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Supplemental Data

CRAF Autophosphorylation of Serine 621

Is Required to Prevent Its

Proteasome-Mediated Degradation

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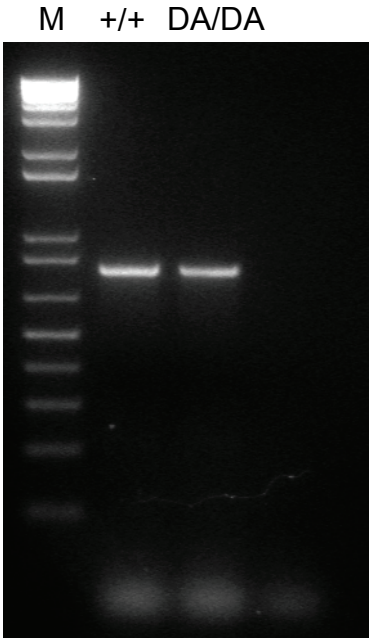

Figure S1. *craf* mRNA splicing is not affected by the targeted DA allele. RNA was prepared from $craf^{+/+}$ and $craf^{DA/DA}$ MEFs and subjected to RT-PCR with primers spanning the targeted region. The primers used were: forward primer 5'-GGAGATGTTGCAGTAAAGATC-3' located within exon 11 and reverse primer 5'-CGTGCAAGCATTGATGTCCTC-3' located within exon 17. The single expected product of 807 bp is obtained in both samples, and there is no evidence of smaller or larger products in the $craf^{DA/DA}$ sample. These results confirm that the presence of the DA mutation and the single loxP site remaining within the targeted allele does not affect *craf* mRNA splicing between exons 11-17. M = 1kb ladder marker (Invitrogen). The data presented in Fig. 1F also shows *craf* mRNA levels are not affected by the mutation.

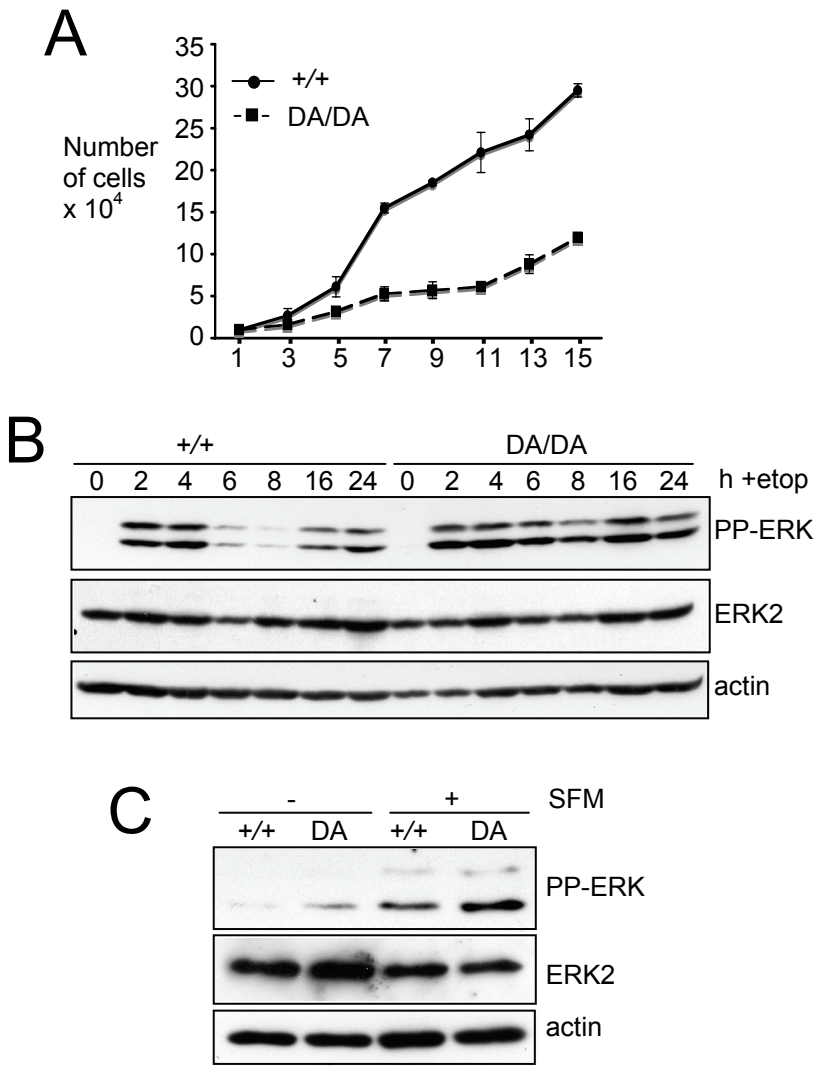

Figure S2. Cell growth is disrupted in *craf*$^{DA/DA}$ cells but ERK phosphorylation is not decreased following treatment with etoposide or serum withdrawal.

(A) Growth of primary MEFs. Growth was assessed by counting cells every two days for a period of up to 15 days following immediate isolation from the embryo. Values are the mean of three independent experiments and error bars represent standard deviation.

(B) *craf*$^{+/+}$ and *craf*$^{DA/DA}$ MEFs were treated with 75 μM etoposide over a time course of 0 to 24 hours, protein lysates were prepared and analysed with antibodies for phosphoERK, ERK2 and actin by western blot techniques.

(C) *craf*$^{+/+}$ and *craf*$^{DA/DA}$ MEFs were either untreated or treated with serum-free media (SFM) for 48 hours, protein lysates were prepared and analysed with antibodies for phosphoERK, ERK2 and actin by western blot techniques.

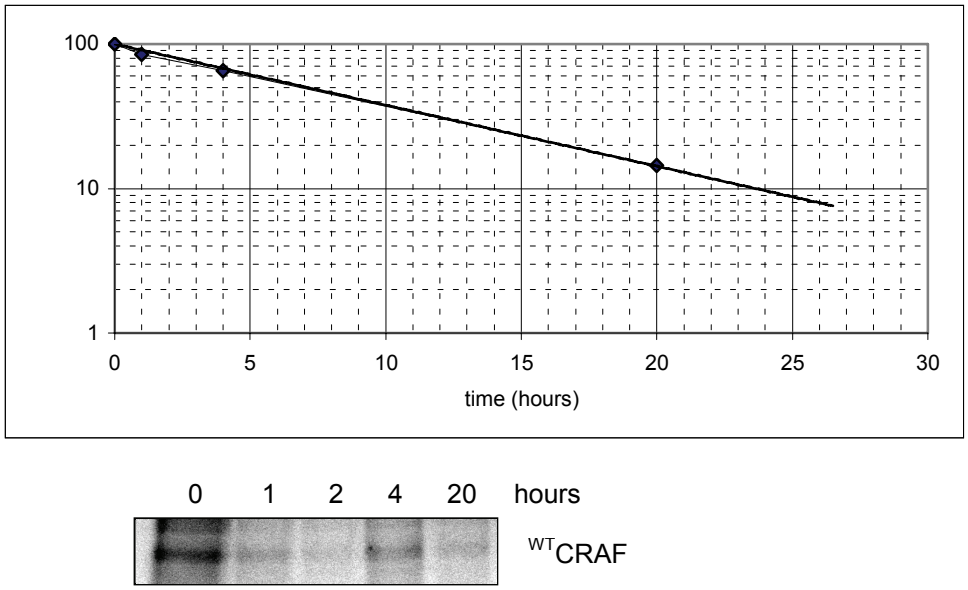

Figure S3. Determination of the half-life of endogenous ^{WT}CRaf in MEFs.
craf$^{+/+}$ MEFs were pulse labelled for 24 hours with ^{35}S-containing media and chased over a time course of up to 20 hours. Protein lysates were harvested, immunoprecipitated with an antibody for CRaf and immunoprecipitated material was electrophoresed through a SDS-PAGE gel, gels were dried and exposed to X-Ray film. The optical density of bands on X-ray film was quantitated using NIH Image J software. The % reduction in optical density compared to optical density at t=0 was determined and plotted on a graph of log optical density vs time. The half-life of CRaf ($t_{1/2}$) was calculated as the time required for the optical density to decrease by 50% and was found to be ~7 hours. A typical example of data obtained from two independent experiments is presented.

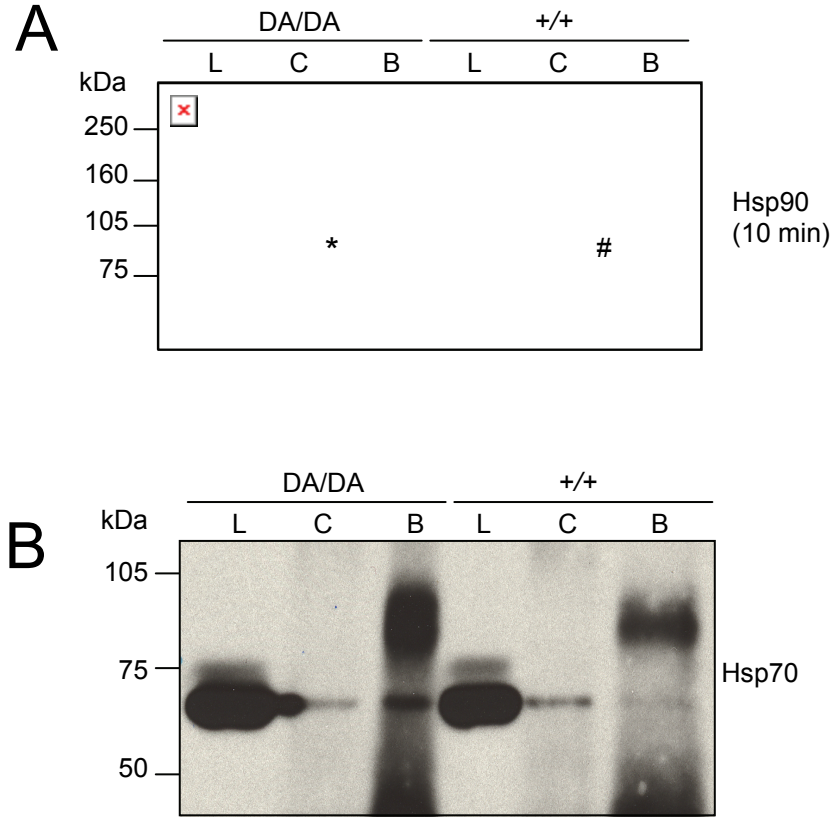

Figure S4. ^{D486A}CRaf is a misfolded protein

(A) ^{DA}CRaf has an increased ability to bind HSP90. Protein lysates were prepared from *craf*$^{+/+}$ and *craf*$^{DA/DA}$ cells and CRaf (C) or BRaf (B) proteins were immunoprecipitated. Immunoprecipitated proteins as well as total protein lysate (L) were analysed with an antibody for Hsp90. A considerably stronger interaction of ^{DA}CRaf than ^{WT}CRaf with HSP90 is detected (marked by *). This is a longer exposure of the autoradiogram presented in figure 3C in which the co-immunoprecipitation of ^{WT}CRaf with Hsp90 in *craf*$^{+/+}$ cells is more easily visible (marked by #), but increased affinity of ^{DA}CRaf with Hsp90 is observed (marked by *).

(B) Interaction of ^{DA}CRaf with HSP70 is not altered. Protein lysates were prepared from *craf*$^{+/+}$ and *craf*$^{DA/DA}$ cells and CRaf (C) or BRaf (B) proteins were immunoprecipitated. Immunoprecipitated proteins as well as total protein lysate (L) were analysed with an antibody for Hsp70. Similar levels of Hsp70 are co-immunoprecipitated with wild-type CRaf and ^{DA}CRaf.

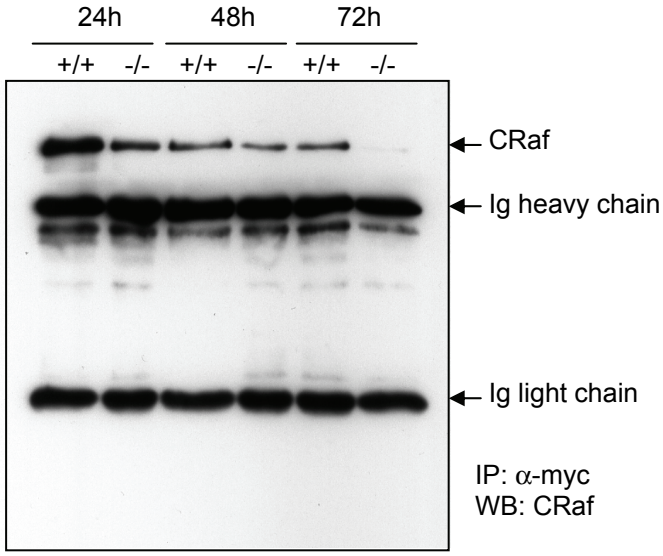

Figure S5. CHIP is not required for the proteasomal degradation of kinase inactive CRaf

^{K375M}CRAF was co-transfected into either wild-type (+/+) or CHIP knockout (-/-) immortalised lung fibroblasts with a vector expressing GFP. Transfection efficiencies were confirmed by directly visualising GFP expression and were found to be ~50% for both cell lines. Protein lysates were harvested 24, 48 and 72 hours post-transfection and CRAF was immunoprecipitated with an antibody for the myc-tag. Immunoprecipitates were electrophoresed on SDS-PAGE gels and CRaf expression levels were assessed by western blotting with an antibody for CRAF. The data confirm the CHIP siRNA data showing that, kinase-inactive CRaf is no more stable in the absence of CHIP than the presence of CHIP.