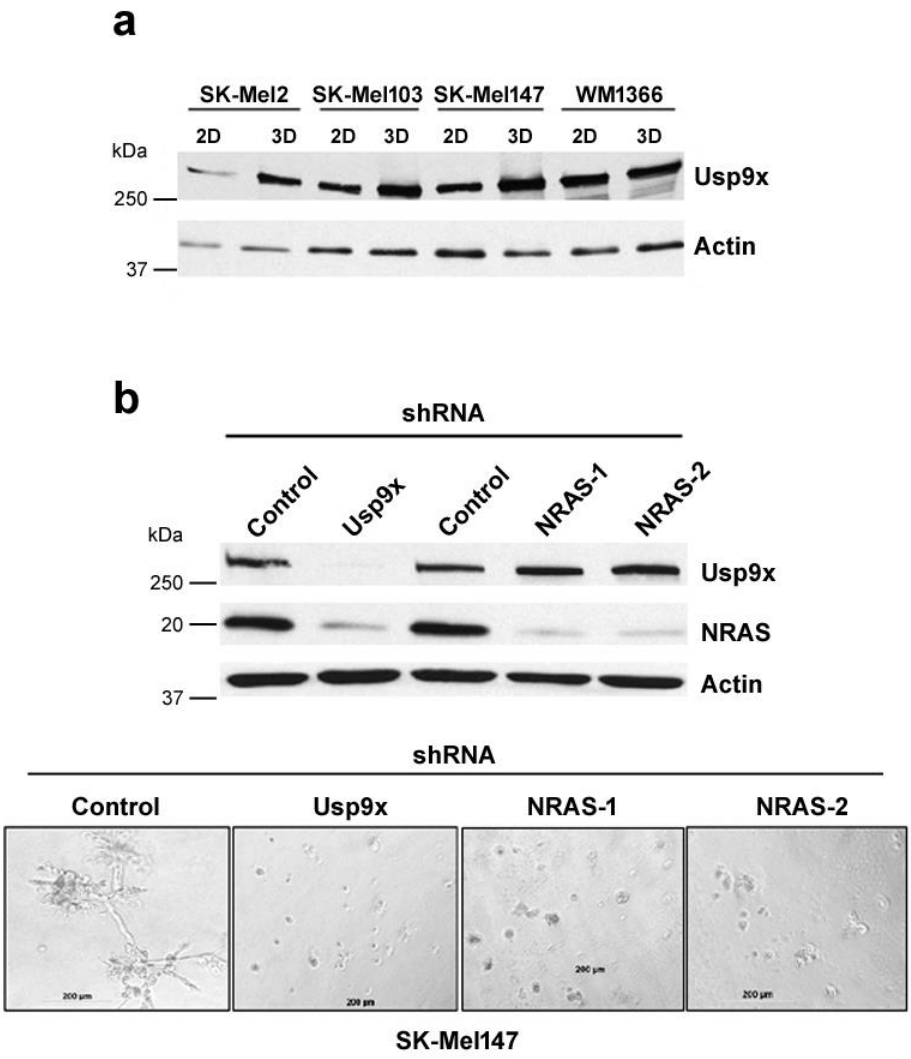
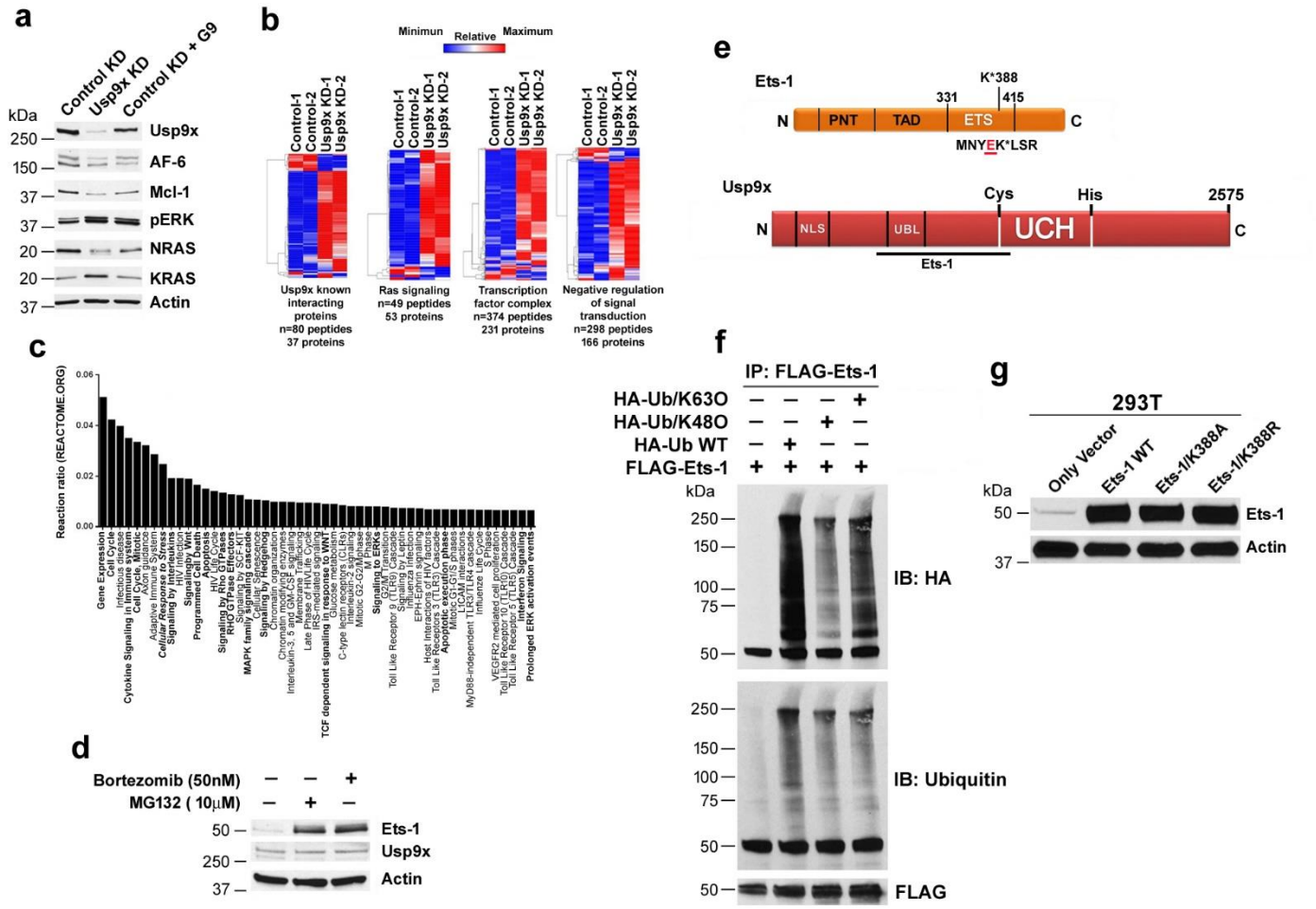
Supplementary Information

Supplementary Fig. 1. Elevated Usp9x in melanoma and NRAS mutant melanoma cells are dependent on NRAS for 3D growth.



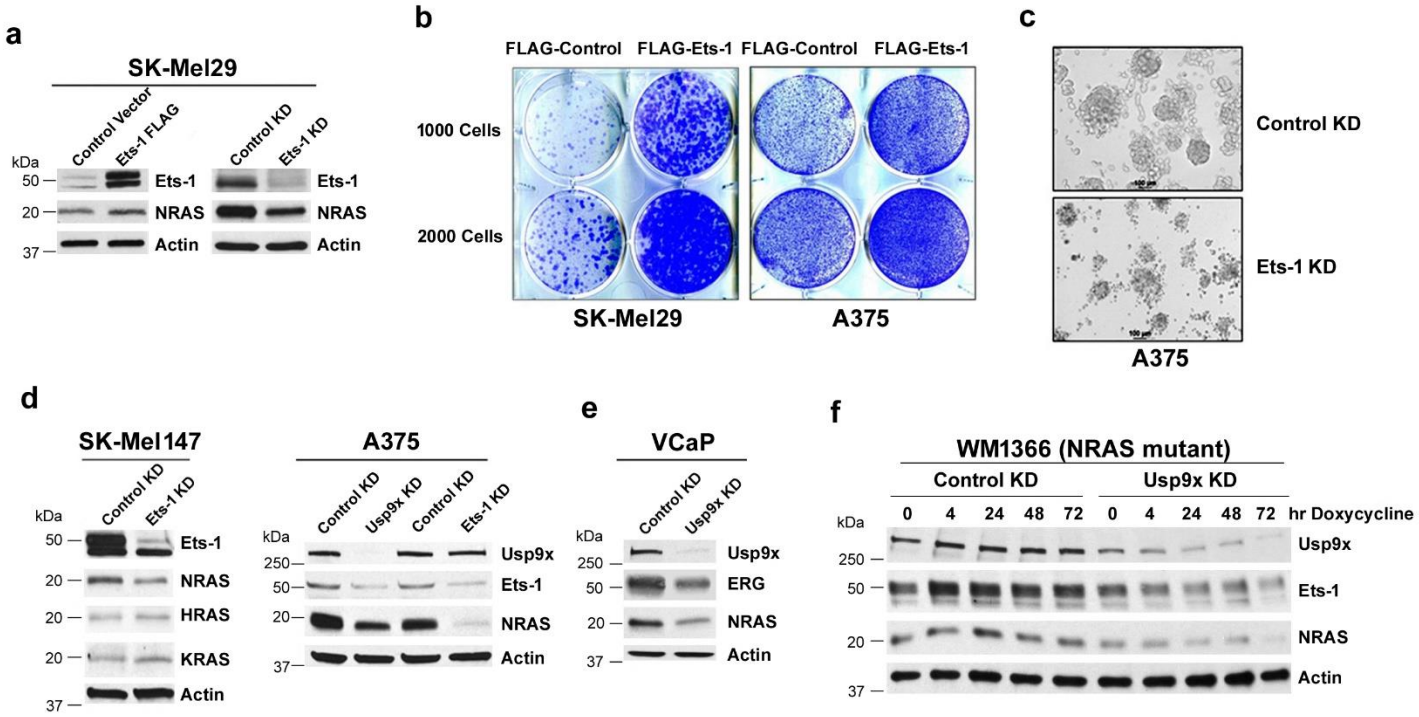
a. Immunoblot for Usp9x protein in NRAS mutant melanoma cells grown in 2D (monolayer) or 3D (agarose) for 3 days. Actin served as a loading control. **b.** Top - Immunoblot for Usp9x and NRAS protein in SK-Mel147 melanoma cells with shRNA-mediated Usp9x or NRAS (NRAS-1 and NRAS-2) KD. Bottom - Colony formation and expansion in control, Usp9x KD or NRAS KD SK-Mel147 cells (200 cells plated) grown on matrigel for 7 days.

Supplementary Fig. 2. Identifying proteins deubiquitinated by Usp9x.



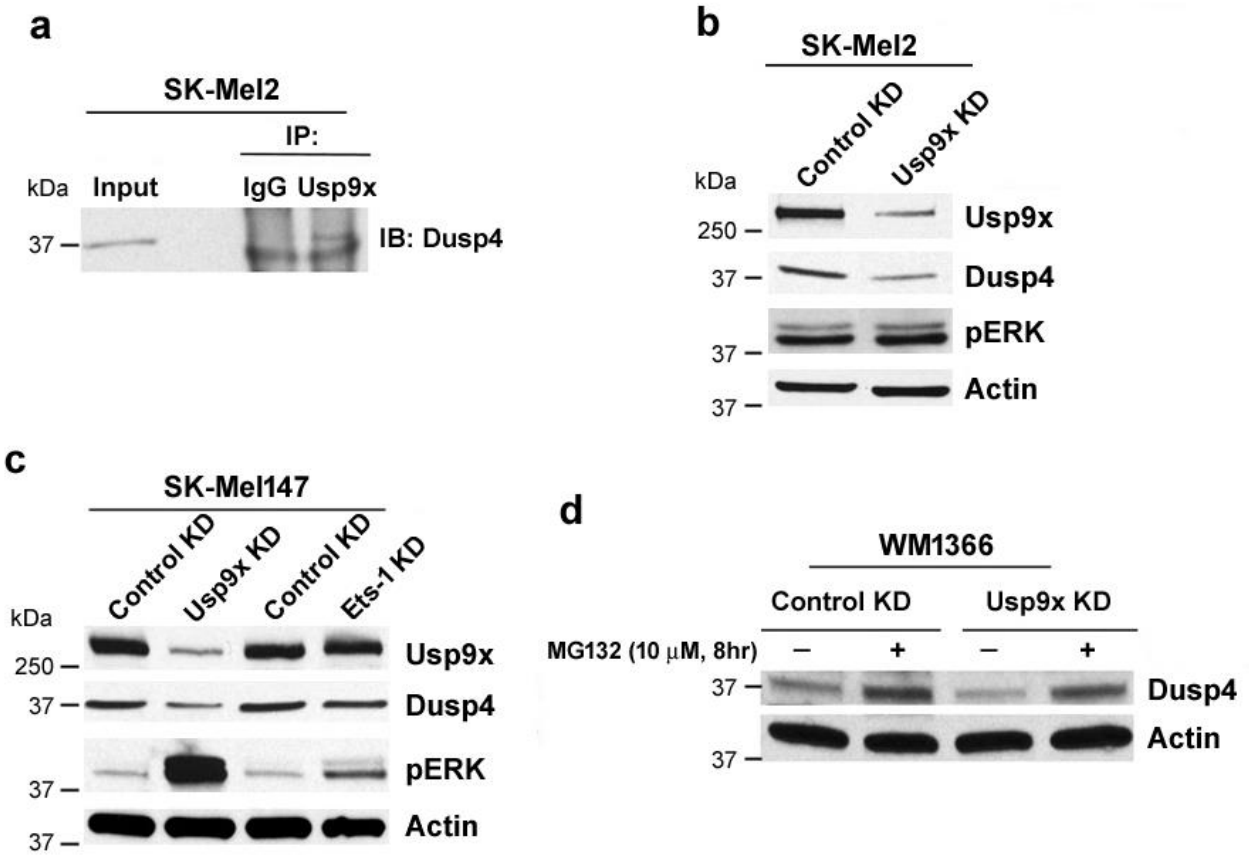
a. Immunoblot for known substrates of Usp9x in NRAS mutant SK-Mel147 melanoma cells after treatment as indicated. Lysates from this assessment were subjected to UbiScan (Cell Signaling Technologies, Inc.) analysis. **b**. Heat map analysis of ubiquitination changes in known Usp9x-interacting proteins (total=104; PubMed, GENE) following Usp9x KD in melanoma cells (37 proteins showed altered ubiquitination). Heat maps of differentially ubiquitinated proteins involved in RAS signaling, transcription factor complexes and negative regulation of signal transduction. Heat maps represent peptides with a coefficient of variation (CV) > 50. Complete protein list provided in the Supplemental Data files 1-7. The number of unique peptides and proteins reproducibly detected in each analysis is shown. **c.** Reactome (molecular pathways) analysis of proteins differentially ubiquitinated after Usp9x KD in NRAS mutant melanoma cells (Supplementary Data file 8). Enriched groups are ranked by the most significant reactome ratio. **d**. SK-Mel147 cells were treated as described for 8 h before assessing Ets-1, Usp9x and actin levels by immunoblotting. **e.** Schematic diagram of the human Ets-1 protein with specific domains and predicted site of Usp9x deubiquitination (K*388). Usp9x (full length 2575 aa) protein showing the location of the NLS (nuclear localization sequence), UBL (ubiquitin-like domain), UCH (ubiquitin C-terminal hydrolase domain) and predicted site of Ets-1 interaction. **f**. K63-linked ubiquitination of Ets-1. HEK293T cells were co-transfected with FLAG-Ets-1 (WT) and pRK5-HA-ubiquitin (WT), pRK5-HA-Ub/K48 only and pRK5-HA-Ub/K63 only expression constructs. After 48 h, FLAG-Ets-1 was immunoprecipitated and immunoblotted with antibodies against HA and Ubiquitin to detect ubiquitinated Ets-1. **g.** HEK293T cells were transfected with an expression vector for HA-Ets-1 WT or specific K388 mutants (as indicated). After 48 h, cell lysates were immunoblotted for HA (Ets-1). Actin served as a protein loading control.

Supplementary Fig. 3. Usp9x controls Ets-1 and NRAS protein levels.



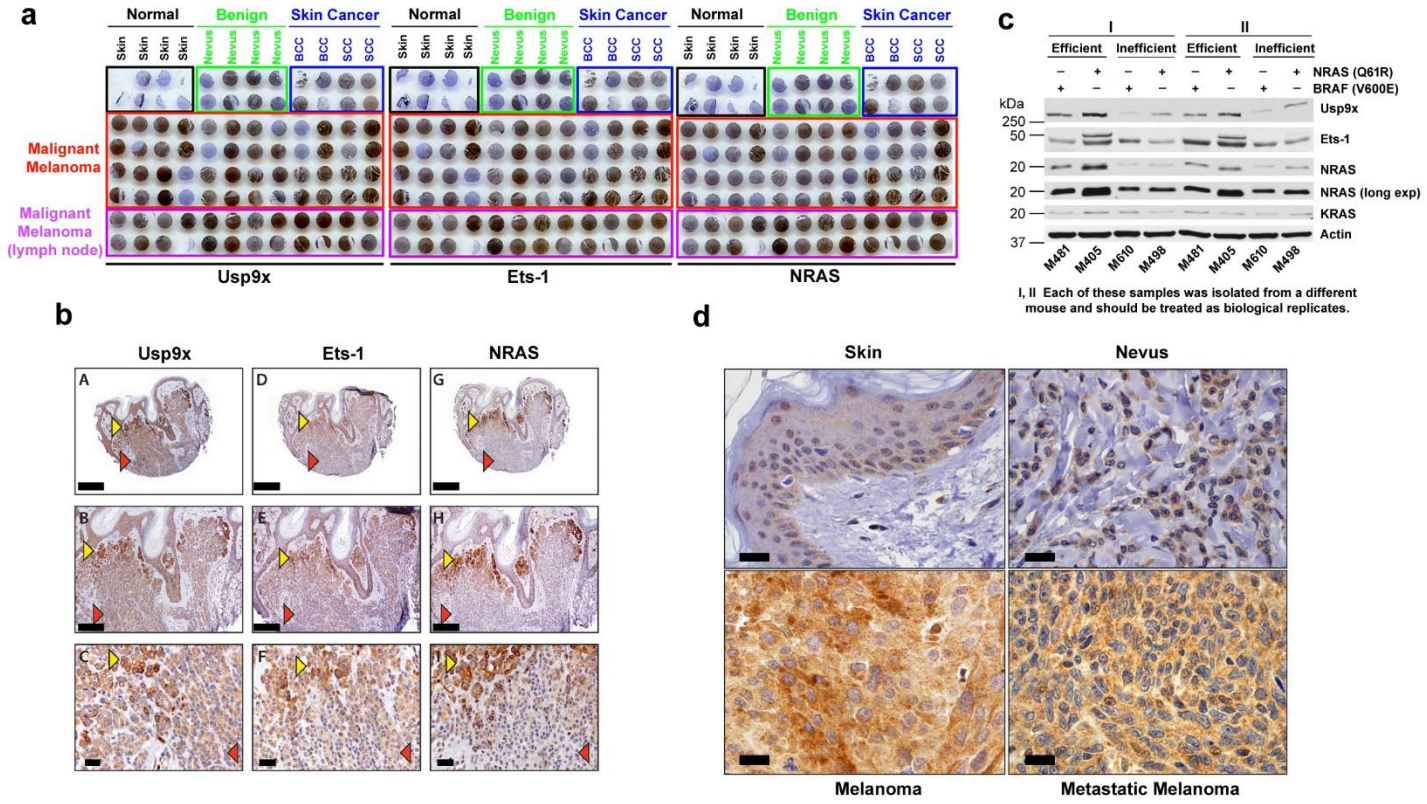
a. Immunoblot for Ets-1 and NRAS in BRAF mutant SK-Mel29 cells with Ets-1-overexpression (Ets-1 FLAG; left) or KD (right). **b**. Crystal violet staining of FLAG-Control or FLAG-Ets-1 SK-Mel29 (left) or A375 (right) cells grown for 3 weeks in 6-well plates. **c**. Phase contrast images of control and Ets-1 KD BRAF mutant A375 cells grown on matrigel (3D) for 7 days (black bar represents 100 μm). **d.** Immunoblot for Ets-1, NRAS, HRAS and KRAS protein in control and Ets-1 KD SK-Mel147 NRAS mutant melanoma cells after 5 days of KD (left); immunoblot for Ets-1, Usp9x, NRAS and actin in control, Usp9x KD and Ets-1 KD BRAF mutant A375 melanoma cells after 5 days of KD (right). **e**. Immunoblot assessment of Usp9x, ERG, NRAS and actin protein levels in control and Usp9x KD VCaP ERG positive prostate cancer cells. **f**. Immunoblot for the protein indicated in lysates from WM1366 cells subjected to infection, selection and induction of shRNA (as indicated) with doxycycline-inducible vectors (TRIPZ). Lysates derived from cells treated with doxycycline (1 μg/ml) for the interval indicated are shown.

Supplementary Fig. 4. Usp9x controls Dusp4 protein levels.



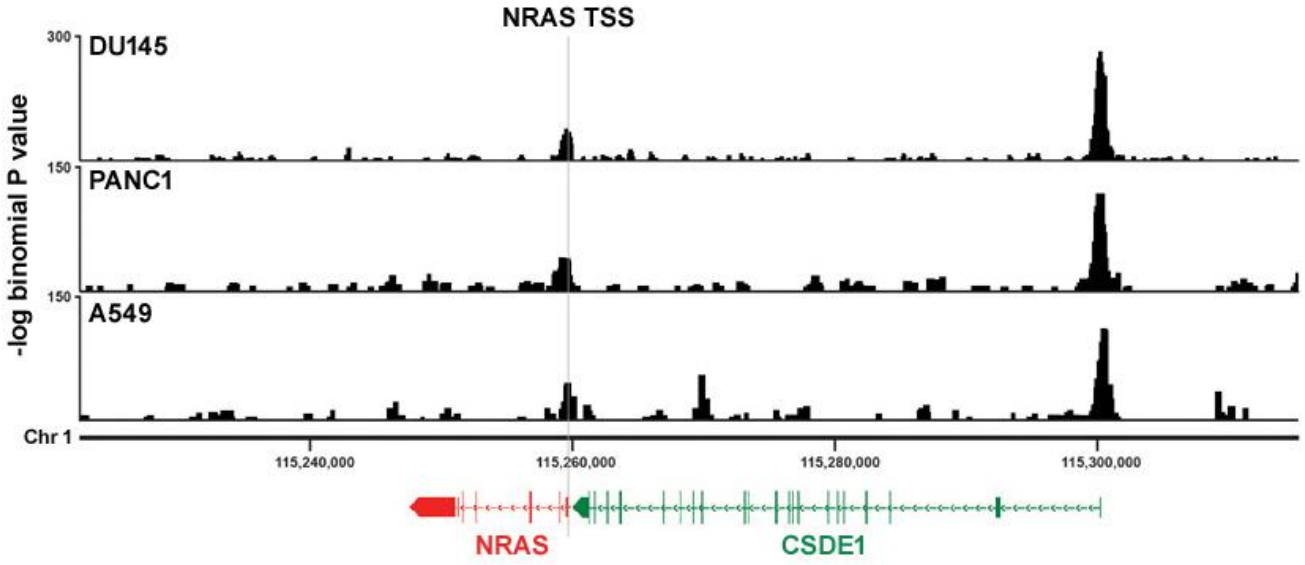
a. Anti-Usp9x or control IgG pulldowns from NRAS mutant SK-Mel2 cells were immunoblotted for Dusp4 and compared to Dusp4 levels in total cell lysates (input) (control IgG band is light chain Ig). **b.** Immunoblot for Usp9x, Dusp4, pERK and actin in control and Usp9x KD NRAS mutant SK-Mel2 melanoma cells after 5 days of KD. **c.** Immunoblot for Usp9x, Dusp4, pERK and actin in SK-Mel147 cells after Usp9x or Ets-1 KD. **d**. Immunoblot for Dusp4 and actin in Usp9x KD NRAS mutant melanoma WM1366 cells treated with MG132 for 8 h.

Supplementary Fig. 5. Primary and metastatic melanoma tumors express high levels of Usp9x and Ets-1.



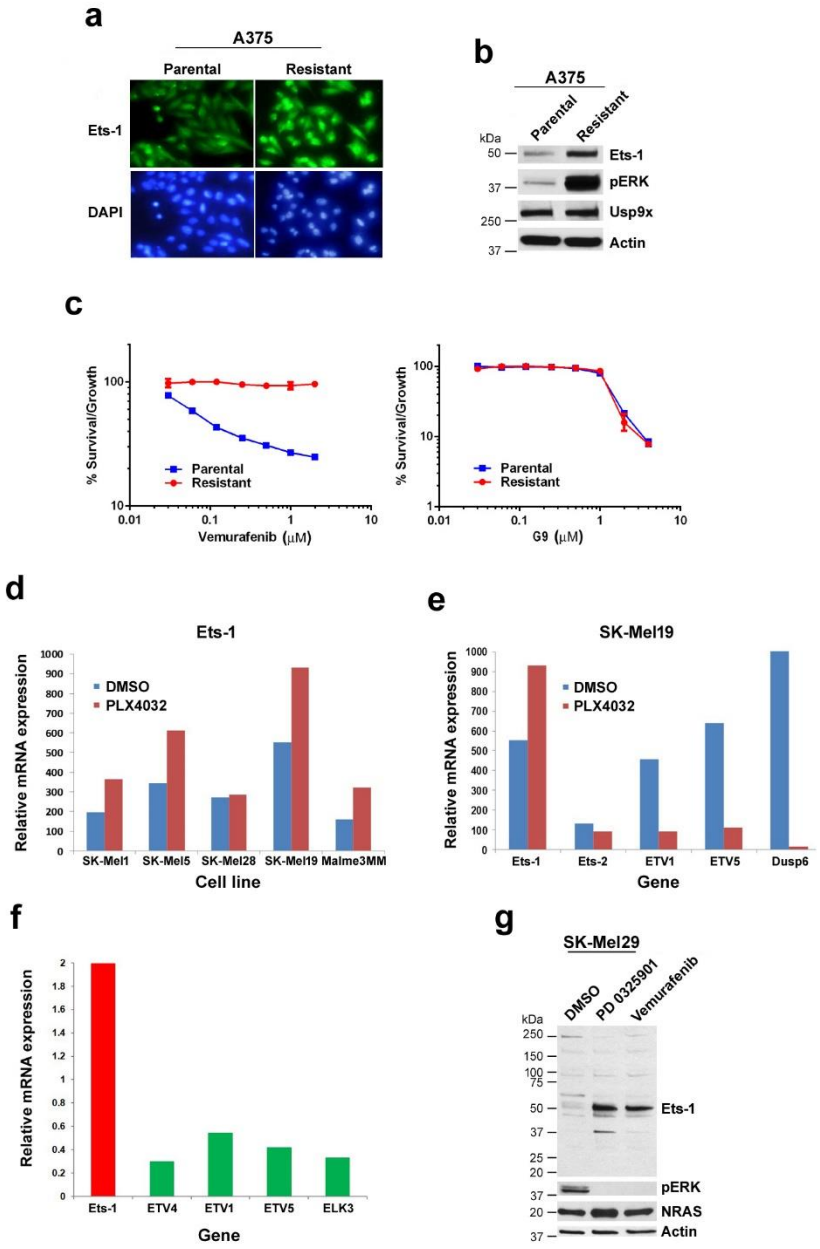
a. Immunostaining for Usp9x, Ets-1 and NRAS protein expression in a tissue microarray of normal skin, benign nevus, basal cell carcinoma, squamous cell carcinoma, malignant melanoma and metastatic melanoma (described in Figure 5). **b**. Stratified expression of Usp9x, Ets-1 and NRAS with dermal maturation in a benign nevus. The superficial aspect of the nevus (yellow arrowheads) demonstrates robust expression of all three proteins, which is diminished in the deeper aspect of the nevus (red arrowheads). The black bar represents 20 μm. **c.** Tumor tissue from primary human melanoma explants established in NSG mice [1] was assessed by immunoblot for Usp9x, Ets-1, NRAS (short and long exposure) and KRAS protein expression (NRAS and BRAF mutation status verified by the University of Texas Southwestern Medical Center, Dallas, TX). **d.** Immunostaining for Ets-1 in normal skin, benign nevus, primary melanoma and metastatic melanoma (from Fig. 5h) photographed under high magnification (1000X). Black bars represent 20 μm.

Supplementary Fig. 6. ChIP-SEQ analysis of Ets-1 occupancy near the NRAS gene.



Ets-1 occupancy near the gene encoding NRAS is plotted by -log binomial *P*-value from ChIP-SEQ data from the prostate (DU145), pancreatic (PANC1), and lung (A549) cell lines. A549 data is from ENCODE (Richard Myers Group, GSM1010829).

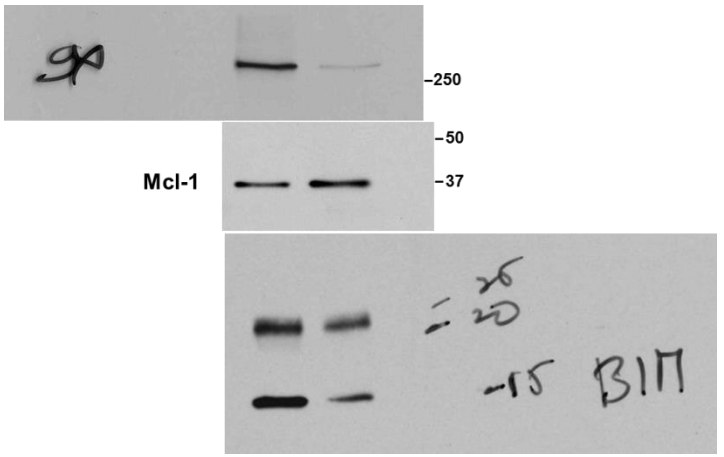
Supplementary Fig. 7. Ets-1 is induced by BRAF and MEK kinase inhibition.



a. Parental and vemurafenib-resistant A375 melanoma cells [2] were plated on glass cover slips, fixed and stained for Ets-1 protein and detected with fluorescently tagged anti-IgG. DAPI staining was used to detect cell nuclei. **b**. Parental and vemurafenib-resistant A375 cells were immunoblotted for the proteins indicated. **c.** Cells from **b** plated in individual wells of a 96-well plate were treated with the indicated dose of compound for 72 h before cell proliferation was assessed by MTT assay. The results represent the average +/- S.D. of 4 replicates. **d**. Ets-1 expression (taken from a microarray probe set, accession no. GSE20051) in BRAF-mutant melanoma cell lines treated with BRAF inhibitor (250 nM PLX4032, 8 h), as previously described [3]. Seventy-three genes were reported to be altered by BRAF inhibition in this microarray. **e**. ETS family member expression in the same microarray described in d. Relative levels are compared to untreated controls. **f**. ETS family member expression (taken from a microarray; GO: transcription factor activity: 00037000) in control and BRAF inhibitor (0.1 μM for 6 hr) treated A375 cells, as recently described [4]. **g**. Total immunoblot for Ets-1, pERK, NRAS and actin in BRAF mutant SK-Mel29 cells treated with Vemurafenib (1 μM) or PD0325901 (0.5 μM) for 24 h.

Supplementary Figure 8 - Representative uncropped Western blots corresponding to the figure designated.

Figure 1



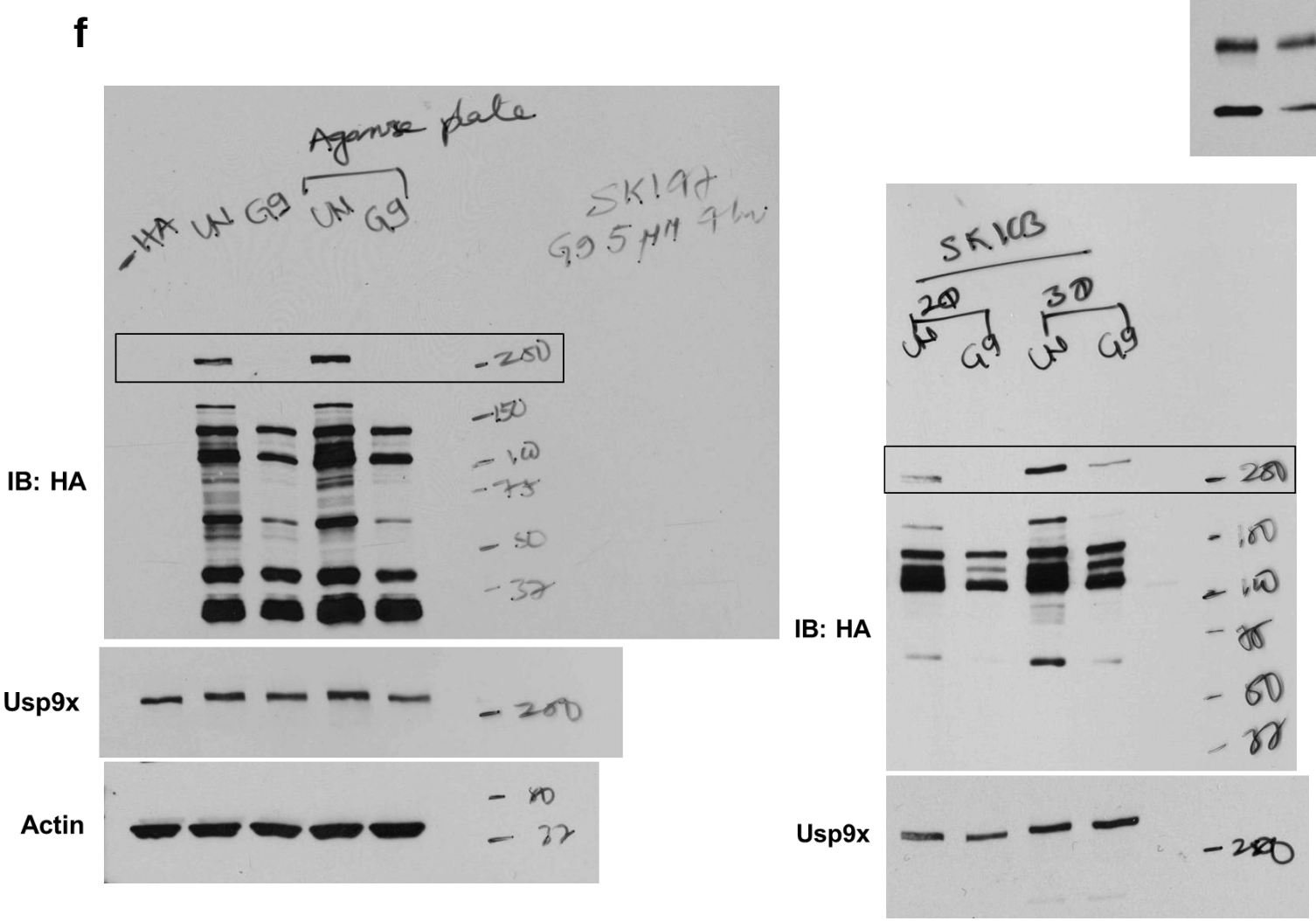
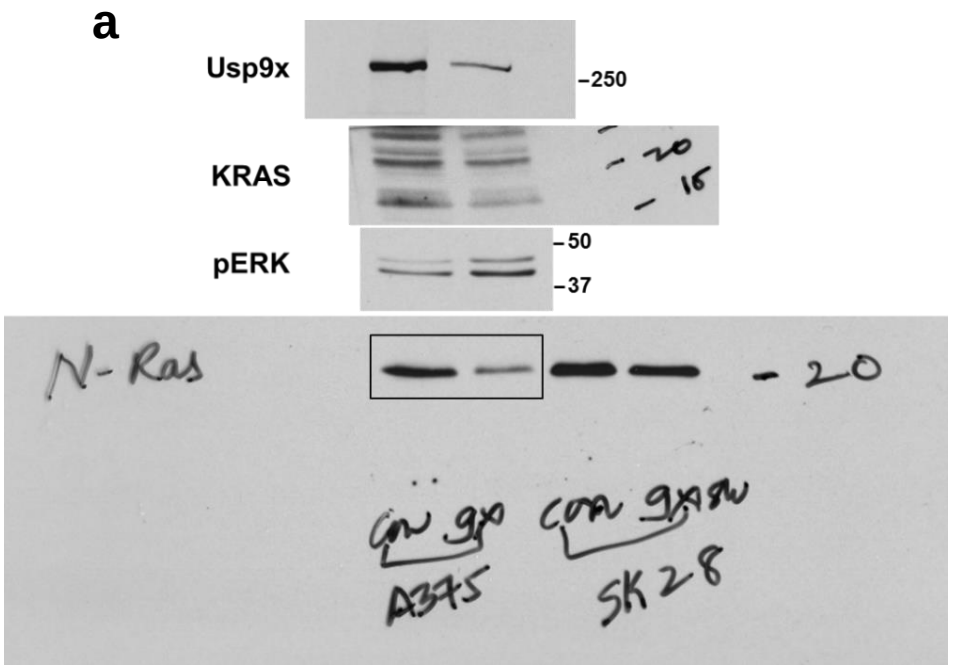


Figure 2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Con 9x
A375
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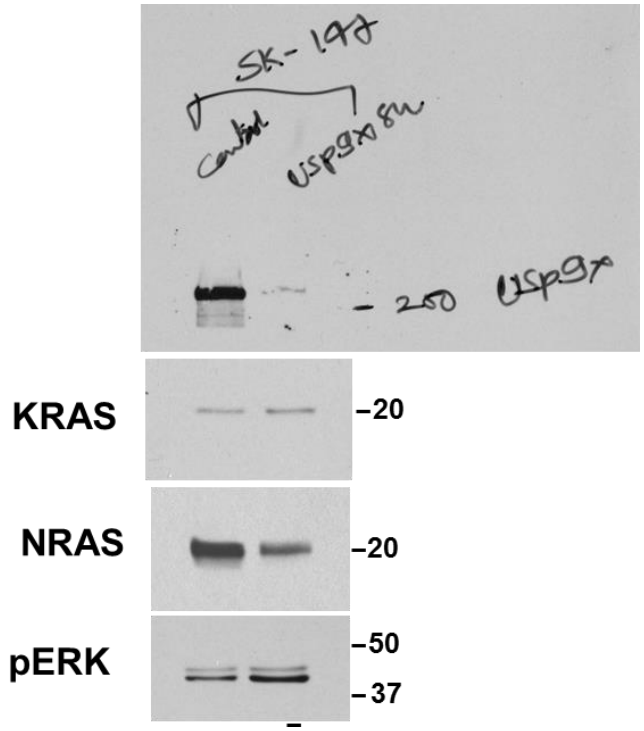
SK28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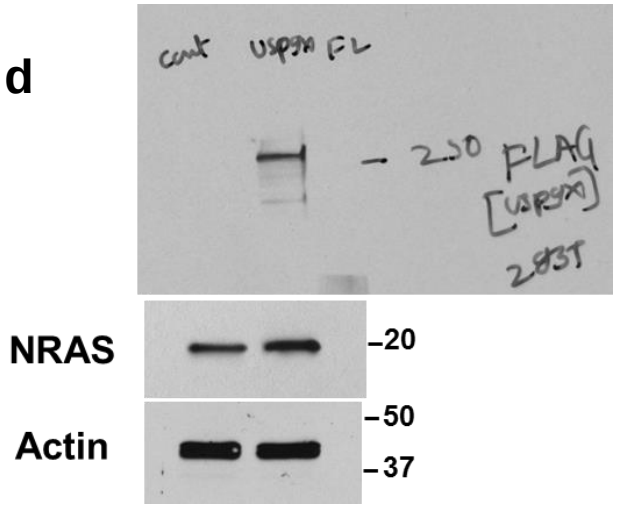
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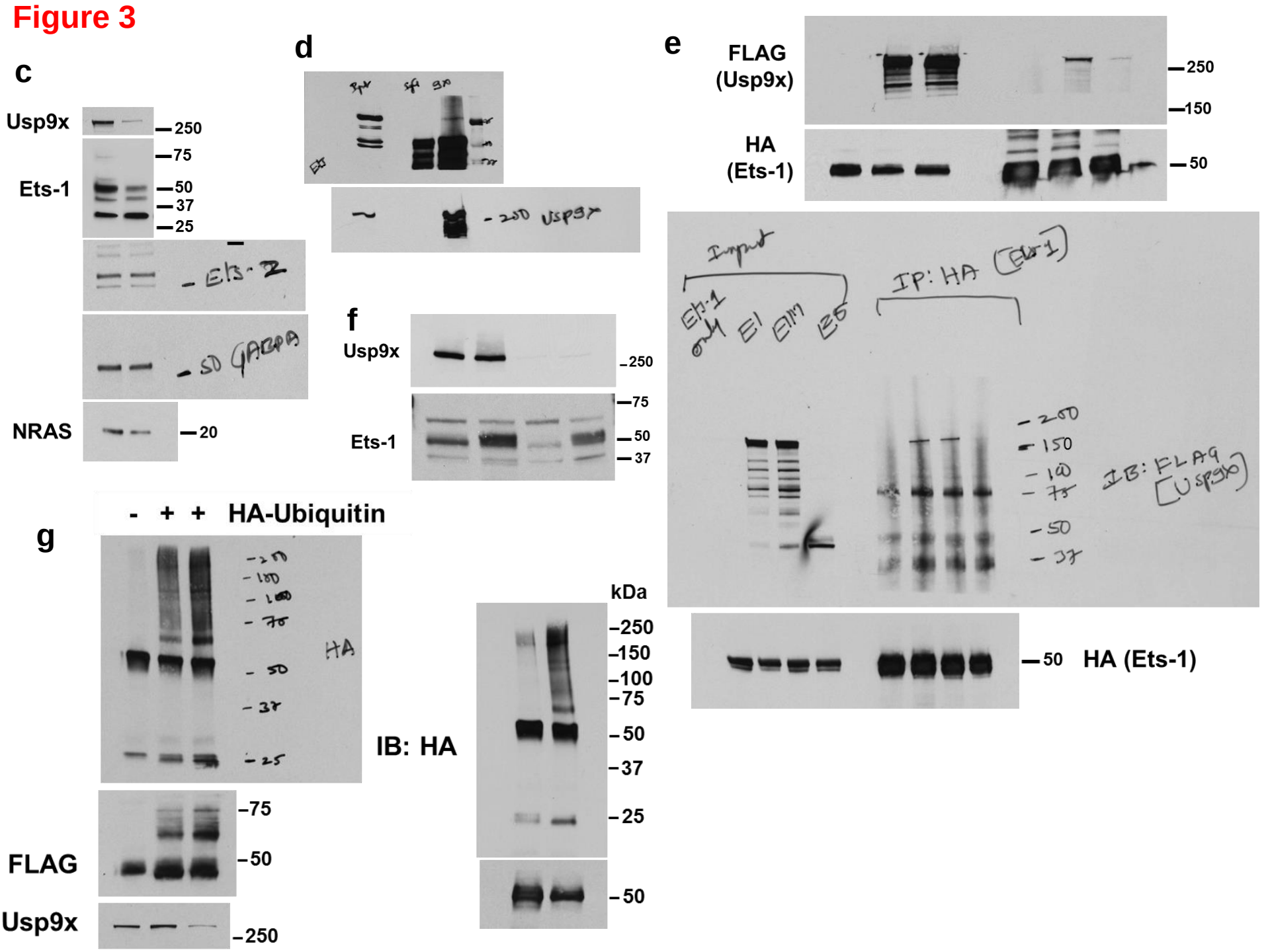
Figure 3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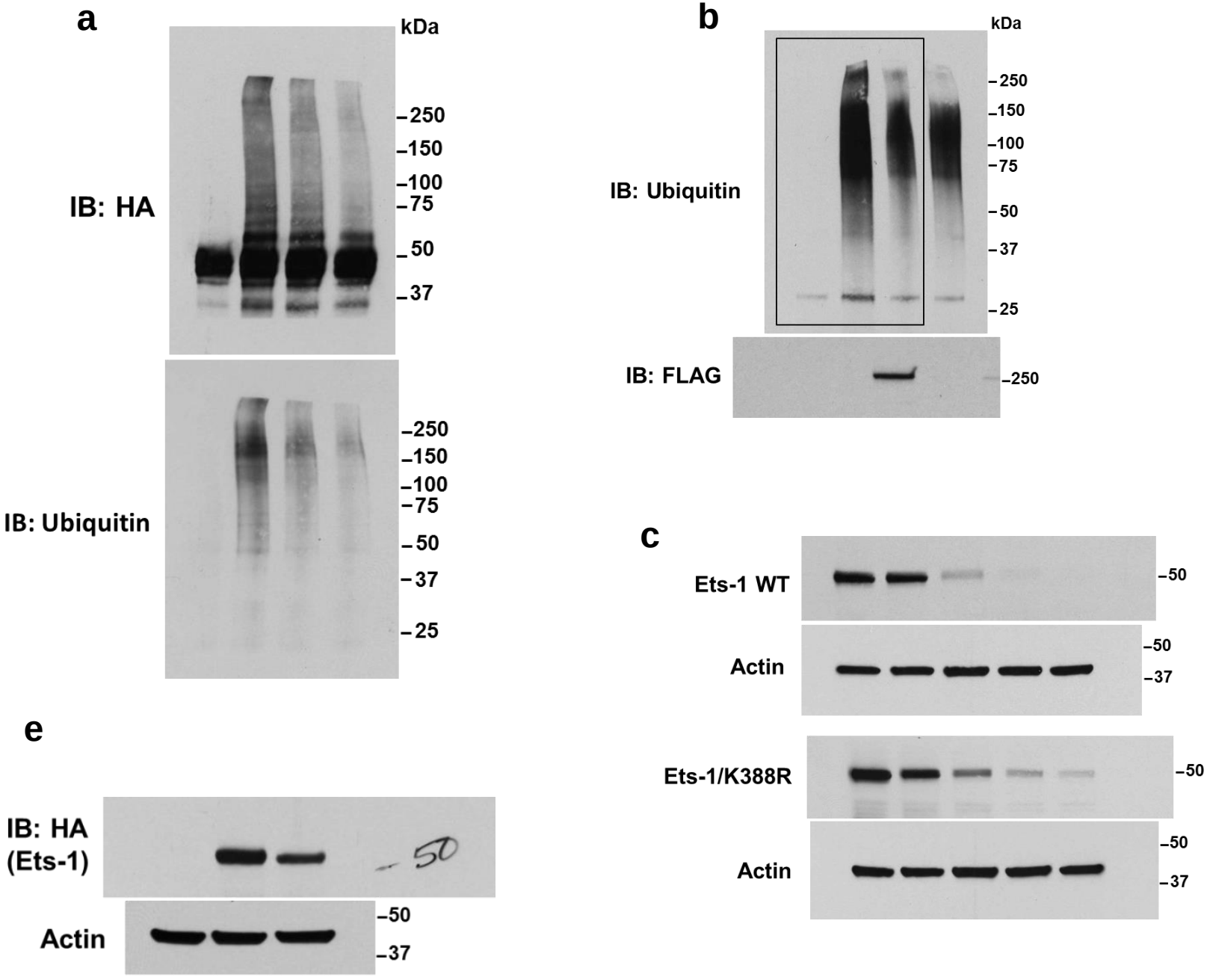
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IB: Ubiquitin
250
150
100
75
50
37
25
IB: FLAG
250
c
Ets-1 WT
50
Actin
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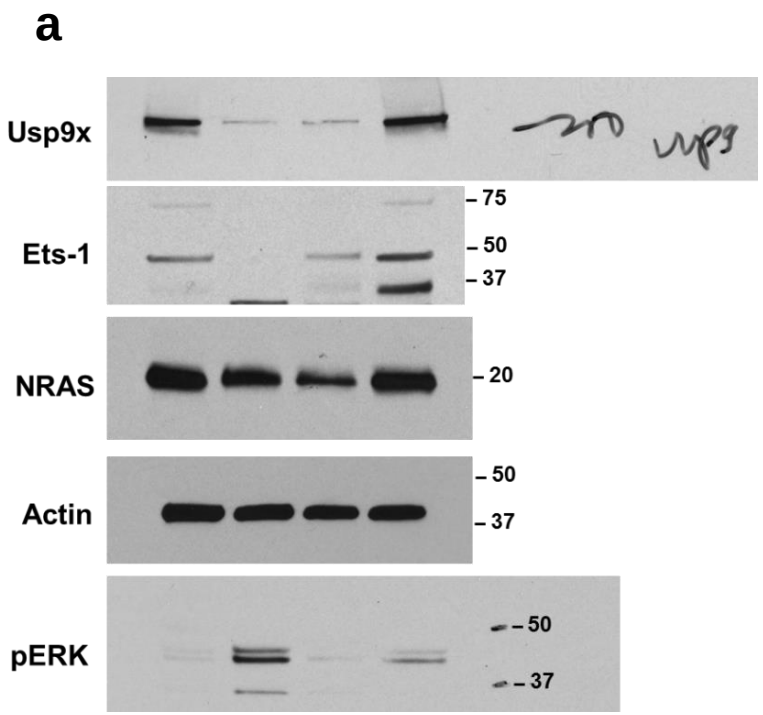
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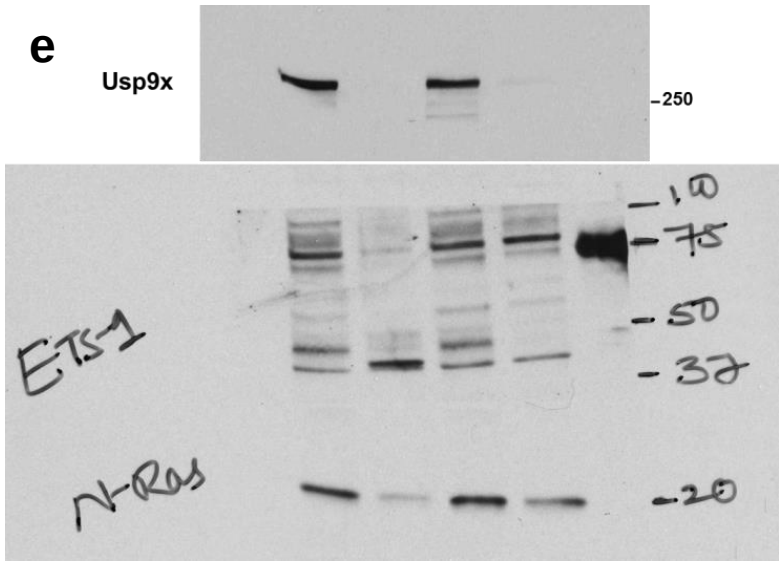
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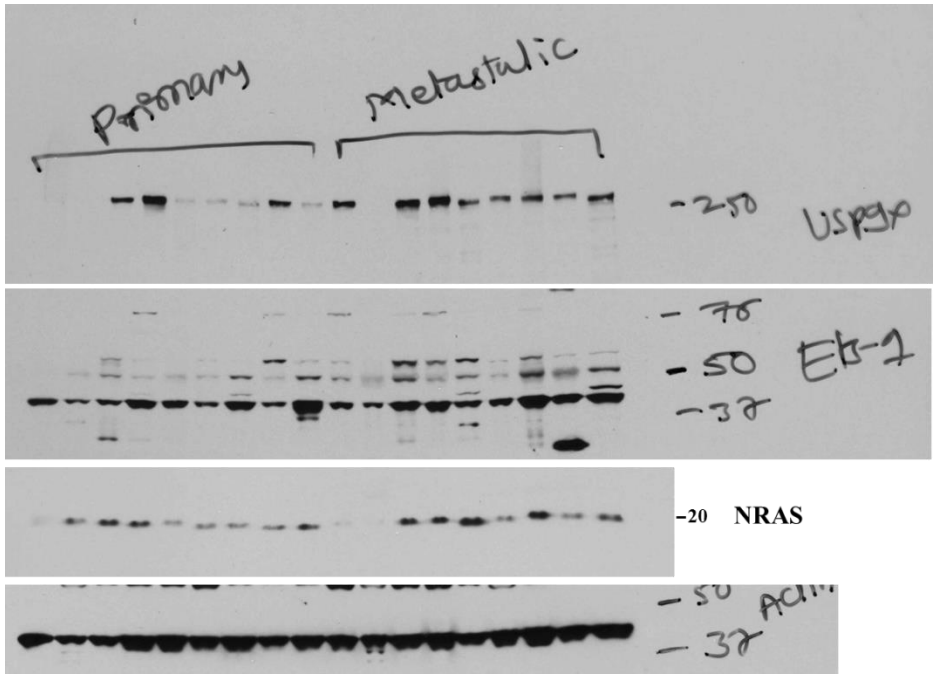
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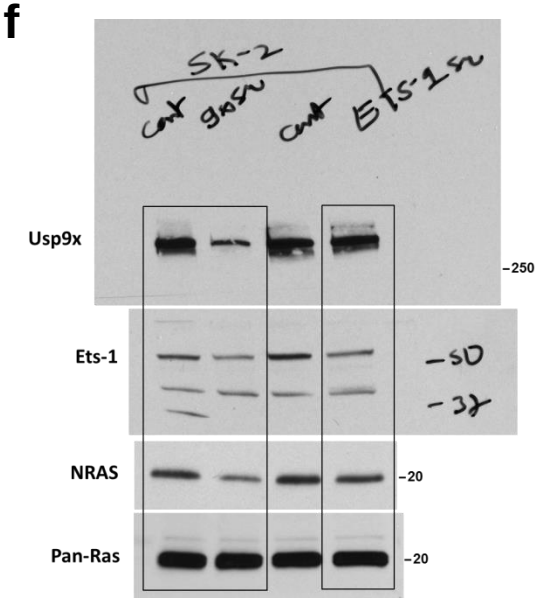
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gastric
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Ets-1 sh
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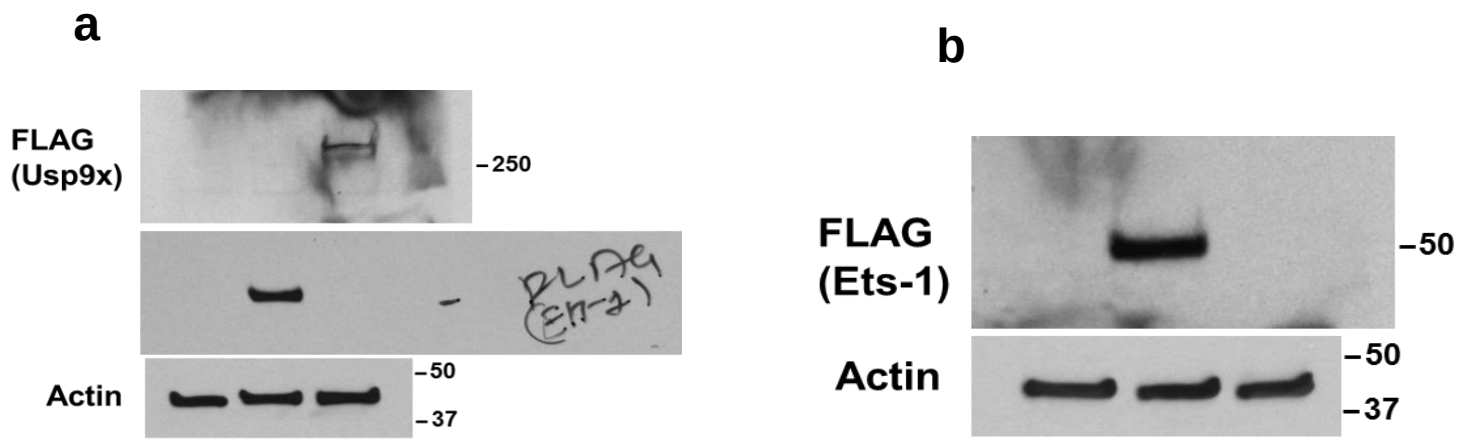
37
NRAS
20
Pan-Ras
20

Figure 6



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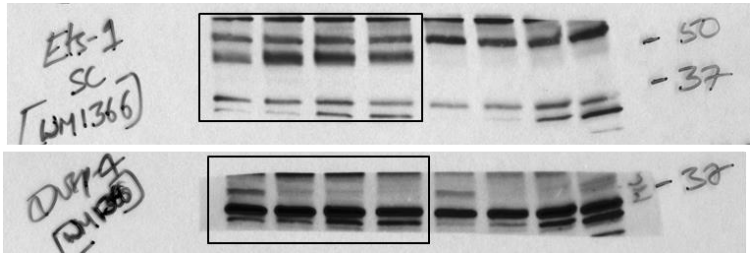
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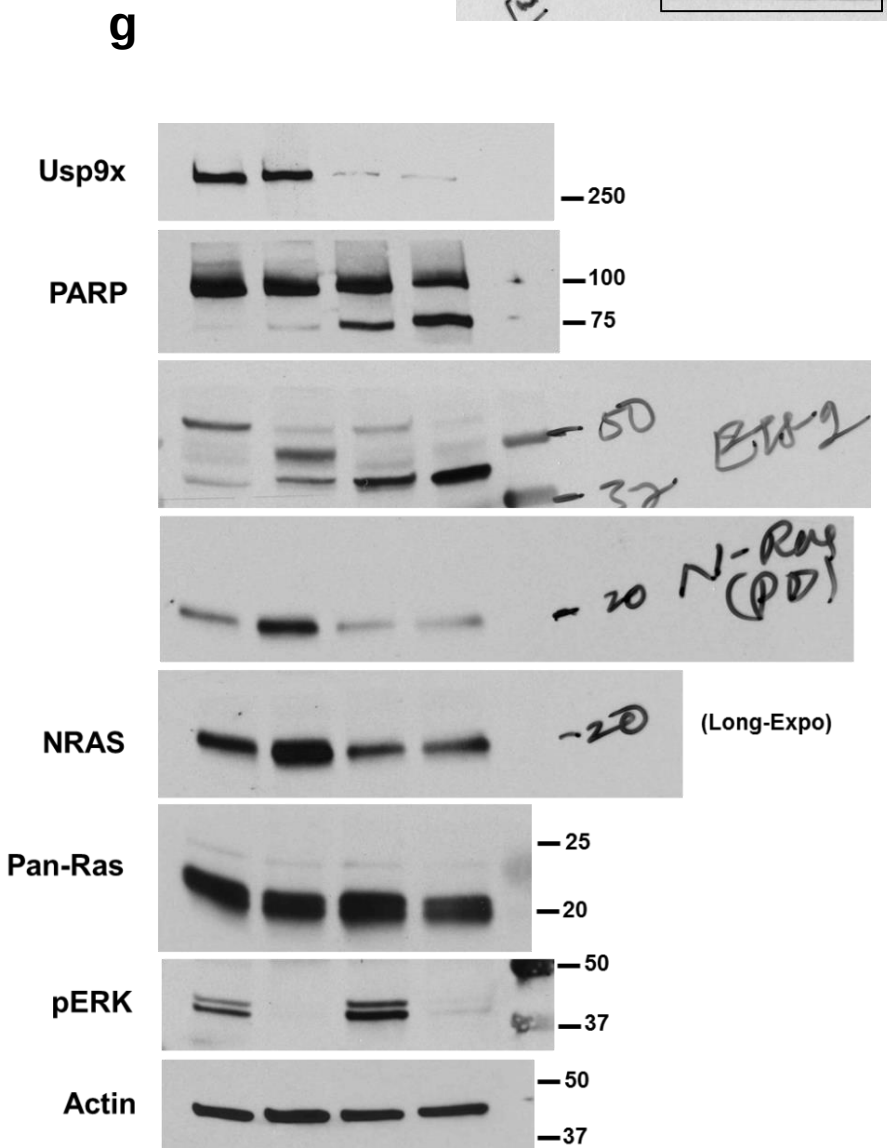
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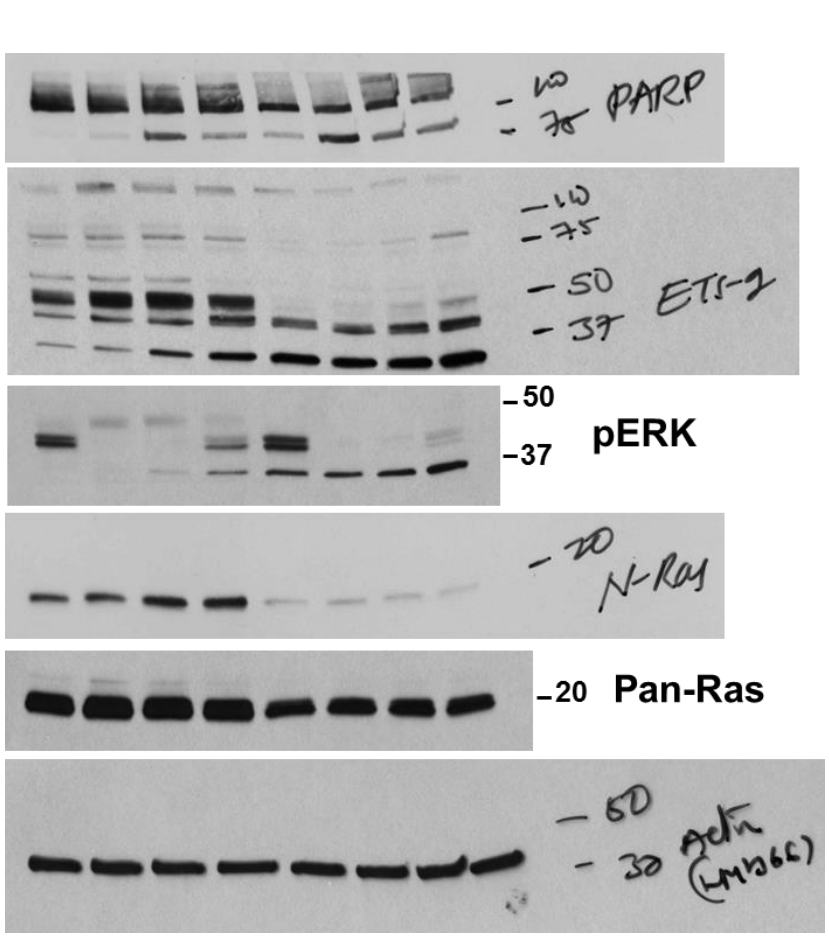
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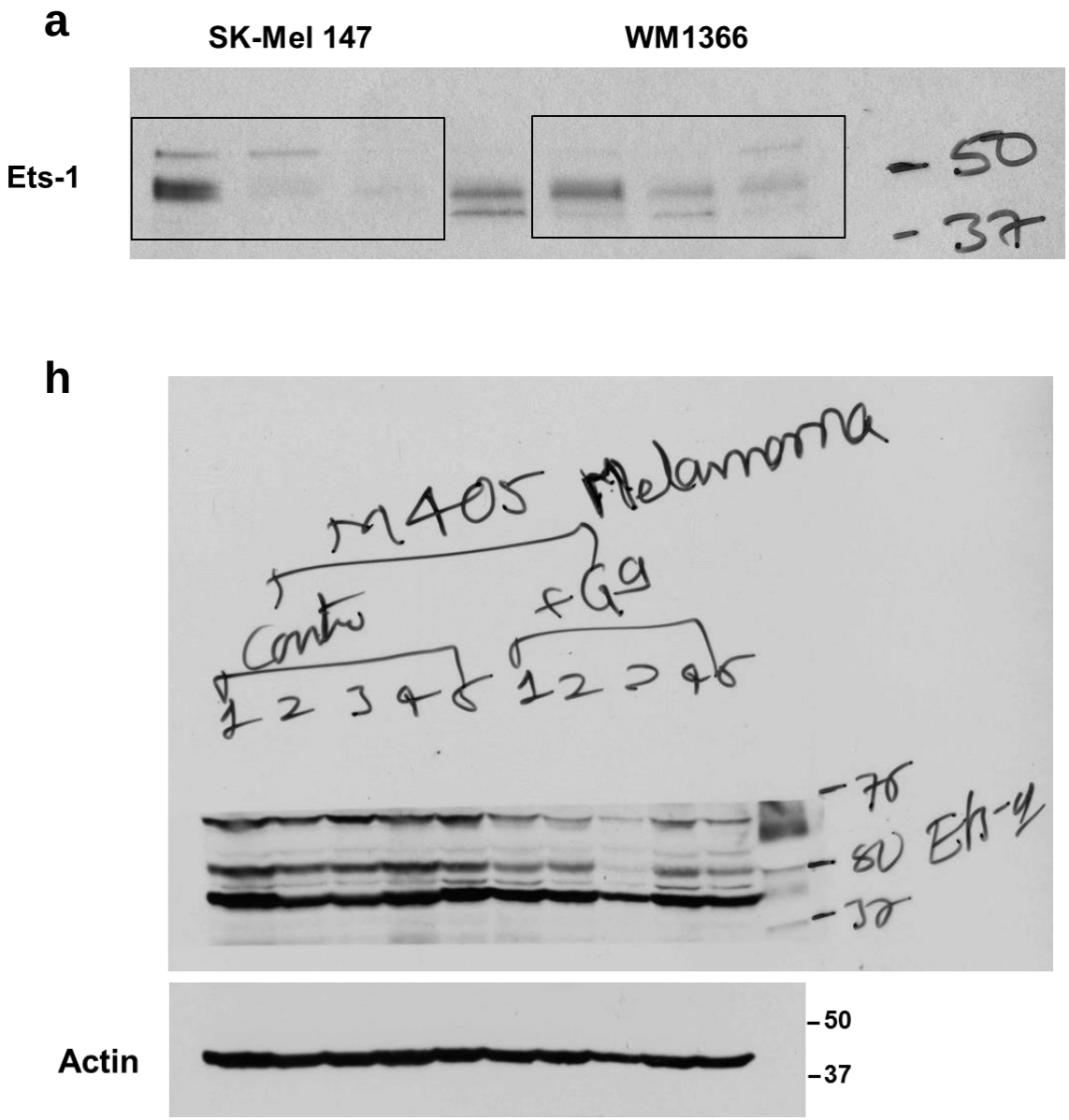
Actin
(LM1366)

Figure 8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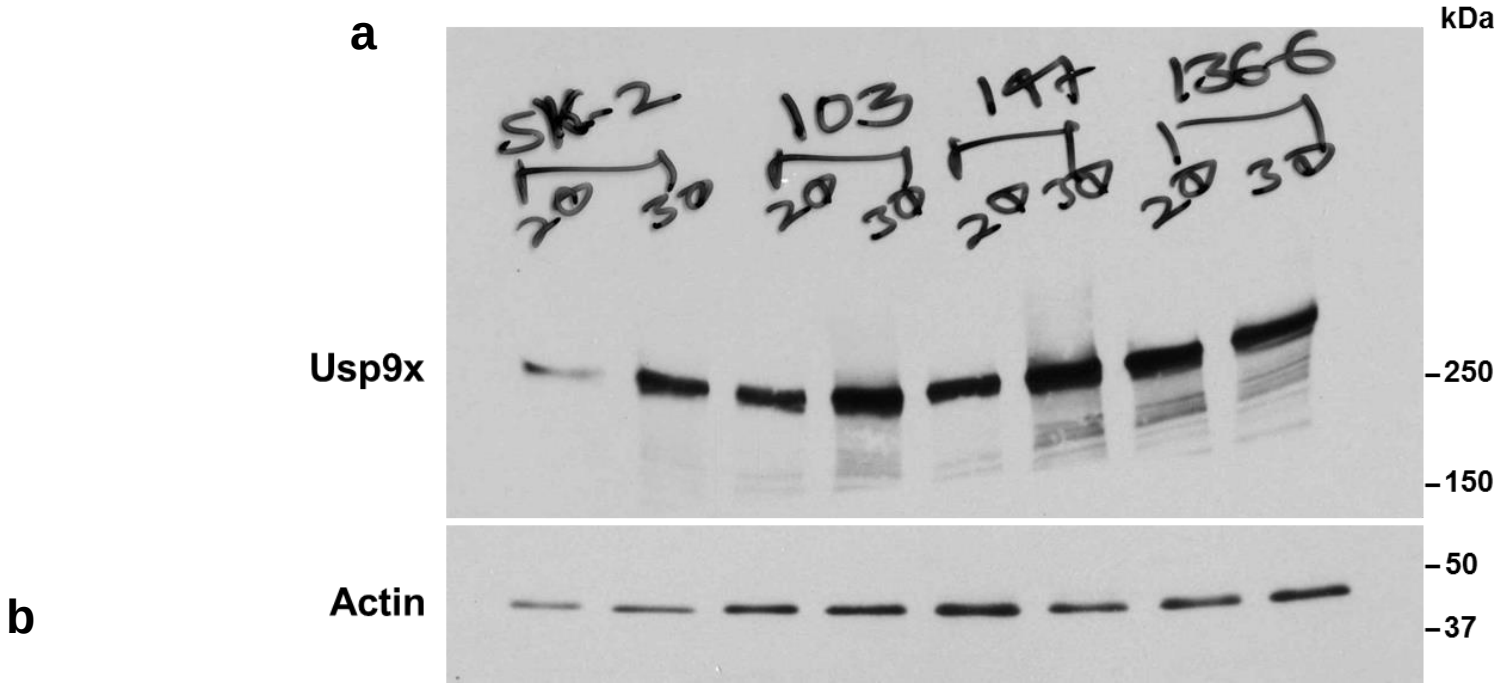
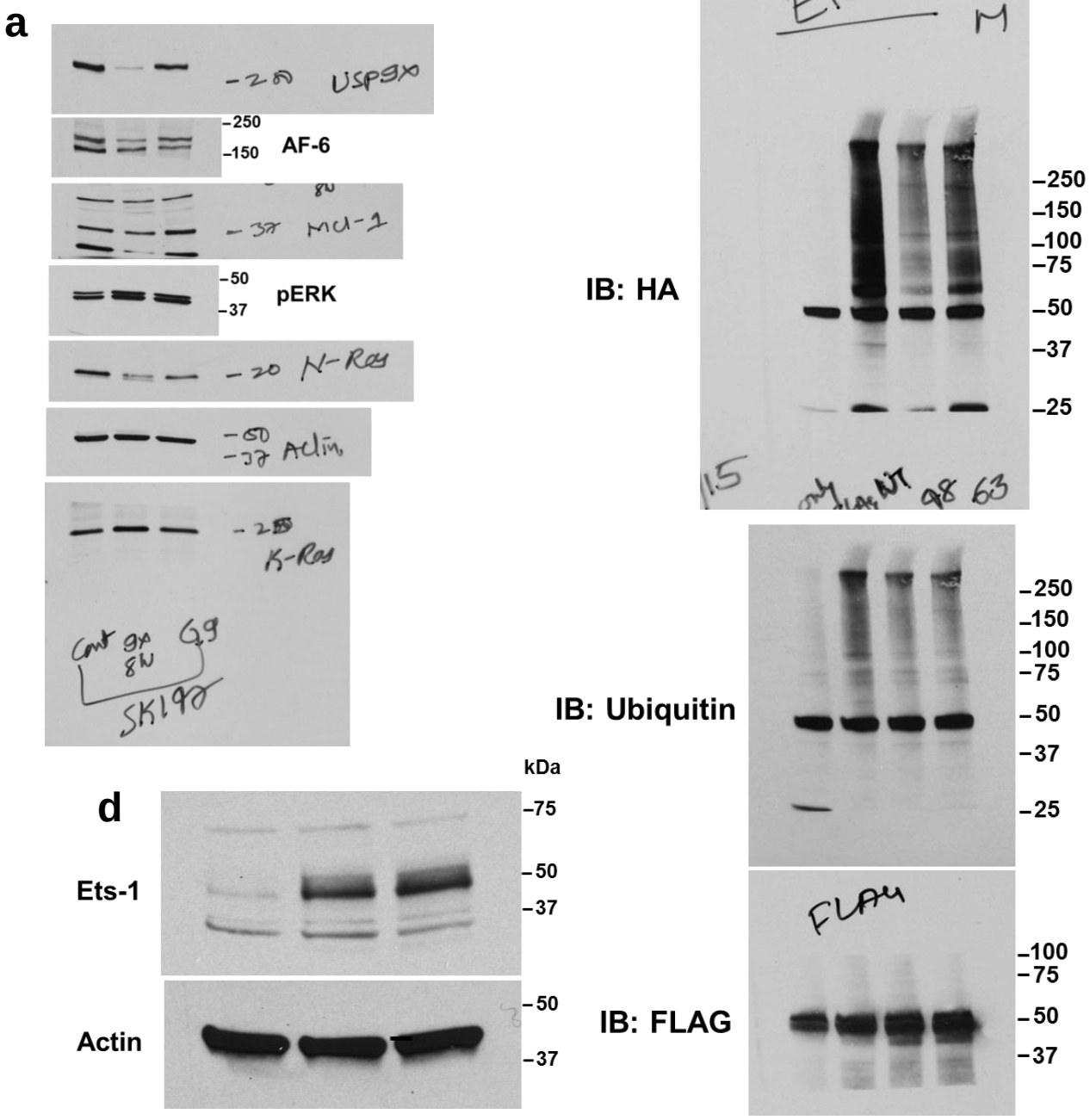
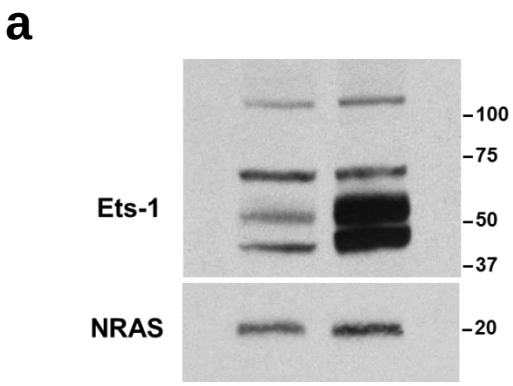
Supplementary Figure 1

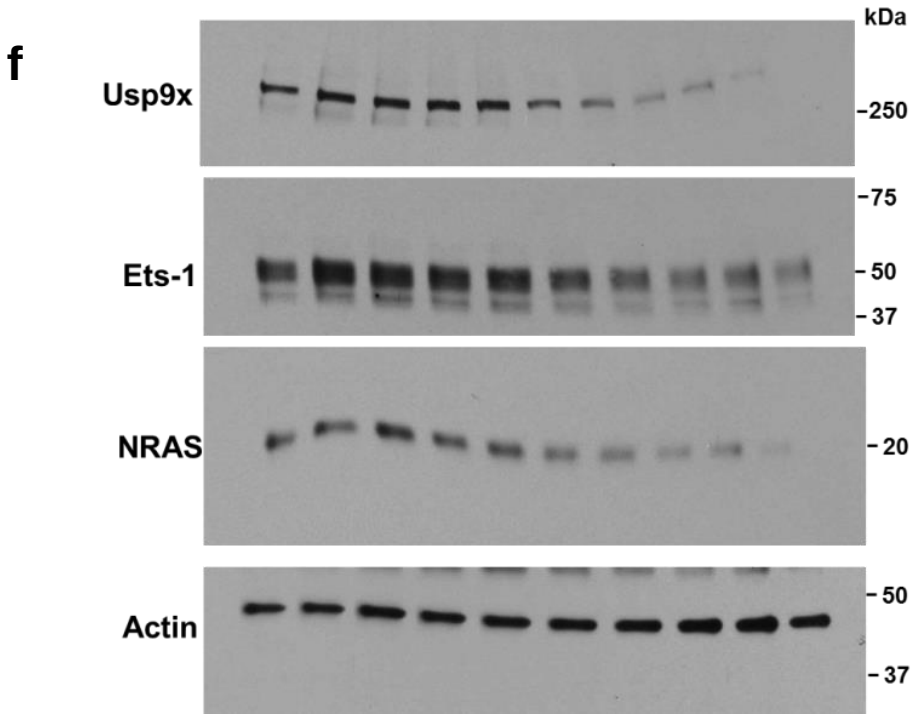


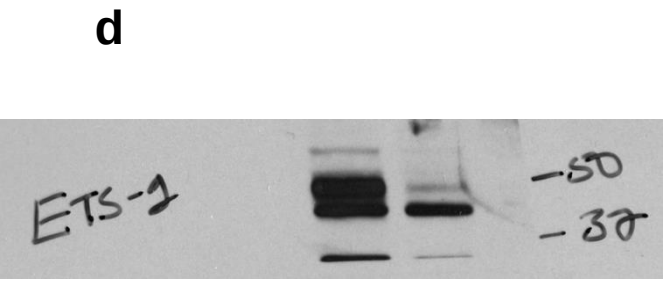
Supplementary Figure 2

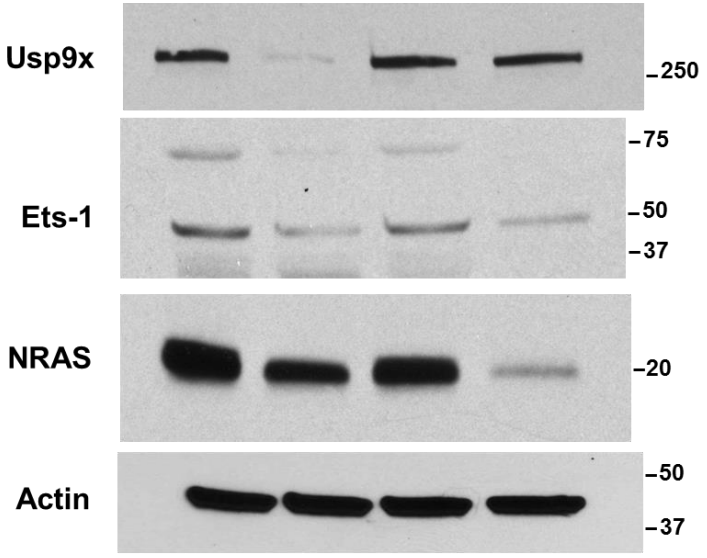


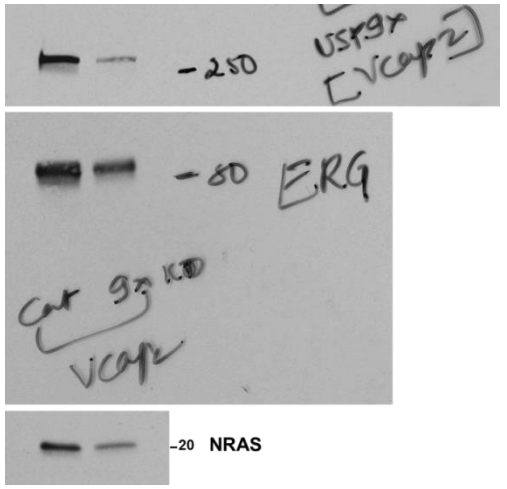
Supplementary Figure 3











Supplementary Figure 4

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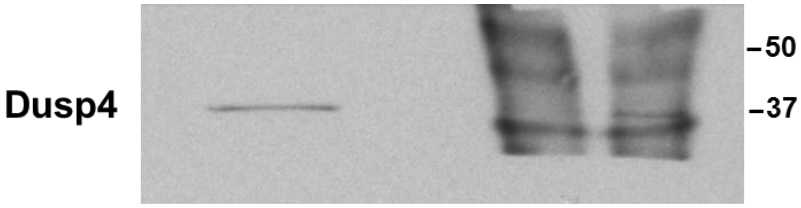

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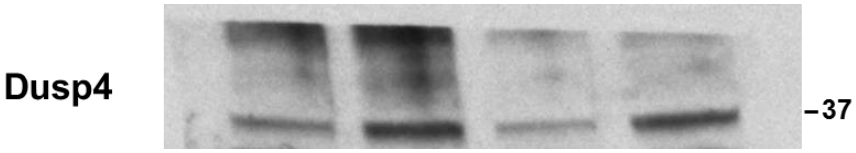

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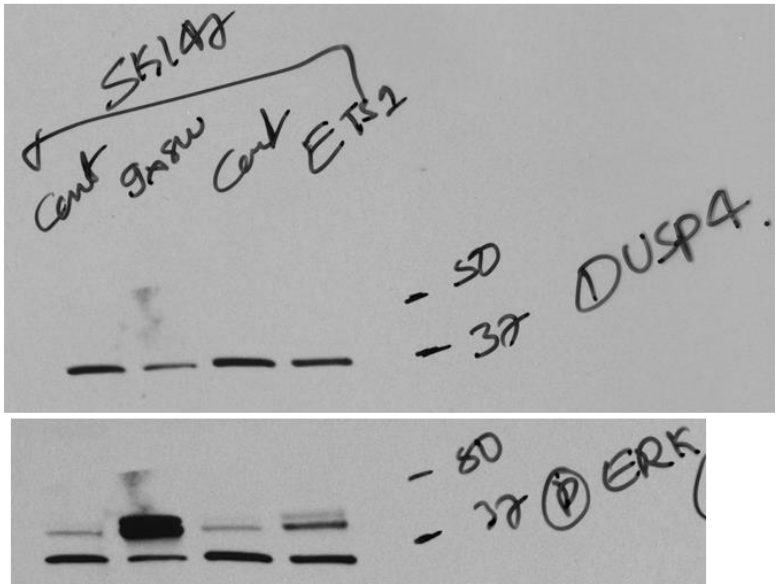

Supplementary Figure 5

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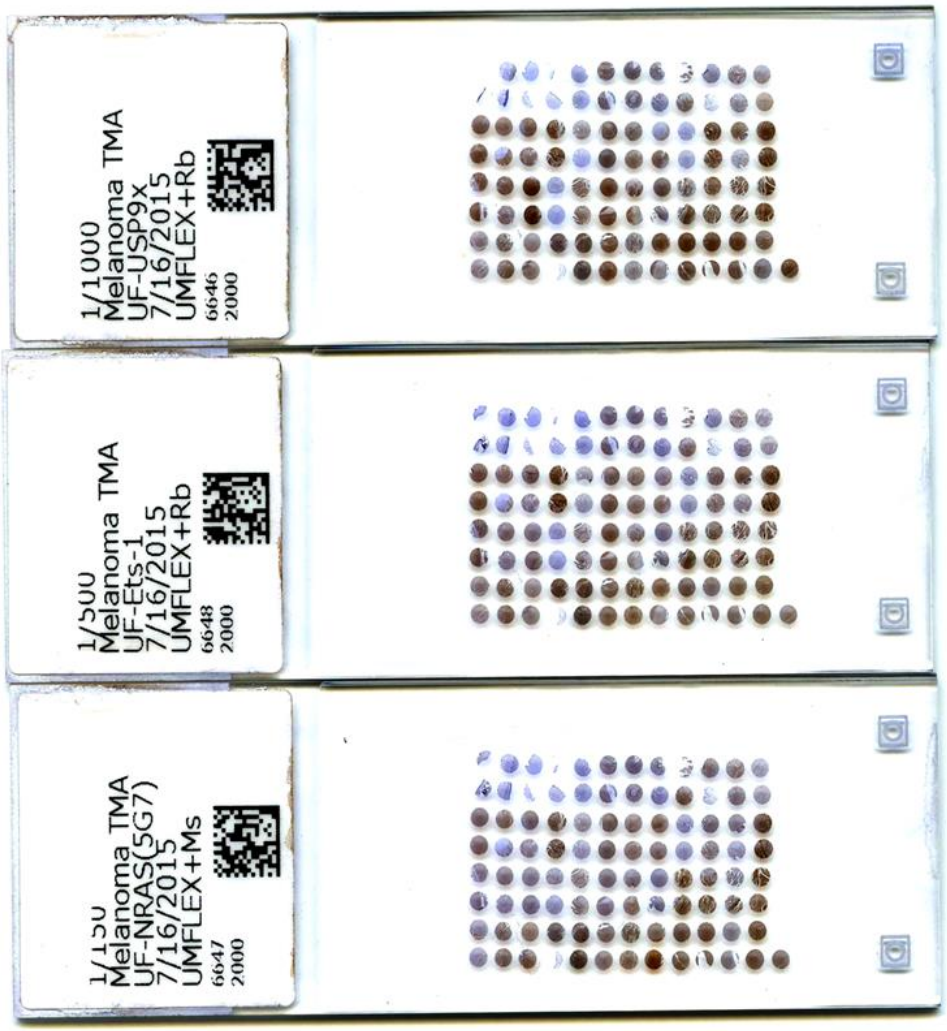

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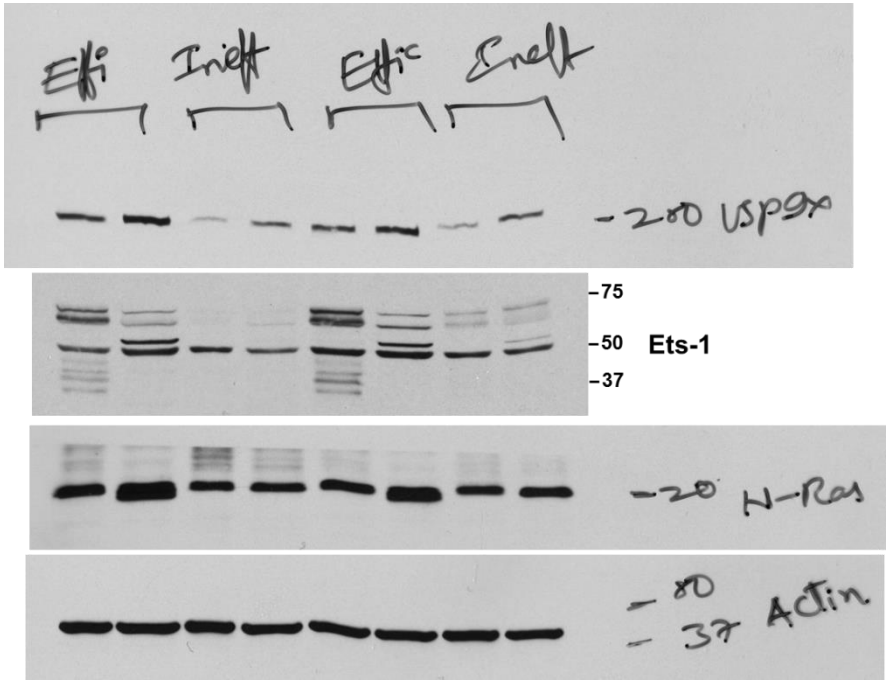

Supplementary Figure 7

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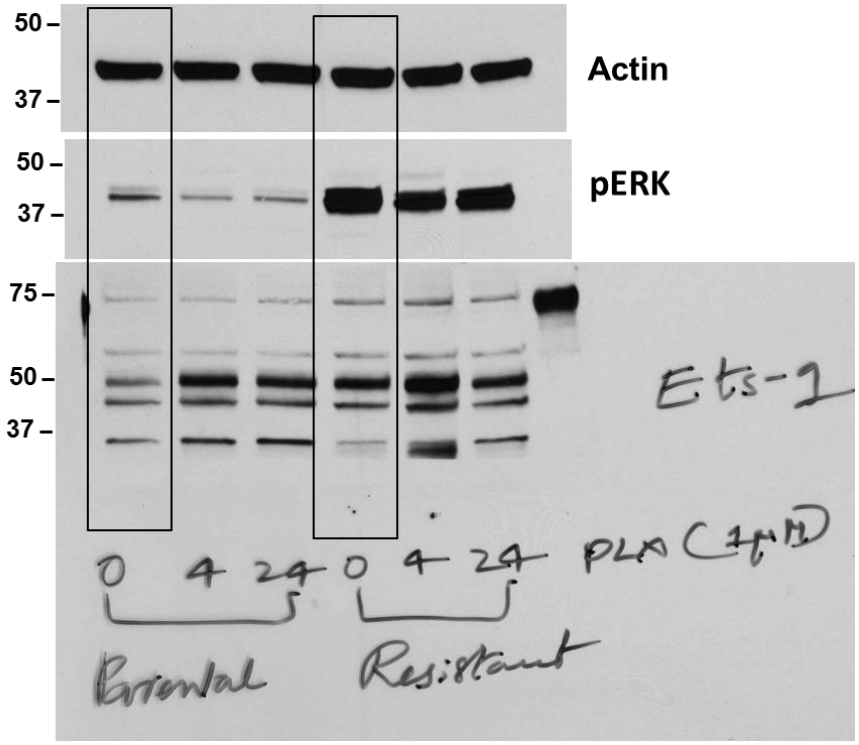

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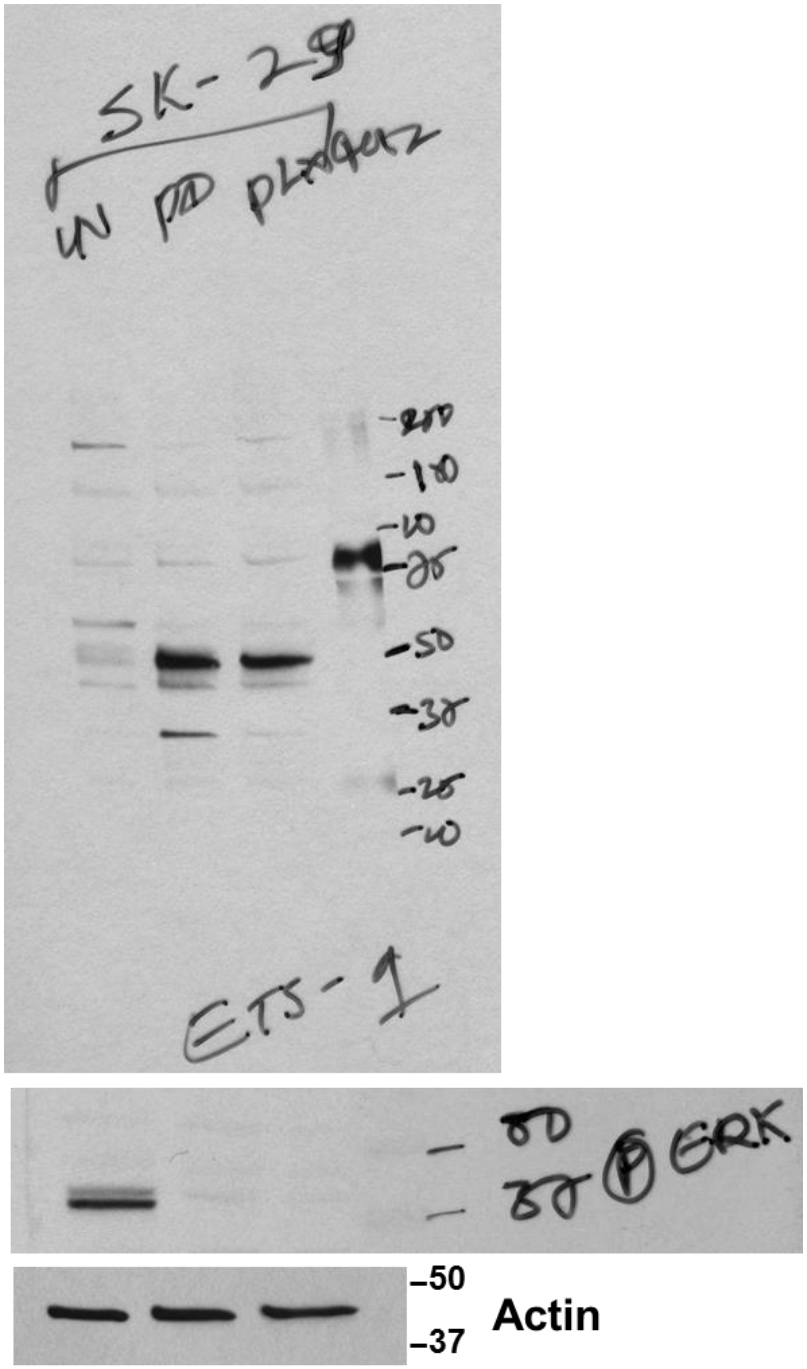

Supplementary Table 1. Characteristics of melanoma patient tumors analyzed in this study

	Tissue#	GENDER	AGE at Tissue Bank	Diagnosis (Breslow depth)	Stage at time of Tissue Bank	BRAF mutation (if known)
Primary	P1	F	64	Primary invasive (3.8 mm)	III	Negative
	P2	M	62	Primary ulcerated (8.8 mm)	IIC	
	P3	M	81	Primary invasive (3.3 mm)	IIB (T3b N0 M0).	Negative
	P4	F	68	Primary invasive (2.2 mm)	IIA (T3a N0 M0).	
	P5	M	70	Primary invasive (3.4 mm)	IIC (T4b N0 M0).	
	P6	M	52	Ulcerated invasive (6.0 mm)	IIIB	
	P7	M	62	Primary melanoma (8 mm)	IIIC (Tx, N3, M0).	Positive
	P8	M	81	Primary invasive (9.0 mm)	IIC (T4b N0 M0),	
	P9	M	87	Primary invasive (6.40 mm)	IIA (T2b N0 M0)	
Metastatic	M1	M	60	Melanoma, probably metastatic (in transit mets)	IIIC (T3b, N2c, M0)	Positive
	M2	M	64	Metastatic melanoma involving lymph node with extranodal extension	IIIC (T3b, N1b, M0)	Negative
	M3	M	48	Metastatic melanoma	III	Positive
	M4	M	39	Metastatic melanoma	IIIC (T3b N1b M0).	Positive
	M5	F	52	Metastatic melanoma	IV	Negative
	M6		91	Metastatic melanoma	III	
	M7	M	76	Metastatic melanoma	IIIB(Tx, N2b, Mx).	Negative
	M8	M	57	Metastatic melanoma	IIIC (T2a N3 M0).	Negative
	M9	M	86	Metastatic melanoma	III	

Supplementary Table 2. Antibody sources and dilutions used in this study.

Protein	Antibody and dilution	Source	Catalogue Number
RAS signaling	Mouse NRAS (1:250)	Santa Cruz	sc-31 (F155)
	Mouse Pan-RAS (1:5000)	Santa Cruz	sc-166691 (C-4)
	Mouse HRAS (1:250)	Santa Cruz	sc-520 (C-20)
	Rabbit Dusp4 (1:1000)	Santa Cruz	sc-1200 (C-18)
	Mouse KRAS (1:500)	Calbiochem	OP24
	Rabbit AF-6 (1:1000)	Bethyl Laboratories	A302-199A
	Rabbit ERK2 (1:5000)	Santa Cruz	Sc-154
	Rabbit pERK (1:2000)	Cell Signaling	9101L
Ubiquitin signaling	Rabbit Usp9x (1:2000)	Bethyl Laboratories	A301-350A
	Mouse Ubiquitin (total) (1:5000)	Santa Cruz	sc-8017 (P4D1)
ETS signaling	Rabbit Ets-1 (1:2000)	Bethyl Laboratories	A303-501A
	Rabbit ERG (1:4000)	Abcam	ab92513
	Rabbit Ets-2 (1:1000)	Santa Cruz	sc-351
	Rabbit GABPAα (1:1000)	Santa Cruz	sc-22810
	Rabbit Ets-1(C-20) (1:2000)	Santa Cruz	sc-350
Apoptosis signaling	Rabbit Mcl-1(1:1000)	Santa Cruz	sc-819 (S-19)
	Rabbit Caspase8 (1:2000)	Cell Signaling	9746S
	Rabbit PARP (1:2000)	Cell Signaling	9542S
	Rabbit BID (1:1000)	Cell Signaling	2002
	Rabbit BIM (1:1000)	Cell Signaling	2933T
	Rat HA (1:2000)	Roche	11867423001
	Rabbit HA (Y-11) (1:2000)	Santa Cruz	sc-805
	Mouse FLAG (M2) (1:4000)	Sigma	F1804-1MG
	Mouse β-Actin (1:250)	Santa Cruz	sc-69879 (AC-15)
Alexa Fluor Conjugated Ab	Alexa Fluor® 488 Goat	Invitrogen	A11034
	Anti-Rabbit IgG (1:5000)	Santa Cruz	sc-2027
Immunohistochemistry	Rabbit Usp9x (1:1000)	Abcam	ab-19879
	Rabbit Ets-1 (1:500)	Bethyl Laboratories	A303-501A
	Mouse NRAS (1:150)	Origene	TA505835 (5G7)

Supplementary Table 3. Primers used in Ets-1 mutagenesis.

KPKMNYEKLSRGLRYY (K*388)	Ets-1 Ubiquitin recognition motif
5'-cctaagatgaattatgagaaactgagccgtggcctacgct-3'	**WT**
5'-cctaagatgaattatgagAGActgagccgtggcctacgct-3' (F) 5'-agcgtaggccacggctcagTCTctcataattcatcttagg-3' (R)	**Arginine**
5'-cctaagatgaattatgagGCCctgagccgtggcctacgct-3' (F) 5'-agcgtaggccacggctcagGGCctcataattcatcttagG-3' (R)	**Alanine**

Supplementary Table 4. Primers used in mutation of the putative ETS binding site in the NRAS promoter.

Primer sequence (5' to 3')	Primer name	N	%GC	%mismatch	Tm
TTCAACTTTATATCACGAGCATGGATGGGTCTGAT	Ets1E1MF	35	40.00	5.7142857	72.90
ATCAGACCCATCCATGCTCGTGATATAAAGTTGAA	Ets1E1MR	35	40.00	5.7142857	72.90
GGAGGGCGGTTTTATCTGCTCCCTTGTCGTTTGAGG	Ets1E2MF	36	55.60	5.5555555	79.99
CCTCAAACGACAAGGGAGCAGATAAAACCGCCCTCC	Ets1E2MR	36	55.60	5.5555555	79.99
GTTAATGGCGAAAGAATAGCAGCGAATAAAGTTTTAC	Ets1E3MF	37	35.10	5.4054054	72.24
GTAAAACTTTATTCGCTGCTATTCTTTCGCCATTAAC	Ets1E3MR	37	35.10	5.4054054	72.24
TACGTAGCGGGCGGGGCCGAACGTGCCGCTCCTTGGTGGG	Ets1E4MF	40	72.50	5	89.35
CCCACCAAGGAGCGGCACGTTCGGCCCCGCCCGCTACGTA	Ets1E4MR	40	72.50	5	89.35
GGGGCTGTTCATGGCGGTGCTGGGGTCTCCAACATTTAAG	Ets1E5MF	40	72.50	5	89.35
CTTAAATGTTGGAGACCCCAGCACCGCCATGAACAGCCCC	Ets1E5MR	40	72.50	5	89.35

Supplementary References

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