Supplemental material

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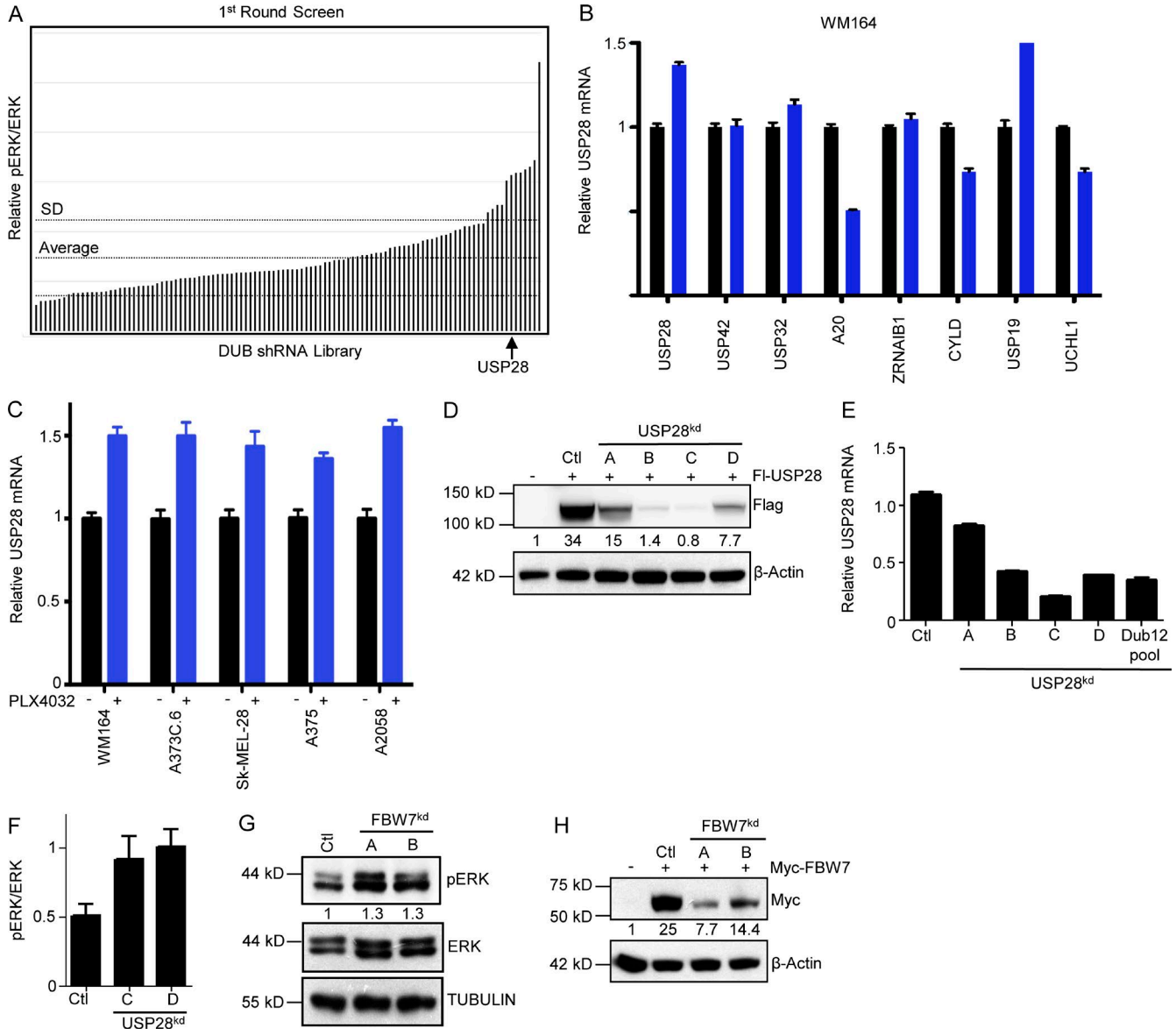


Figure S1. **USP28 is a novel regulator of MAPK signaling. (A)** Graph representing relative mean pERK/ERK values from two individual DUB screens. 293T cells were transfected with the individual pools from the DUB library. 72 h later, cell lysates were collected and probed for pERK and ERK. Proteins levels were quantified by ImageJ and a relative value of pERK/ERK was obtained. **(B)** WM164 cells were treated with 1 µM vemurafenib (PLX4032) for 3 h. USP28, USP42, USP32, A20, ZRNAIB1, CYLD, USP19, and UCHL1 mRNA levels relative to GAPDH are shown as evaluated by qRT-PCR. Data are mean ± SD of triplicate samples from a representative experiment performed three times. **(C)** WM164, A373 C.6, SK-MEL-28, A375, and A2058 cells were treated with vemurafenib (PLX4032) for 3 h. USP28 mRNA levels relative GAPDH are shown as evaluated by qRT-PCR. Data are mean ± SD of triplicate samples from a representative experiment performed three times. **(D)** Immunoblot analysis of 293T cells expressing Flag-USP28 and shRNA vectors (A–D) targeting USP28. Whole cell extracts were probed with the indicated antibodies. Data shown are representative of three independent and reproducible experiments. Respective proteins levels were quantified by ImageJ comparing indicated proteins to relevant controls. **(E)** 293T cells expressing shRNA vectors (A–D) targeting USP28 or DUB pool 12 (USP28). USP28 mRNA levels relative GAPDH are shown as evaluated by qRT-PCR. Data are mean ± SD of triplicate samples from a representative experiment performed three times. **(F)** Quantification of relative band intensities of pERK/ERK from three independent experiments. Respective proteins levels were quantified by ImageJ. **(G)** Immunoblot analysis of 293T cells expressing shRNA vectors (A and B) targeting FBW7. Whole cell extracts were probed with the indicated antibodies. **(H)** Immunoblot analysis of 293T cells expressing myc-FBW7 and shRNA vectors (A and B) targeting FBW7. Whole cell extracts were probed with the indicated antibodies. For G and H data shown are representative of three independent and reproducible experiments. Respective proteins levels were quantified by ImageJ comparing indicated proteins to relevant controls.

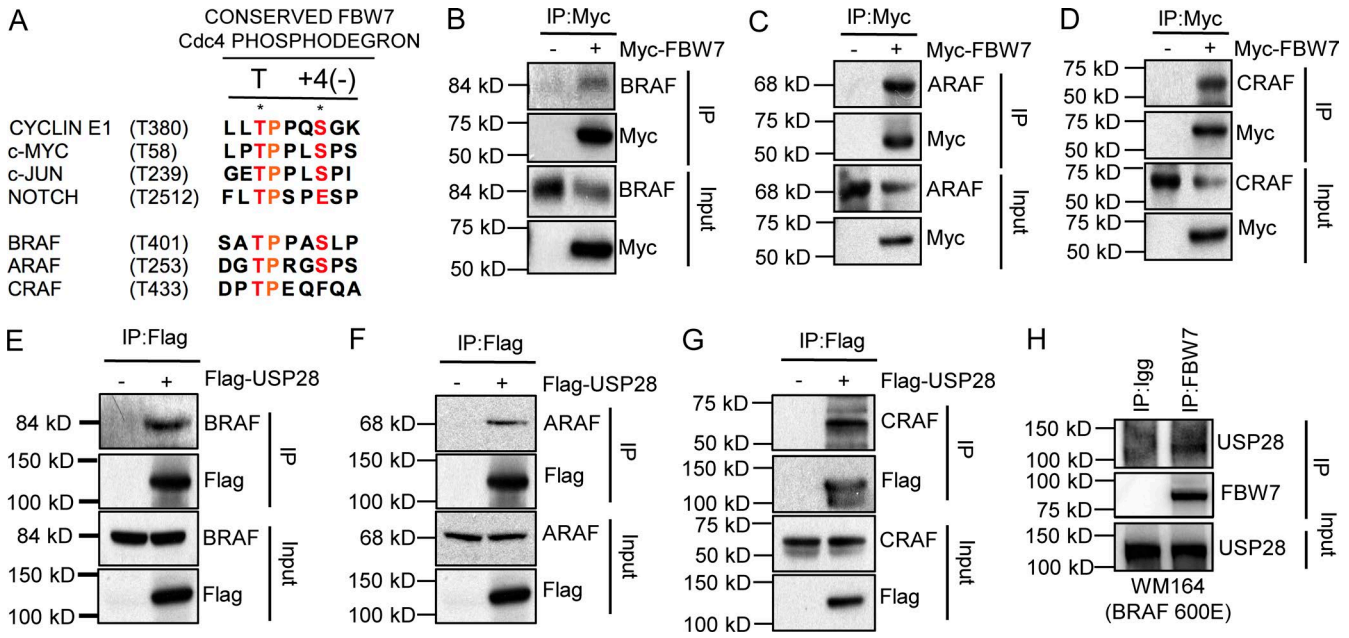


Figure S2. **USP28/FBW7 forms a complex with RAF family members. (A)** Conserved FBW7 Cdc4 phosphodegron domain highlighting conserved phosphoepitope and negative charge at the +4 position of known FBW7 substrates and BRAF, ARAF, and CRAF. **(B–D)** Immunoprecipitation with anti-Myc resin from lysates of 293T cells expressing Myc-FBW7 and an immunoblot analysis of indicated proteins including BRAF (B), ARAF (C), and CRAF (D). **(E–G)** Immunoprecipitation with anti-Flag resin from lysates of 293T cells expressing Flag-USP28 and an immunoblot analysis of indicated proteins including BRAF (E), ARAF (F), and CRAF (G). **(H)** Immunoprecipitation with anti-FBW7 resin from lysates of WM164 melanoma cells and an immunoblot analysis of indicated proteins. For B–H, data shown are representative of two independent and reproducible experiments.

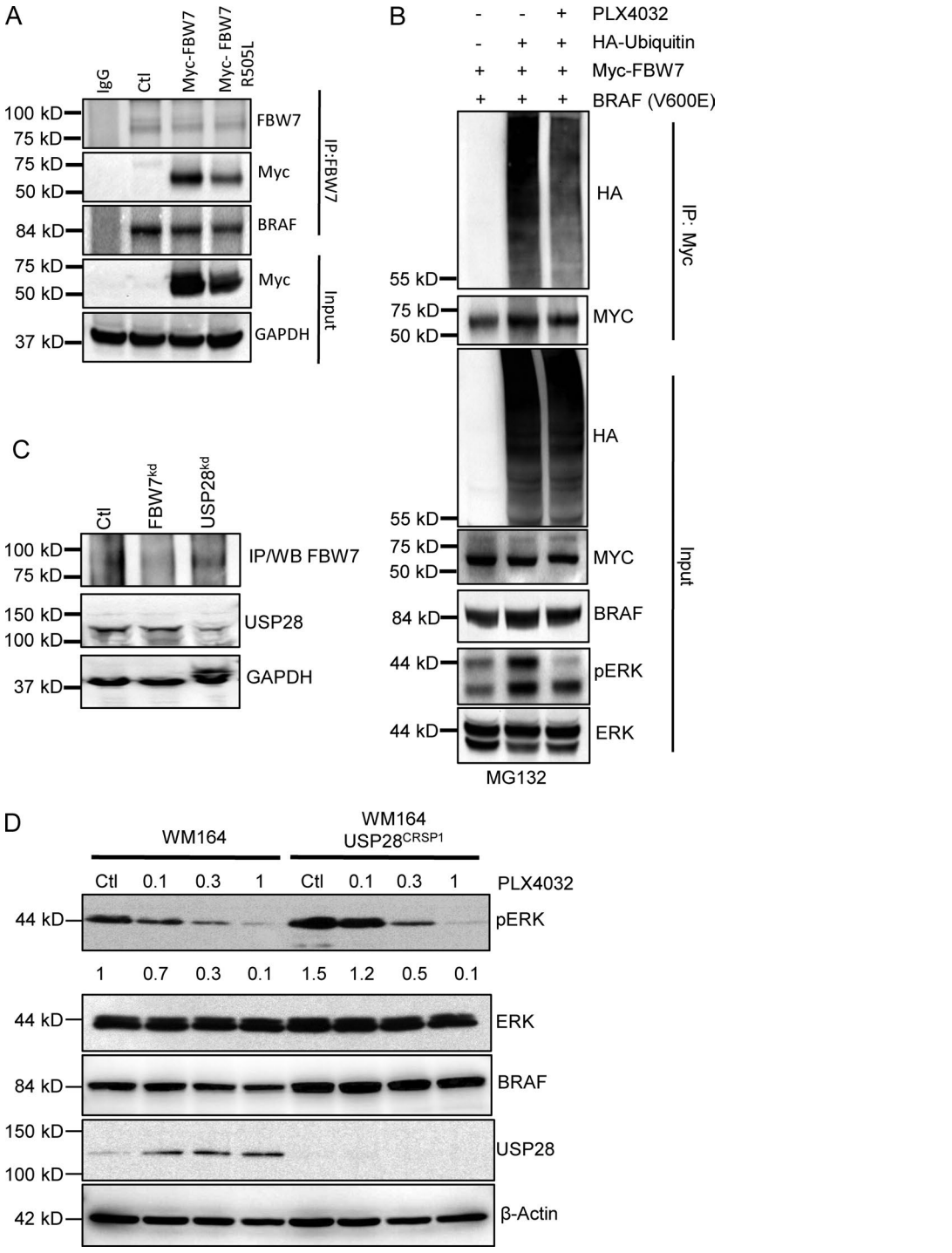


Figure S3. **Vemurafenib alters FBW7 ubiquitination. (A)** Immunoprecipitation with an N-terminal anti-FBW7 resin from lysates of 293T cells expressing Myc-FBW7 or Myc-FBW7(R505L). Immunoblot analysis of indicated proteins is shown. **(B)** Immunoprecipitation with anti-Myc resin in 293T cells stably expressing BRAF(V600E), Myc-FBW7, and HA-Ub, treated with proteasome inhibitor, MG132 (5 µM) and vemurafenib (PLX4032; 1 µM) overnight. Immunoblot analysis of indicated proteins is shown. **(C)** Immunoblot analysis of WM164 melanoma cells stably expressing shRNA vectors targeting USP28 and FBW7. Whole cell extracts were derived from Fig. 2 K and probed with the indicated antibodies. **(A–C)** Data shown are representative of two independent and reproducible experiments. **(D)** Immunoblot analysis of WM164 or WM164 USP28 CRISPR knockout cells treated with different concentrations of vemurafenib (PLX4032) for 1 h. Whole cell extracts were probed with the indicated antibodies. Data shown are representative of two independent and reproducible experiments. For respective immunoblots, proteins levels were quantified by ImageJ comparing indicated proteins to relevant controls.

A

Pathological Characteristics of Study cohort	
TNM stage	N=424
Stage 0	6
Stage I	77
Stage II	140
Stage III	169
Stage IV	23
Stage I/II NOS	9

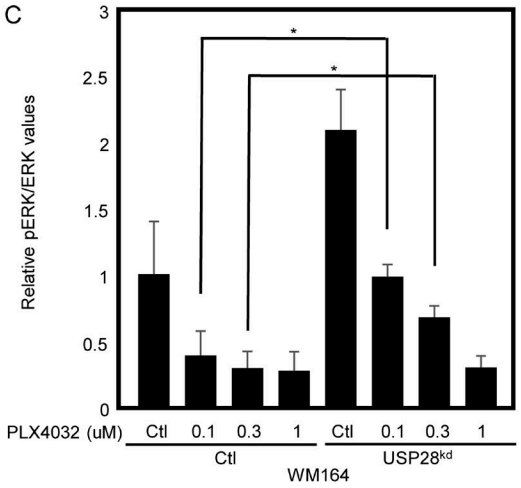
B

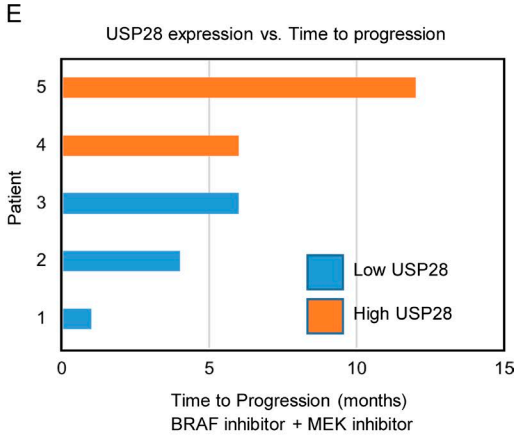
Multivariate Analysis					
Response Survival:	Degrees of freedom	Sum of Squares	Mean of Squares	F-value	P-value
USP28 expression	1	473.7	473.71	21.047	5.8e-006 *
Residuals	422	9493.5	22.50		
Response Gender:					
USP28_expression	1	0.002	0.002315	0.0098	0.9212
Residuals	422	99.620	0.236067		
Response Age :					
USP28_expression	1	1244	1243.84	5.1099	0.0243
Residuals	422	102723	243.42		
Response Tumor stage :					
USP28_expression	1	50.5	50.502	3.9717	0.04691
Residuals	422	5365.9	12.715		

Figure S4. **Characteristics of study cohort. (A)** Pathological characteristics of study cohort (*n* = 424; TCGA). This cohort contains 424 patients of which the disease stages are as follows: 1.4% for Stage 0, 18.2% for Stage I, 33.0% for Stage II, 39.9% for Stage III, 5.2% for Stage IV, and 2.3% for Stage I/II NOS. **(B)** Multivariate analysis comparing USP28 expression to survival ($P = 5.88 \times 10^{-6}$), gender (P = 0.9212), age (P = 0.0243), and tumor stage (P = 0.04691).

A

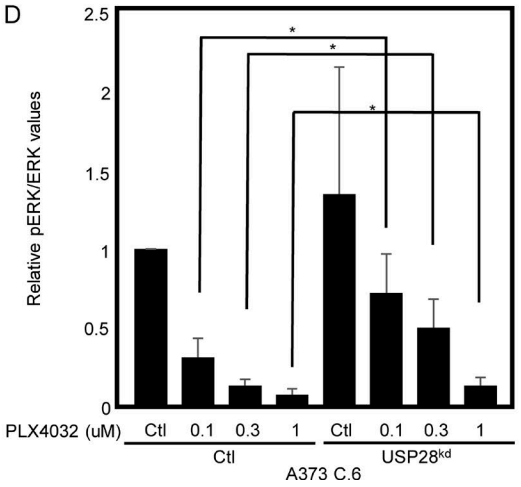
Cells	IC50 (nM)
WM164	301
WM164 CRISPR28	1230





B

Cells	IC50 (nM)
A373	119.4
A373 shUSP28-1	818.5
A373 shUSP28-2	856.2
A373 shUSP28-3	1052



F

Cells	IC50 (nM)
A373	302.1
A373 shUSP28-1	162.3
A373 shUSP28-3	214.3

Figure S5. **Sensitivity of parental cell lines and USP28 knockdown cells to vemurafenib. (A)** Quantification of IC50 values (nM) of Fig. 4 F. **(B)** Quantification of IC50 values (nM) of Fig. 4 G. **(C)** Graph representing relative pERK levels after vemurafenib treatment from three independent experiments of WM164 or WM164 knockdown cells after indicated vemurafenib treatment for 1 h as in Fig. 4 H. A two-tailed Student's *t* test compares the treated populations. **(D)** Graph representing relative pERK levels after vemurafenib treatment from three independent experiments of A373C.6 USP28 or A373C.6 USP28 knockdown cells after indicated vemurafenib treatment for 1 h as in Fig. 4 I. A two-tailed Student's *t* test compares the treated populations; *, $P < 0.05$. **(E)** Bar graph comparing USP28 expression versus time to progression (months) in patients receiving either dabrafenib plus trametinib or vemurafenib plus cobimetinib ($n = 5$). Mean USP28 expression in all of the samples was used as a cutoff for low (<100) versus high (>100) USP28 expression. **(F)** Quantification of IC50 values (nM) of Fig. 7 B.

Tables S1, S2, and S3 are included as separate Excel files. Table S1 shows a deubiquitinating enzyme screen analyzing altered pERK/ERK ratios. Table S2 shows USP28 copy number variation and NRAS, BRAF, NF1, and FBW7 mutation status in 118 melanoma patients. Table S3 shows the chemical compound screen in A373 cells and A373 USP28 knockdown cells.