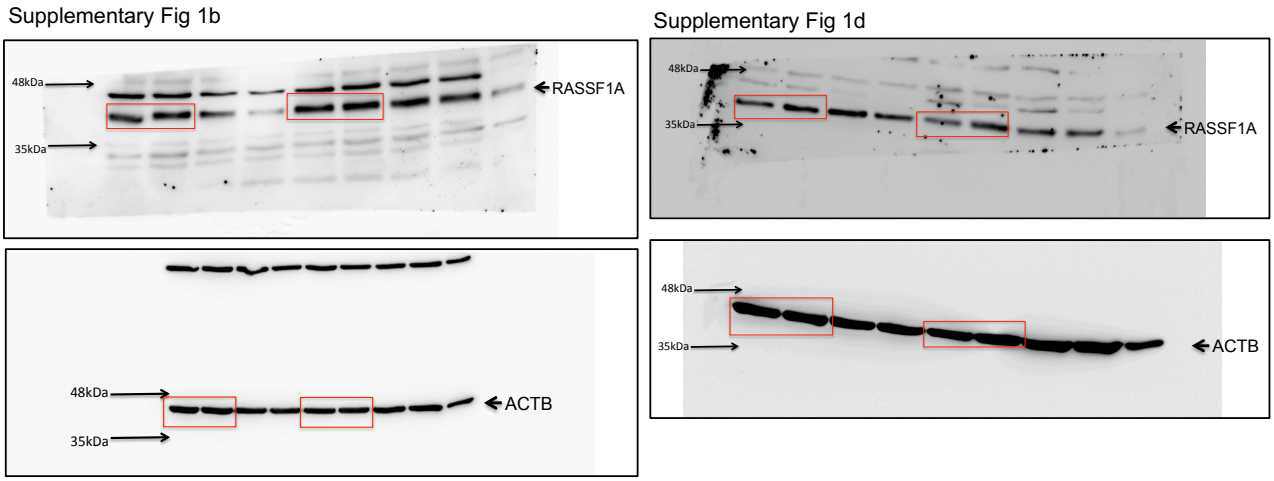






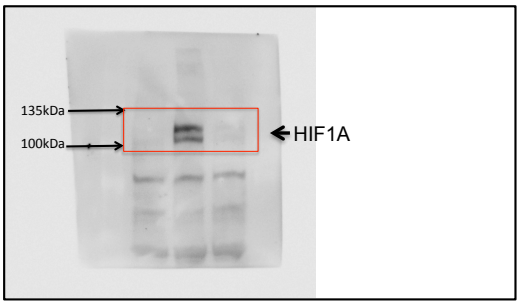


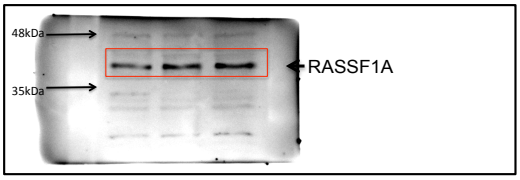
Supplementary Fig. 19: Uncropped scans of western blots for Fig. 8a, 8c, 8e, 8g, 8i.

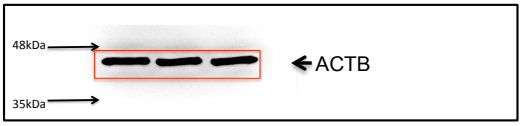


Supplementary Fig. 20: Uncropped scans of western blots for Supplementary Fig. 1b and 1d.

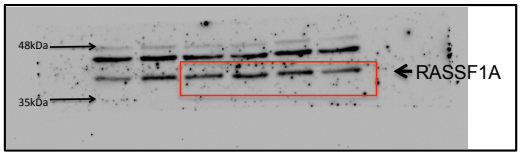
Supplementary Fig 2a

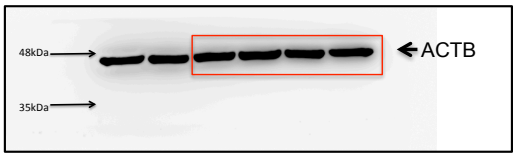




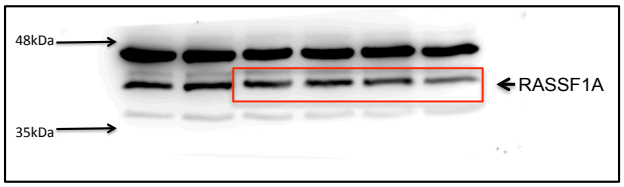


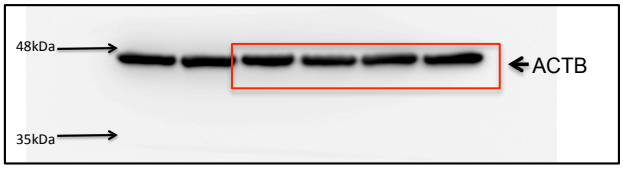
Supplementary Fig 2b



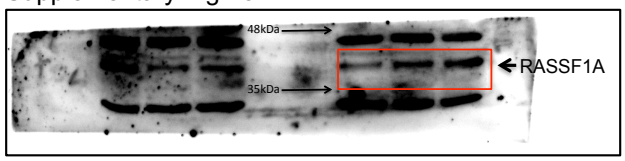


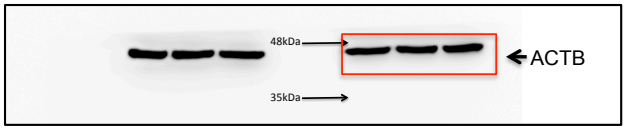
Supplementary Fig 2c



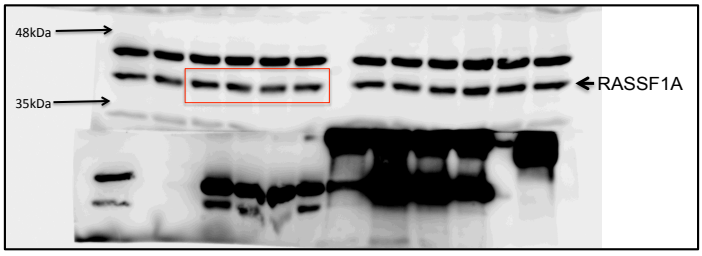


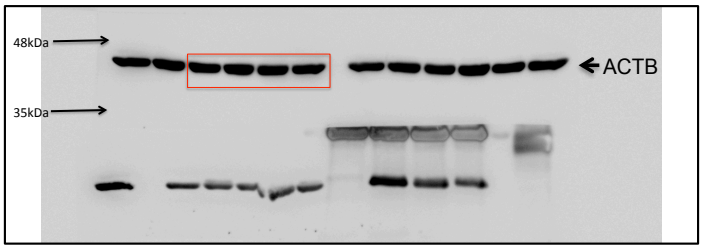
Supplementary Fig 2e

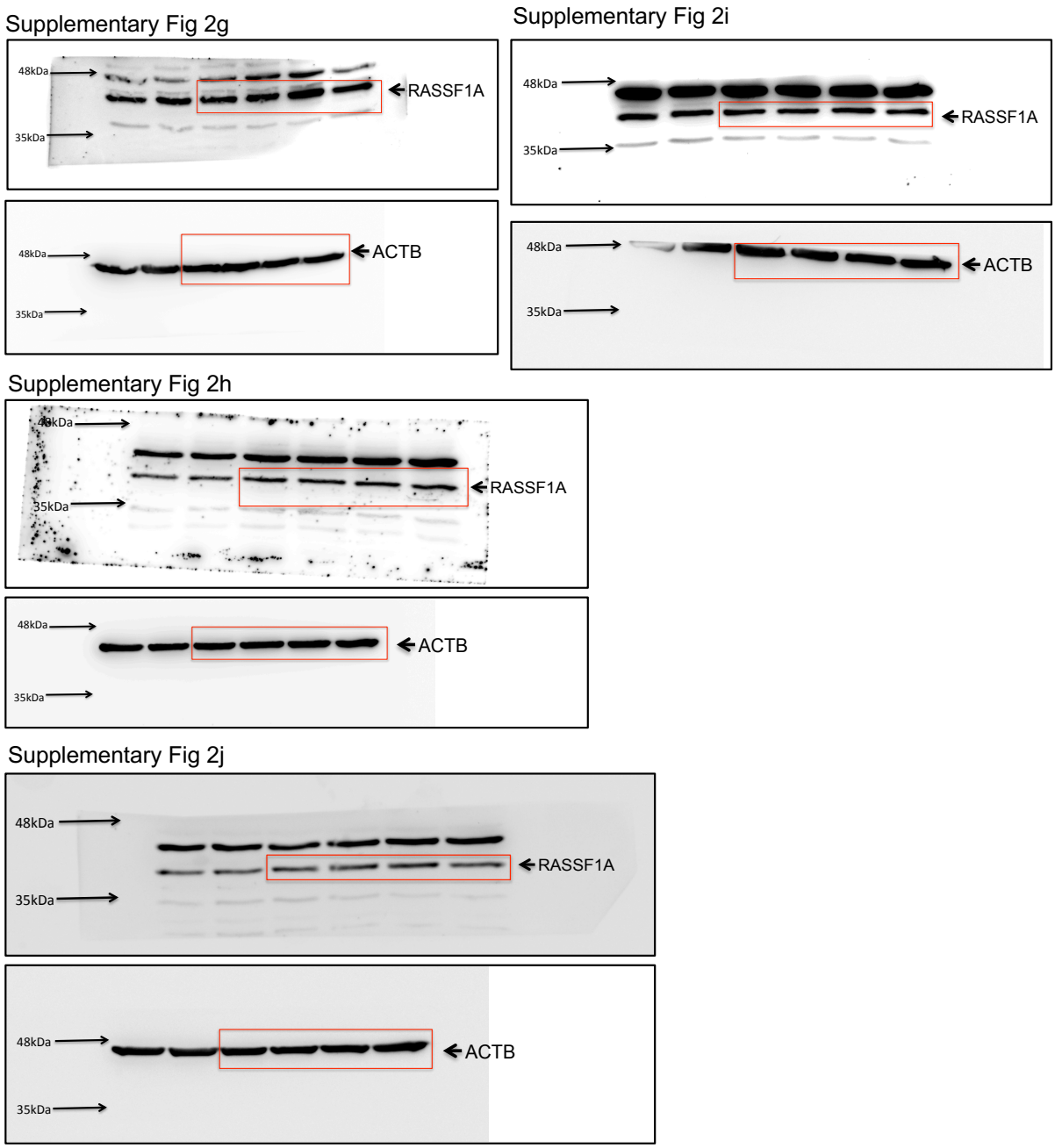




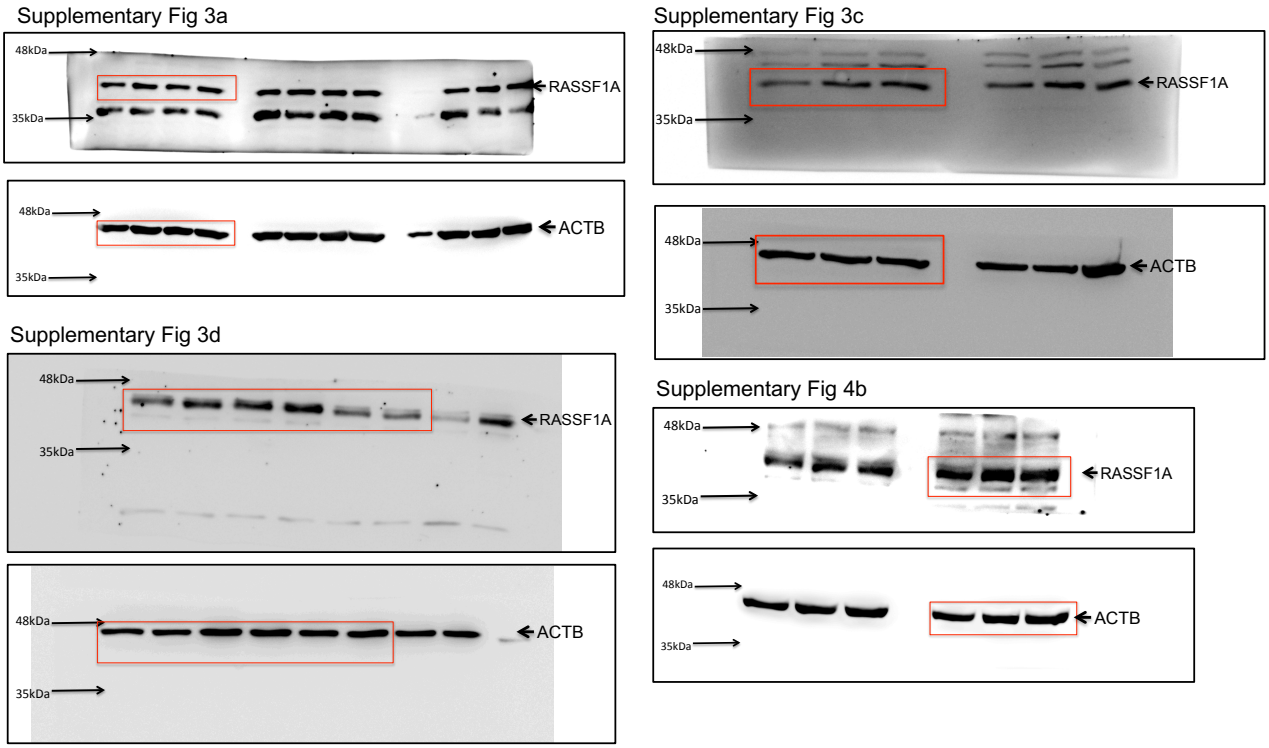
Supplementary Fig 2f





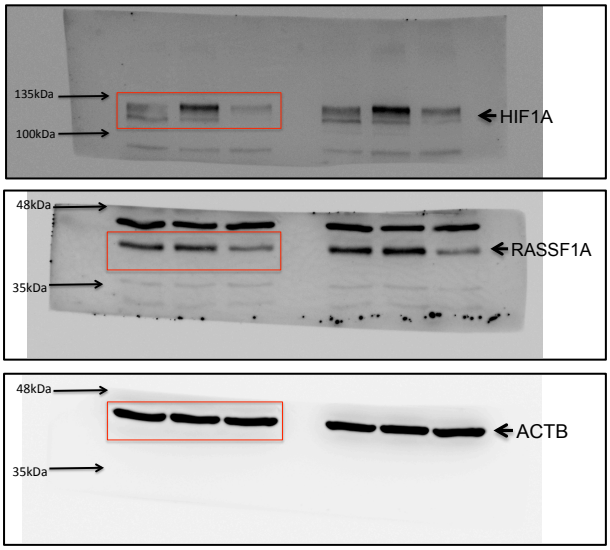


Supplementary Fig. 21: Uncropped scans of western blots for Supplementary Fig. 2a -2j.

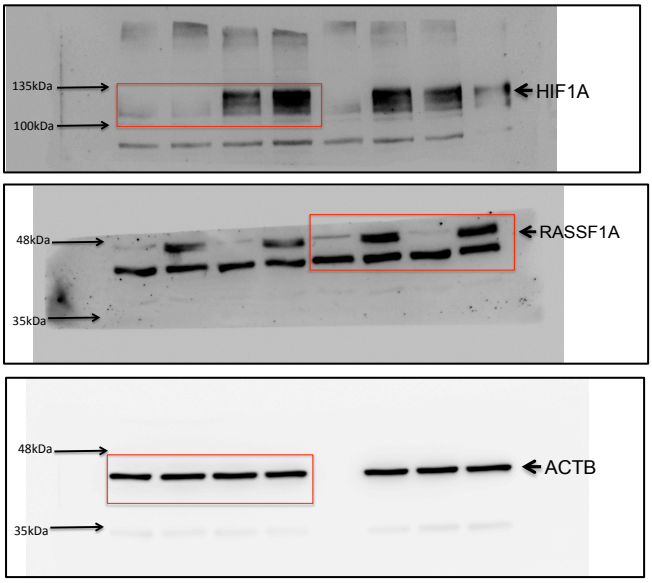


Supplementary Fig. 22: Uncropped scans of western blots for Supplementary Fig. 3a, 3c, 3d and 4b.

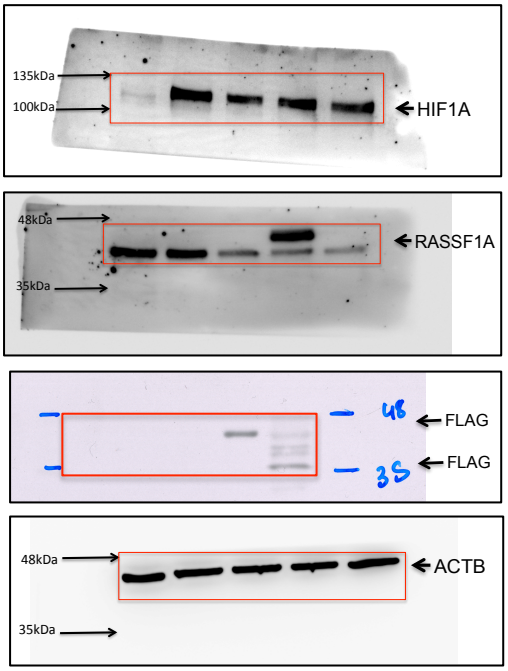
Supplementary Fig 6a



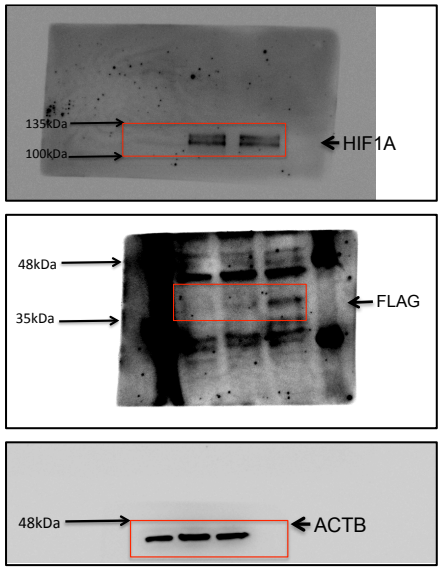
Supplementary Fig 6b



Supplementary Fig 6c

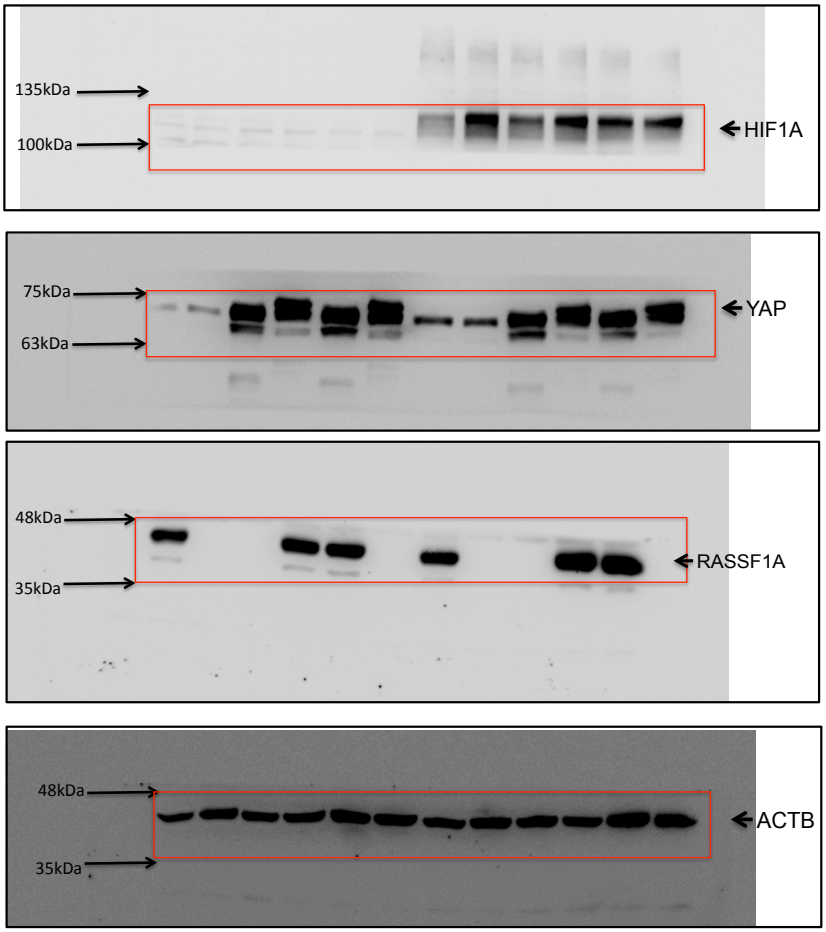


Supplementary Fig 6e



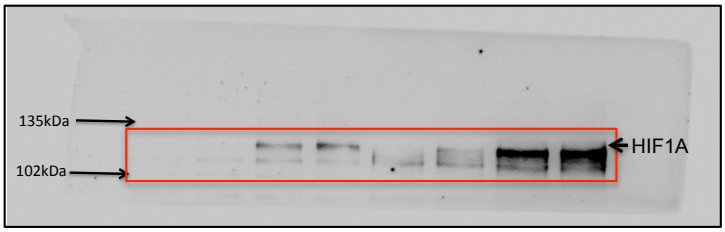
Supplementary Fig. 23: Uncropped scans of western blots for Supplementary Fig. 6a – 6c and 6e.

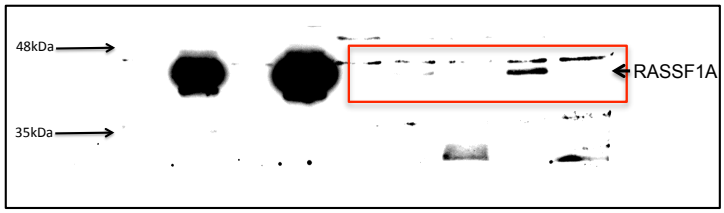
Supplementary Fig 7b

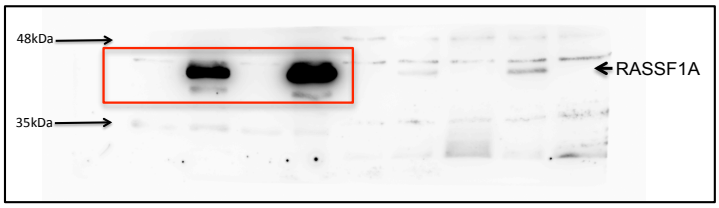


Supplementary Fig. 24: Uncropped scans of western blots for Supplementary Fig. 7b.

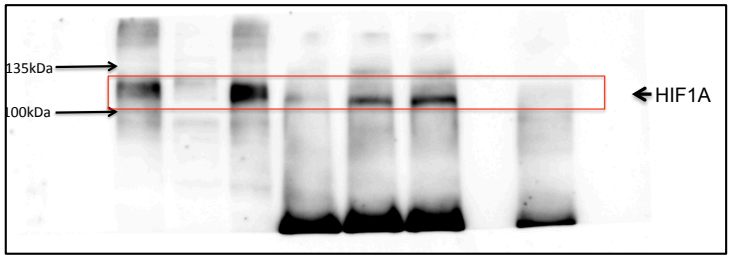
Supplementary Fig 8a

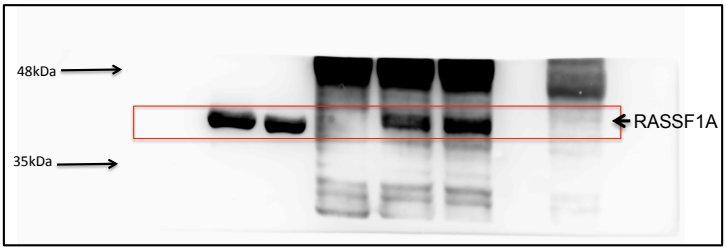




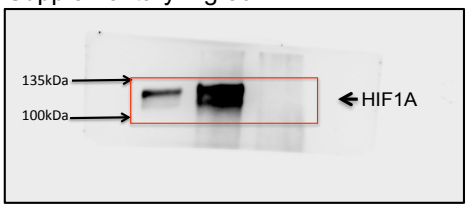


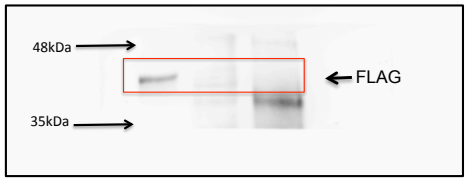
Supplementary Fig 8b



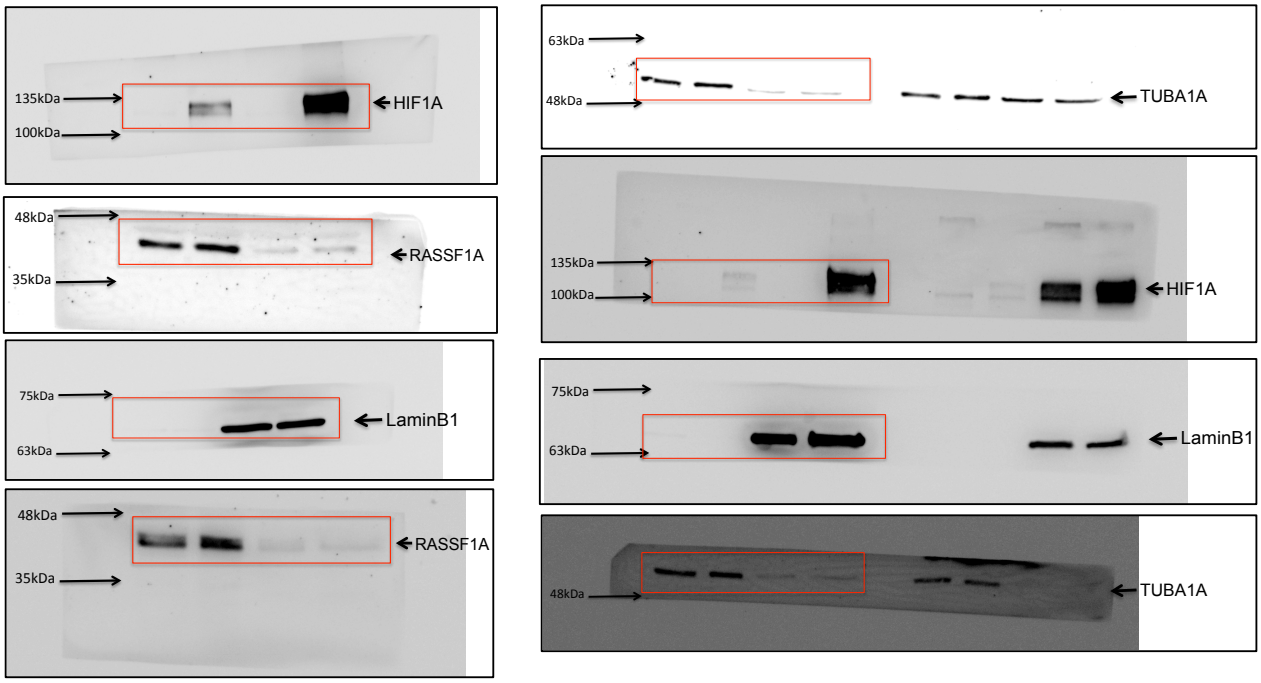


Supplementary Fig 8c

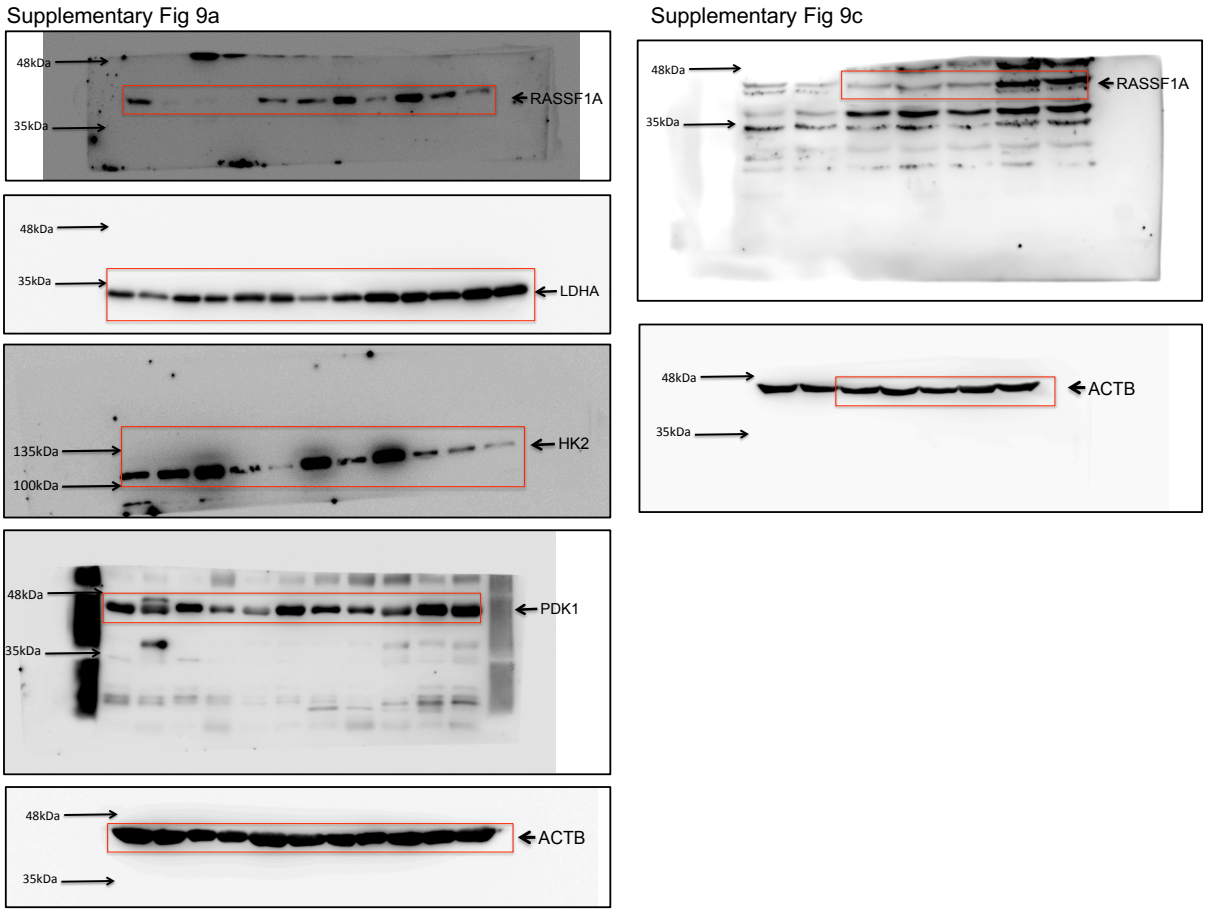




Supplementary Fig 8d

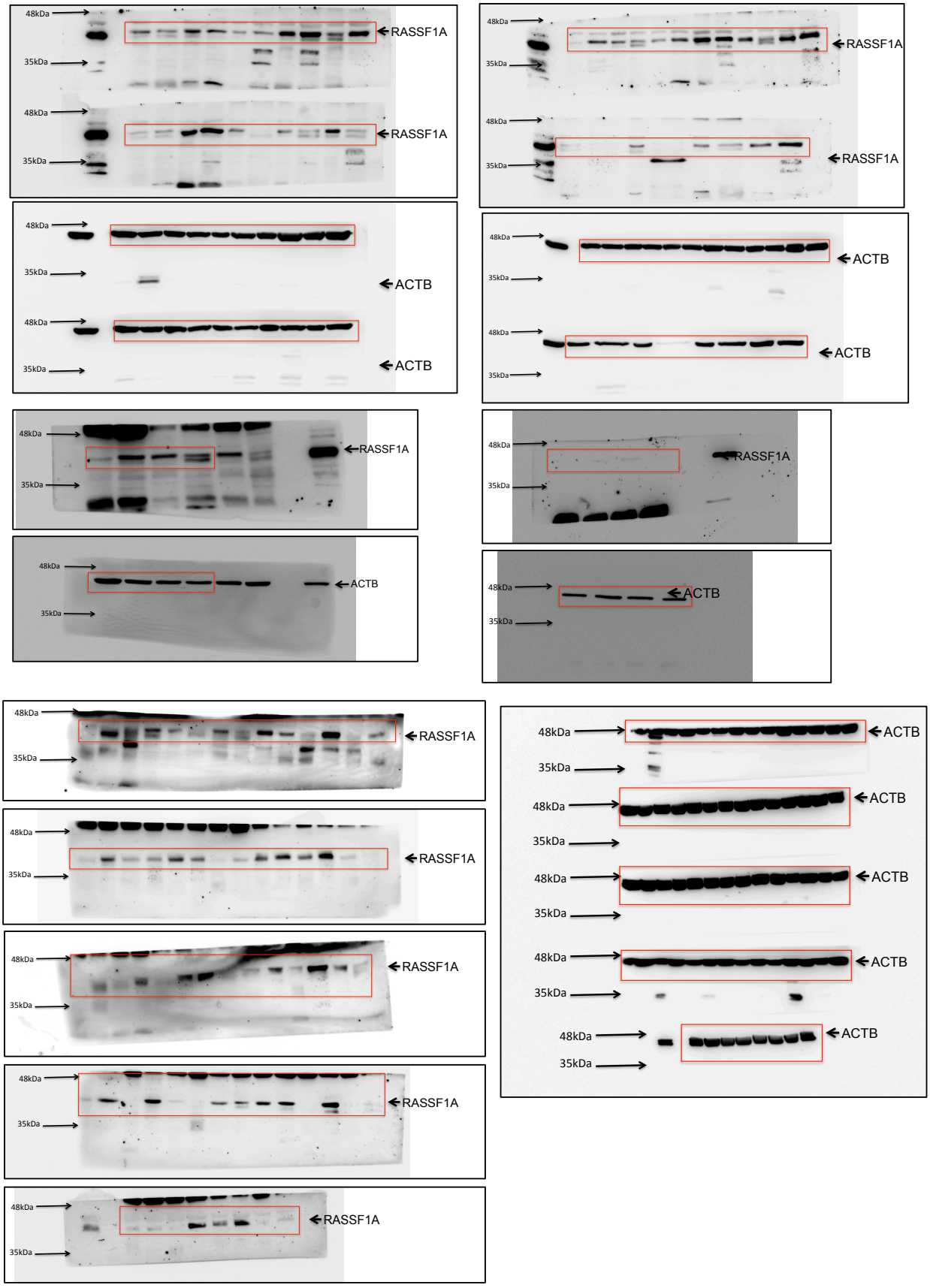


Supplementary Fig. 25: Uncropped scans of western blots for Supplementary Fig. 8a-8d.



Supplementary Fig. 26: Uncropped scans of western blots for Supplementary Fig. 9a and 9c.

Supplementary Fig 11a



Supplementary Fig. 27: Uncropped scans of western blots for Supplementary Fig. 11a.

Supplementary Table 1: Real time PCR Primers

	Species	Forward Primer	Reverse Primer
RASSF1A	Human	GGCTGGGAACCCGCGGTG	TCCTTGCAAGGAGGGTGGCTTCT
HIF1A	Human	TAAAGGAATTTCAATATTTGATGGG	AAAGGGTAAAGAACAAAACACACAG
HIF2A	Human	GATCTTTCTGTCAGGAAACATCAGC	GTTGACAGTACGGCCTCTGTTG
PDK1	Human	CTCAGGACACCATCCGTTCA	ATCTTGCAGGCCATACAGCA
LDHA	Human	GAAGATAAGTGGTTTTCCCAAAAA	CTTTGAGTTTGATCACCTCATAAGC
HK2	Human	GCAGGATGATTGCCTCGC	ACTCTCCGTGTTCTGTCC
CA9	Human	TTTGCCAGAGTTGACGAGGC	CTGAGCCTTCCTCAGCGATT
PRKCA	Human	GCCTATGGCGTCCTGTTGTA	CTCCTTTGCCACACACTTTGG
PRKCB	Human	CACTCCAGACTACATCGCCC	TCTCTTGTCTCTAGCTTTTGGCT
NOX1	Human	TGTTTGTGGATGCCTTCCTG	AAGGACAGCAGATTGCGACA
B2M	Human	AGATGAGTATGCCTGCCG	TCATCCAATCCAAATGCG
HPRT	Human	TGACACTGGCAAAACAAT	GGTCCTTTTCACCAGCAA
RASSF1A	Mouse	GCGACCTCTGTGGAGACTTC	CTCTAGTGCAGAGTCCCAGC
18s rRNA	Mouse	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
RASSF1A	Rat	CACAACACGCAATCCGTCAC	CGACATCGGTAATGGCAGGT
HPRT	Rat	GACTTTGCTTTCCTTGGTCA	AGTCAAGGGCATATCCAACA
RASSF1A_HRE1_CHIP	Human	CACAGTAAAGCTGGCCTCCA	CTCCTTCGTCCCCTCCTCA
RASSF1A_HRE2_CHIP	Human	TGCAGGTGGGTTGAGGTTAC	GAGGGCCTTCAGTGCAATCT
LDHA_CHIP	Human	ACTCAGGCTCATGGCTC	GGCTGGGGGTGGATG
PDK1_CHIP	Human	CGCGTTTGGATTCCGTG	CCAGTTATAATCTGCCTTCCCTATTATC
HK2_CHIP	Human	GCCCCGCAGGTAGTCAG	AGCCACGATTCTCTCCACG

Supplementary Table 2: siRNA target sequences

Gene	Target Sequence
RASSF1	AAGCACCGAAGCGAAACTTAA
HIF1A	AACCAAGTAGCCTGTTATCAA
HIF2A	CCCGGATAGACTTATTGCCAA
PRKCA	CGCAGTGGAATGAGTCCTTTA
PRKCB	CCGGATGAAACTGACCGATTT
NOX1	CTGAATCTTCCCTGTTGCCTA
Control siRNA	AATTCTCCGAACGTGTCACGT

Supplementary Table 3: Antibodies

Antibody	Company	Application
RASSF1A	Abcam (ab23950)	Western blotting
RASSF1	Santa Cruz (sc-18722)	Immunohistochemistry
RASSF1A	Biorbyt (orb11328)	Proximity ligation assay
RASSF1A	Abcam (ab91212)	Immunoprecipitation
HIF1A	BD biosciences (610-727)	Western blotting/IHC/PLA
HIF1A	Abcam (ab2185)	IP/ChIP
HK2	Abcam (ab104836)	Western blotting
PDK1	Abcam (ab110025)	Western blotting
LDHA	Abcam (ab101562)	Western blotting
FLAG M2	Sigma (F1804)	IP/Western blotting
PHD2	Novus bioloigicals (NB100-2219)	IP/Western blotting
Hydroxy-proline	Abcam (ab37067)	Western blotting
K48 ubiquitin	Abcam (ab140601)	Western blotting
ACTB	Sigma (A5441)	Western blotting