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Supplementary Materials for

Ubiquitination of K-Ras Enhances Activation and Facilitates Binding to Select Downstream Effectors

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Model of Ras signaling

We considered a model of Ras signaling consisting of enzymatic reactions involving conversion of the GDP-bound form RasGDP to the GTP-bound form RasGTP by a GEF. RasGTP can be reverted to RasGDP by a GAP. Parameter values for Ras, GEF, and GAP abundance, and GEF and GAP enzyme kinetics were taken from a recent modeling study of Ras signaling (1). In addition, Ras ubiquitination by an E3 ubiquitin ligase (E3) and a deubiquitinating enzyme (DUB) were incorporated into the model. The model assumes that ubiquitinated Ras exists in its GTP-bound form and that deubiquitination of Ras can revert Ras to its GDP or GTP-bound form with equal probability. Each forward and backward enzymatic reaction is of the form:

$$\begin{aligned} E^+ + Ras_i &\xleftrightarrow{kon_i^+ / koff_i^+} C_i^+ \xrightarrow{kcat_i^+} E^+ + Ras_{i+1} \\ E^- + Ras_i &\xleftrightarrow{kon_i^- / koff_i^-} C_i^- \xrightarrow{kcat_i^-} E^- + Ras_{i-1} \end{aligned} . \qquad (1)$$

where E^+ is a GEF or E3 ligase, E^- is a GAP or DUB, $C_i^{+/-}$ are the respective enzyme-substrate complexes. $i \to i+1$ denotes transition of Ras from its GDP-bound to its GTP-bound form or transition of its GTP-bound form to its GTP-bound, ubiquitinated form. $i \to i-1$ denotes transition of Ras from its GTP-bound to its GDP-bound form or transition of its GTP-bound, ubiquitinated form to its GTP-bound form. With the following definitions,

$$\begin{aligned} x_1 &= RasGDP \\ x_2 &= RasGTP \\ x_3 &= RasUbGTP \\ x_4 &= GEF \\ x_5 &= GAP \\ x_6 &= E3 \\ x_7 &= DUB \end{aligned} ,$$

mass action kinetics results in a system of differential equations,

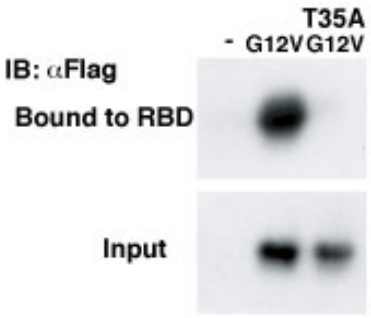
$$
\begin{aligned}
\frac{dx_1}{dt} &= -\left(kon_1^+ x_4 + kon_3^+ x_6\right)x_1 + koff_1^+ C_1^+ + koff_3^+ C_3^+ + kcat_1^- C_1^- + kcat_3^- C_3^- \\
\frac{dx_2}{dt} &= -\left(kon_1^- x_5 + kon_2^+ x_6\right)x_2 + koff_1^- C_1^- + koff_2^+ C_2^+ + kcat_1^+ C_1^+ + kcat_2^- C_2^- \\
\frac{dx_3}{dt} &= -\left(kon_3^- x_7 + kon_2^- x_7\right)x_3 + koff_3^- C_3^- + koff_2^- C_2^- + kcat_3^+ C_3^+ + kcat_2^+ C_2^+ \\
\frac{dx_4}{dt} &= -kon_1^+ x_4 x_1 + \left(koff_1^+ + kcat_1^+\right)C_1^+ \\
\frac{dx_5}{dt} &= -kon_1^- x_5 x_2 + \left(koff_1^- + kcat_1^-\right)C_1^- \\
\frac{dx_6}{dt} &= -\left(kon_3^+ x_1 + kon_2^+ x_2\right)x_6 + \left(kcat_2^+ + koff_2^+\right)C_2^+ + \left(kcat_3^+ + koff_3^+\right)C_3^+ \\
\frac{dx_7}{dt} &= -\left(kon_3^- x_3 + kon_2^- x_2\right)x_7 + \left(kcat_3^- + koff_3^-\right)C_3^- + \left(kcat_2^- + koff_2^-\right)C_2^- \\
\frac{dC_1^+}{dt} &= kon_1^+ x_4 x_1 - \left(koff_1^+ + kcat_1^+\right)C_1^+ \\
\frac{dC_1^-}{dt} &= kon_1^- x_5 x_2 - \left(koff_1^- + kcat_1^-\right)C_1^- \\
\frac{dC_2^+}{dt} &= kon_2^+ x_6 x_2 - \left(koff_2^+ + kcat_2^+\right)C_2^+ \\
\frac{dC_2^-}{dt} &= kon_2^- x_7 x_3 - \left(koff_2^- + kcat_2^-\right)C_2^- \\
\frac{dC_3^+}{dt} &= kon_3^+ x_6 x_1 - \left(koff_3^+ + kcat_3^+\right)C_3^+ \\
\frac{dC_3^-}{dt} &= kon_3^- x_7 x_3 - \left(koff_3^- + kcat_3^-\right)C_3^-
\end{aligned}
\tag{2}
$$

Conservation of mass leads to four additional constraints:

$$
\begin{aligned}
x_4 + C_1^+ &= GEF \\
x_5 + C_1^- &= GAP \\
x_6 + C_2^+ + C_3^+ &= E3 \\
x_7 + C_2^- + C_3^- &= DUB \\
x_1 + x_2 + x_3 + \sum_i C_i^+ + \sum_i C_i^- &= Ras_{Tot}
\end{aligned}
\tag{3}
$$

where GEF, GAP, $E3$, DUB, and are the total enzyme amounts. Equations were solved numerically using a Range-Kutta method implemented in MATLAB. Steady-state values of the model were used to produce the result obtained in the main text figure.

A



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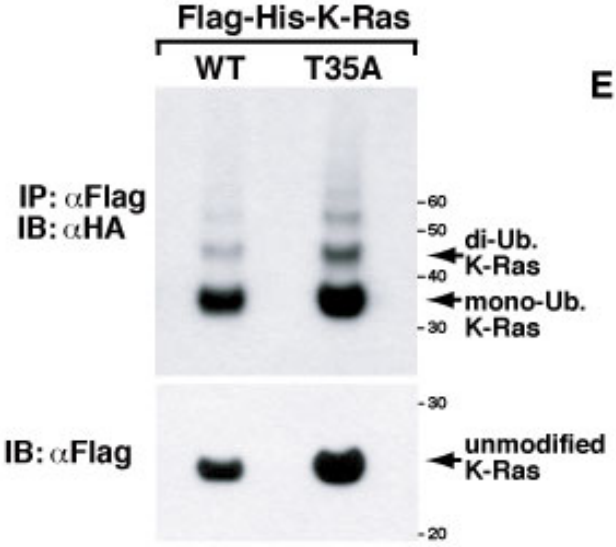

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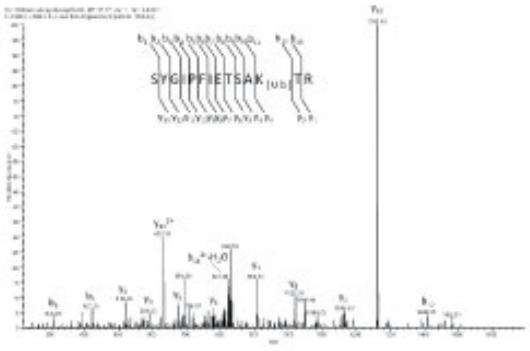
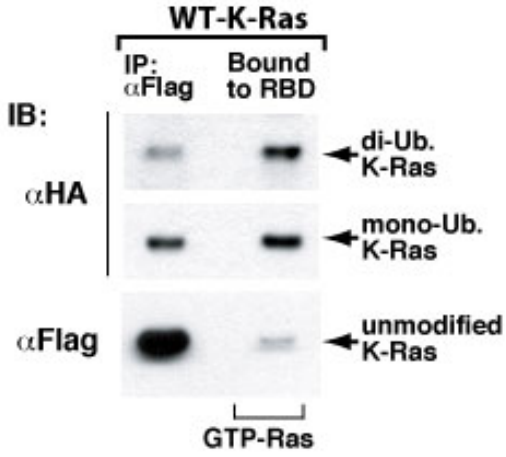


	Ub.site	Number of times identified 1xUb-Ras	2xUb-Ras
KRas	K104	nd	1
	K147	3	1
HRas	**K117**	3	6
	K147	2	8
	K170	2	1
	K185	nd	2

D



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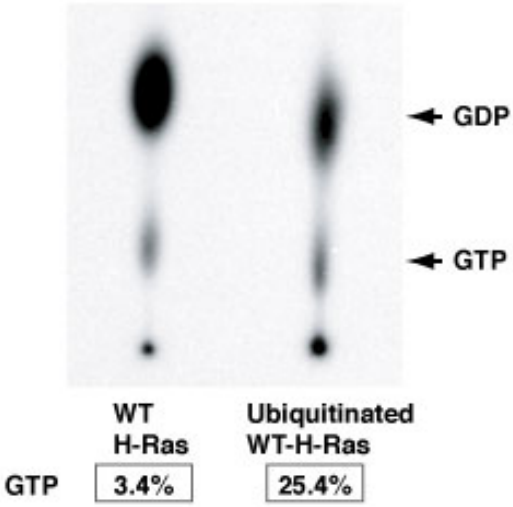
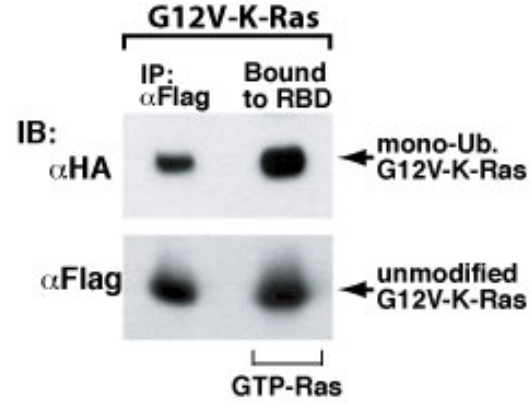


F



Supplemental Fig.1

Figure S1. Characterization of ubiquitinated Ras. (**A**) An effector domain mutant, T35A-G12V-K-Ras, abolishes binding to the Ras binding domain of Raf1. Flag-His-tagged K-Ras constitutively active (G12V) or effector domain mutant (G12V/T35A) was expressed in HEK293T cells, and the binding activity to GST-tagged Ras binding domain of human Raf1 was analyzed. G12V-K-Ras, but not G12V/T35A-K-Ras, bound to the GST-RBD. The blot is representative of two separate experiments. (**B**) Ras ubiquitination occurs independently of its downstream effectors. Flag-His-tagged WT-K-Ras or T35A-K-Ras mutant was coexpressed with HA-tagged ubiquitin (9.4 kD) in HEK293T cells. Ras proteins were immunoprecipitated with an anti-Flag antibody and analyzed with the indicated antibodies. The degree and pattern of ubiquitination of WT-K-Ras and T35A-K-Ras was comparable. Consistent with ubiquitination of endogenous K-Ras (Fig. 1A), the mono-ubiquitinated form was dominant. The blot is representative of two separate experiments. (**C**) Lys^{147} is one of the major ubiquitination sites in K-Ras and H-Ras. For microcapillary/tandem mass spectrometry (LC/MS/MS) experiments, purified ubiquitinated Ras bands were excised from analogous Coomassie blue-stained gels, digested with trypsin, and analyzed by LC/MS/MS using a hybrid linear ion trap-Orbitrap mass spectrometer in data dependent acquisition mode through CID. The MS/MS spectrum for the Ras peptide $SYGIPFIETSAK_{[Ub]}TR$ containing ubiquitinated lysine at position 12 (Lys^{147} in full length protein) that was acquired through CID using a hybrid linear ion trap-Orbitrap mass spectrometer. The **y-** ion series shifts by 114.1 D starting at position $\mathbf{y_3}$ and $\mathbf{b_{12}}$ in the modified peptide representing a –GlyGly- ubiquitin tag after tryptic digestion. The table summarizes the result of mass spectrometry analyses. (**D**) Both mono- and di-ubiquitinated K-Ras4B show increased activity. Flag-His-tagged wild-type K-Ras was expressed with HA-ubiquitin and analyzed as described in Fig.2C. The blot is representative of four separate experiments. (**E**) GTP binding to ubiquitinated H-Ras is enhanced. WT- and ubiquitinated WT-H-Ras were purified from ^{32}P-phosphate labeled cells and the relative quantities of ^{32}P-GTP and ^{32}P-GDP co-purifying with each Ras species was determined by TLC. (**F**) The minimal Ras-binding domain (RBD) from Raf binds similarly to ubiquitinated and non-ubiquitinated forms of G12V mutant K-Ras. Anti-Flag antibody or a GST-fusion of the minimal RBD (residues 1-149 from Raf) were used to purify G12V-K-Ras from cells coexpressing HA-ubiquitin and Flag-His-G12V-K-Ras. GST-RBD domain pulled down mono-ubiquitinated G12V-K-Ras (visualized as migrating) and non-ubiquitinated G12V-K-Ras with equal efficiency. The blot is representative of two separate experiments.

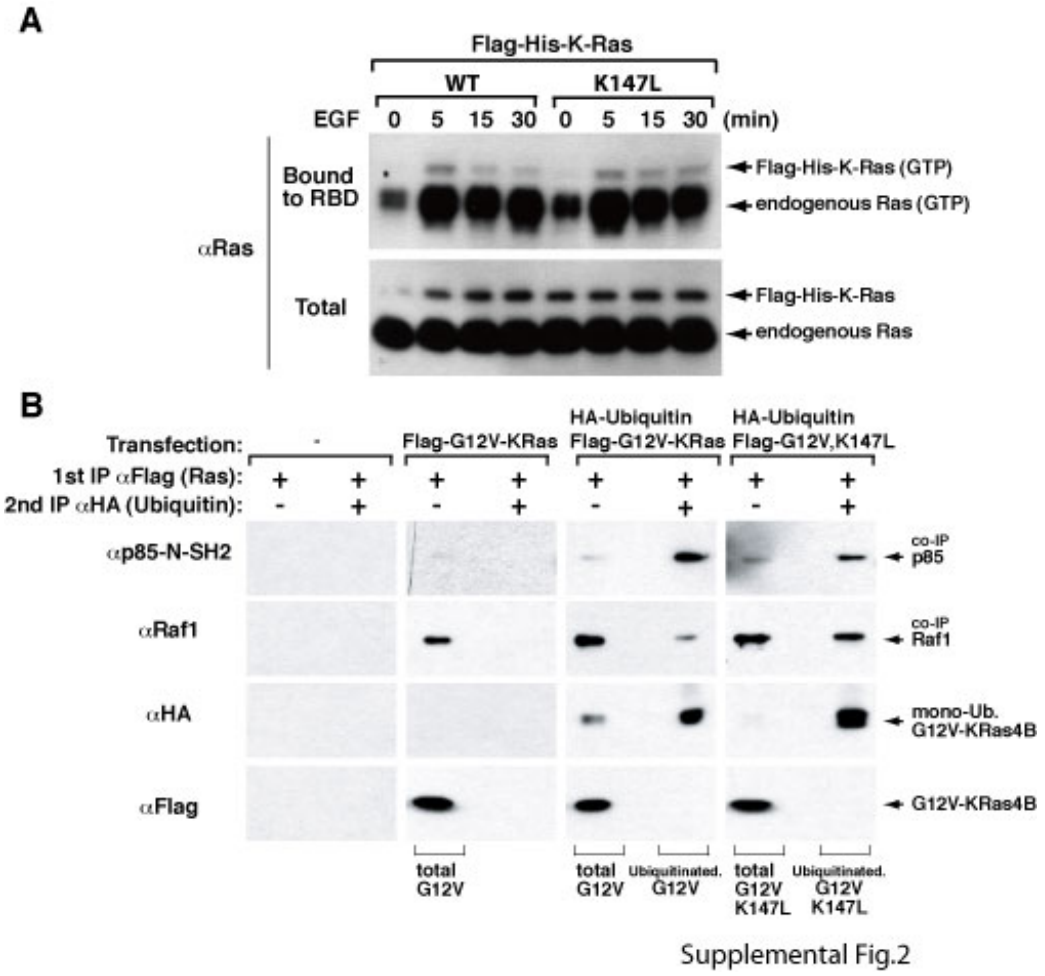


Figure S2. More Raf and PI3K bind to ubiquitinated Ras than to non-ubiquitinated Ras. (**A**) The K147L-K-Ras mutant has comparable biochemical properties to the wild-type K-Ras. K-Ras wild-type (WT) or K147L mutant were expressed in HEK293T cells, and their degree of activation in response to EGF (100 ng/ml) were analyzed by a GST-RBD pull-down assay of Ras. The abundance of both the WT and the K147L mutant were less than that of endogenous Ras. The result shows that the kinetics of WT and K147L-KRas activation are similar. (**B**) More Raf and PI3K bind to ubiquitinated Ras than to non-ubiquitinated Ras. The indicated plasmid was transfected in HEK293T cells and subjected to anti-Flag immunoprecipitation and sequential anti-HA antibody purification. The result shows specific binding of ubiquitinated G12V-K-Ras and G12V/K147L to PI3K and Raf, which was not seen in the control (no plasmid, Flag-G12V-K-Ras only). Blots in all panels are representative of two separate experiments.

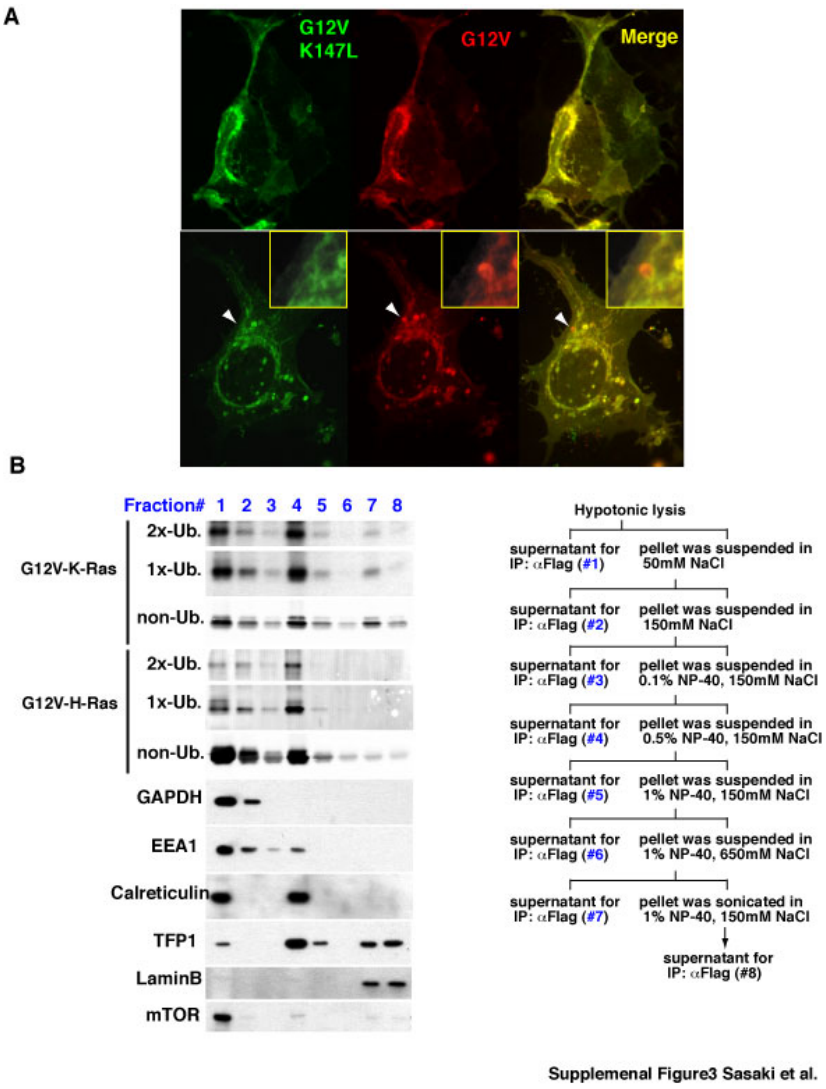


Figure S3. Subcellular localization of ubiquitinated K-Ras. (**A**) Subcellular localization of GFP-G12V/K147L-K-Ras and RFP-G12V-K-Ras in the same cells was assessed by confocal microscopy. A majority of cells displayed indistinguishable localization of GFP-G12V/K147L-K-Ras and RFP-G12V-K-Ras (upper panel), whereas some cells displayed RFP-G12V-K-Ras specific localization (lower panel). The images of RFP-G12V-K-Ras specific localization are representative of five cells. (**B**) The biochemical properties of non-, mono-, and di-ubiquitinated K-Ras and H-Ras. A schematic diagram of the biochemical fractionation protocol is shown on the right. GAPDH (cytosol), EEA1 (early endosome), calreticulin (cytoplasmic and endoplasmic reticulum), mitochondrial trifunctional protein (TFP1), (mitochondria), and laminB (nucleus) show distinctive fractionation patterns. Non-ubiquitinated H-Ras was found in fractions #1 and #4, whereas the abundance of ubiquitinated H-Ras in fraction #1 was lower than that of non-ubiquitinated H-Ras. Ubiquitinated and non-ubiquitinated K-Ras showed similar patterns. Slightly less ubiquitinated-K-Ras was found in fraction #7. The blots are representative of two separate experiments.

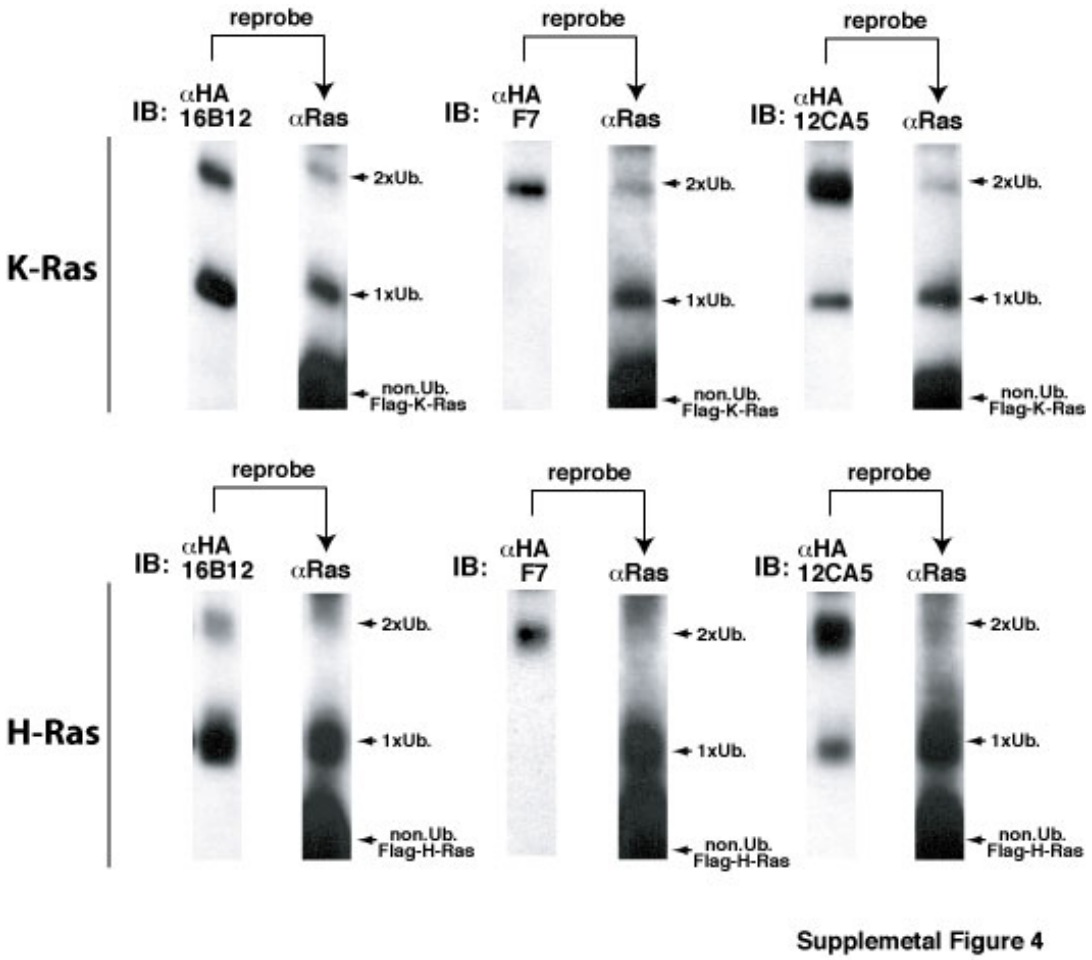


Figure S4. Anti-HA antibody, clone 16B12, faithfully recognizes HA-ubiquitin-Ras. Flag-His-K-Ras or Flag-His-H-Ras was expressed with HA-ubiquitin in HEK293T cells. Cells were lysed with 8 M urea and Flag-His-Ras proteins were purified by Ni-NTA agarose. The purified Flag-His-Ras was subjected to 12% SDS-PAGE and immunoblotted with three different anti-HA monoclonal antibodies: clone 16B12 (Covance), F-7 (Santa Cruz), and 12CA5 (Santa Cruz). The membrane was stripped with Reblot-Strong (Chemicon International) and reprobed with anti-Ras antibody (reprobe). The anti-Ras blot demonstrates that the amount of mono-ubiquitinated Ras was more than that of di-ubiquitinated form. The anti-HA (16B12) recognized mono- and di-ubiquitinated Ras as did the anti-Ras blot, whereas clones F-7 and 12CA5 recognized di-ubiquitinated Ras preferentially.

References

1. J. Das, M. Ho, J. Zikherman, C. Govern, M. Yang, A. Weiss, A. K. Chakraborty, J.P. Roose, Digital signaling and hysteresis characterize ras activation in lymphoid cells. *Cell* **136**, 337-351 (2009).