Supplementary Figures and Legends 1-14

Suppl. Figure 1. a, List of the RAF mutations used and their properties. b, Model of the effects of RAF inhibitors in the presence of wild-type or mutant BRAF. When BRAF is wild-type and RAS activity is elevated promoting RAF dimer formation, an ATP-competitive RAF inhibitor binds to RAF molecules and inhibits them. However, binding of the inhibitor induces transition to the active phosphorylated state, which is transferred via direct interaction to inhibitor-free RAF molecules causing a marked increase in catalytic activity. As the concentration of the inhibitor increases, the fraction of dimer with both sites bound to drug increases and total cellular RAF activity declines. In cells with $BRAF^{V600E}$, the drug inhibits $BRAF^{V600E}$ and low RAS activity precludes transactivation, so ERK signaling is inhibited.

Suppl. Figure 2. Treatment with PLX4032 inhibits MEK/ERK in cells harboring $BRAF^{V600E}$, but activates ERK signaling in cells with wild-type BRAF. a, Exponentially growing cells were treated with the indicated doses of PLX4032 for 1 hour and lysates were immunoblotted for pMEK and pERK. **b,** Cells with wild-type BRAF (Calu-6) or mutant BRAF (SKMEL28) were treated with vehicle or PLX4032 (1μM/1 hour). Phosphorylation and expression of the indicated proteins were assayed by immunoblotting.

Suppl. Figure 3. Chemical structures of compounds.

Suppl. Figure 4. PLX4032 inhibits all RAF isoforms *in vitro*. Full length myc-tagged ARAF, FLAG-tagged BRAF, V5-tagged CRAF and FLAG-tagged $BRAF^{V600E}$ were overexpressed in 293H cells, immunoprecipitated and subjected to kinase assay in the presence of the indicated concentrations of PLX4032. Kinase activity was determined by immunoblotting for pMEK.

Suppl. Figure 5. MEK/ERK activation by RAF inhibitor depends on upstream signaling. a, SKBR3 cells carrying HER2 amplification were pre-treated with either vehicle (lanes 1 & 3) or 100 nM lapatinib (100nM – lanes 2 & 4) for 1 hour, then 1 μM PLX4032 was added to lanes 3 & 4 for an additional hour**. b**, 293H cells were transfected with EGFP, HA-tagged wild-type RAS or RAS^{G12V}. The cells were treated with vehicle or PLX4032 (1μM) for 1 hour and the lysates were immunoblotted for phosphorylation and expression levels of the indicated proteins. **c**, Similar to **b**, with FLAG-tagged $BRAF^{V600E}$ transfected instead.

Suppl. Figure 6. CRAF expression is required for MEK/ERK activation by RAF inhibitor. a, Wild-type (+/+), BRAF knock-out (BRAF -/-) or CRAF knock-out (CRAF -/-) mouse embryonic fibroblasts (MEFs) were treated with increasing concentrations of PLX4032 for 1 hour. Cell lysates were immunoblotted for pMEK and pERK. b, Similarly to 293H cells (**Fig 2a**), over-expression of active HA-tagged RAS in wild-type MEFs enhances MEK/ERK activation by PLX4032 (1μM/1 hour). **c,** Re-expression of CRAF in CRAF (-/-) MEFs potentiates MEK/ERK induction by PLX4032. Coexpression of active

RAS with CRAF in CRAF(-/-) MEFs results in pronounced MEK/ERK induction upon treatment with PLX4032 (1μM/1 hour).

Suppl. Figure 7. MEK/ERK activation by PLX4032 requires binding to the catalytic domain of RAF. a, 293H cells overexpressing V5-tagged full-length CRAF, or the catalytic domain of CRAF (catC) were treated with vehicle or PLX4032 (1μM/1 hour). **b**, Mutation of the gatekeeper residue abolishes MEK/ERK activation by PLX4032. 293H cells overexpressing V5-tagged catC or $catC^{T421M}$ were treated with the indicated doses of PLX4032 for 1 hour. Cell lysates were immunoblotted for pMEK and pERK.

Suppl. Figure 8. Evaluation of the effect of the gatekeeper mutation on the sensitivity of CRAF to RAF inhibitors *in vitro*. catC or the gatekeeper mutant $catC^{T421M}$ were over-expressed in 293H cells, immunoprecipitated and subjected to kinase assay in the presence of the indicated concentrations of PLX4720 (**a**), PLX4032 (**b**) or Sorafenib (**c**). RAF activity was estimated by immunoblotting for pMEK. The first lane in panel **c** is immunoprecipitation with IgG (negative control).

Suppl. Figure 9. PLX4032 induces the active, phosphorylated state of wild-type and kinase-dead RAF. a, 293H cells over-expressing catC were treated with the indicated amounts of PLX4032 for 1 hour. CatC was immunoprecipitated from the cell lysates, washed extensively and subjected to kinase assay. Kinase activity was determined by immunoblotting for pMEK. **b**, Calu-6 cells were treated with PLX4032 (1μM/1 hour). Endogenous BRAF and CRAF were immunoprecipitated, washed and

assayed for kinase activity. **c**, Treatment with RAF inhibitor results in elevated activating phosphorylation on RAF. V5-tagged wild-type CRAF or kinase-dead $CRAF^{D486N}$ were overexpressed in 293H cells. After 24 hours cells were treated with vehicle or PLX4032 (5μM/1 hour) and lysates were immunoblotted for p338CRAF and p621CRAF. The gatekeeper mutant $CRAF^{T421M}$ was used as negative control.

Suppl. Figure 10. Evaluation of the effect of introduction of a Cysteine at position S428 on the sensitivity of CRAF to quinazoylacrylamide inhibitors. catC (**a**) or $catC^{S428C}$ (**b**) were over-expressed in 293H cells, immunoprecipitated and subjected to kinase assay in the presence of the indicated concentrations of JAB34 or JAB13. RAF activity was measured by immunoblotting for pMEK. The first lane in panel (**a**) is immunoprecipitation with IgG (negative control).

Suppl. Figure 11. JAB compounds activate ERK signaling at lower concentrations. 293H cells expressing V5-tagged catC or $catC^{S428C}$ were treated with PLX4032, JAB13 or JAB34 at the indicated doses for 1 hour. The cell lysates were immunoblotted for pMEK, pERK, total MEK and V5.

Suppl. Figure 12

Activation in the context of full-length CRAF occurs *in trans.* 293H cells co-expressing FLAG-tagged full-length wild-type CRAF and either V5-tagged full-length kinase-dead CRAF ($CRAF^{D486N}$) or JAB34-sensitive, kinase-dead CRAF

(CRAF$^{S428C/D486N}$) were treated with vehicle or 10μM JAB34 for 1 hour and lysates were immunoblotted for pMEK and pERK.

Suppl. Figure 13. R401A mutation in catC diminishes homodimerization. 293H cells were transfected with JAB-sensitive V5-tagged catC^{S428C} and FLAG-tagged catC, or with the same constructs carrying the R401A mutation and treated with vehicle, JAB34 (10μM/1 hour) or EGF (100ng/ml for 5 minutes). The extent of V5-catC/FLAG-catC interaction was determined by immunoprecipitation of FLAG-tagged proteins and immunoblot for V5.

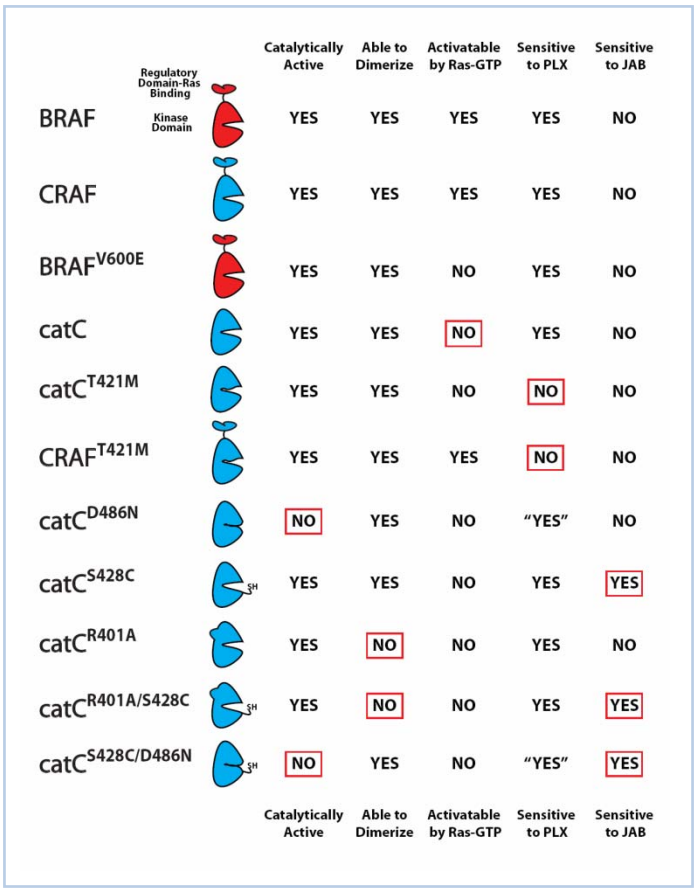
Suppl. Figure 14. **Coexpression of active RAS in BRAF^{V600E} expressing cells renders ERK signaling insensitive to PLX4032. a,** 293H cells coexpressing FLAG-tagged BRAF^{V600E} with EGFP or HA-tagged N-RAS^{G12V} were treated with the indicated doses of PLX4032, or the MEK inhibitor PD325901. **b,** HT-29 cells (colorectal – BRAF^{V600E}) transfected with EGFP or HA-tagged N-RAS^{G12V} were treated with the indicated doses of PLX4032, or the MEK inhibitor PD325901. Lysates were blotted for pMEK and pERK.

Supplementary Table 1

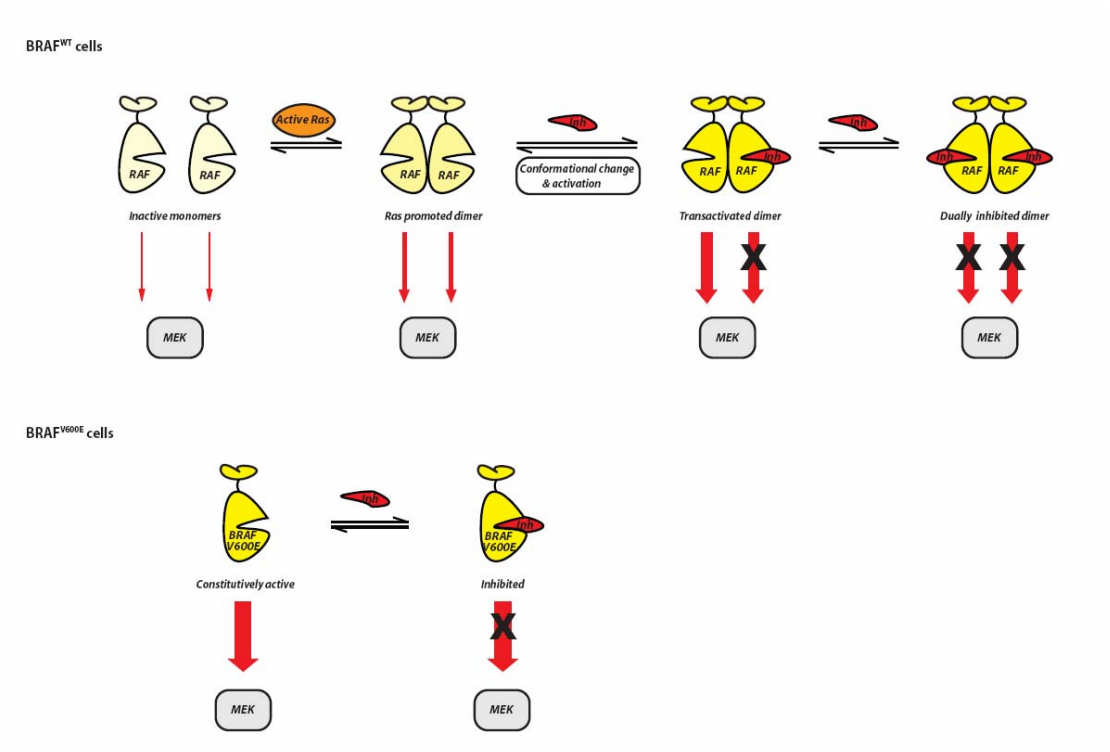
Suppl. Table 1. *In vitro* IC50s of inhibition of the indicated recombinant kinases by PLX4032.

Supplementary Figure 1

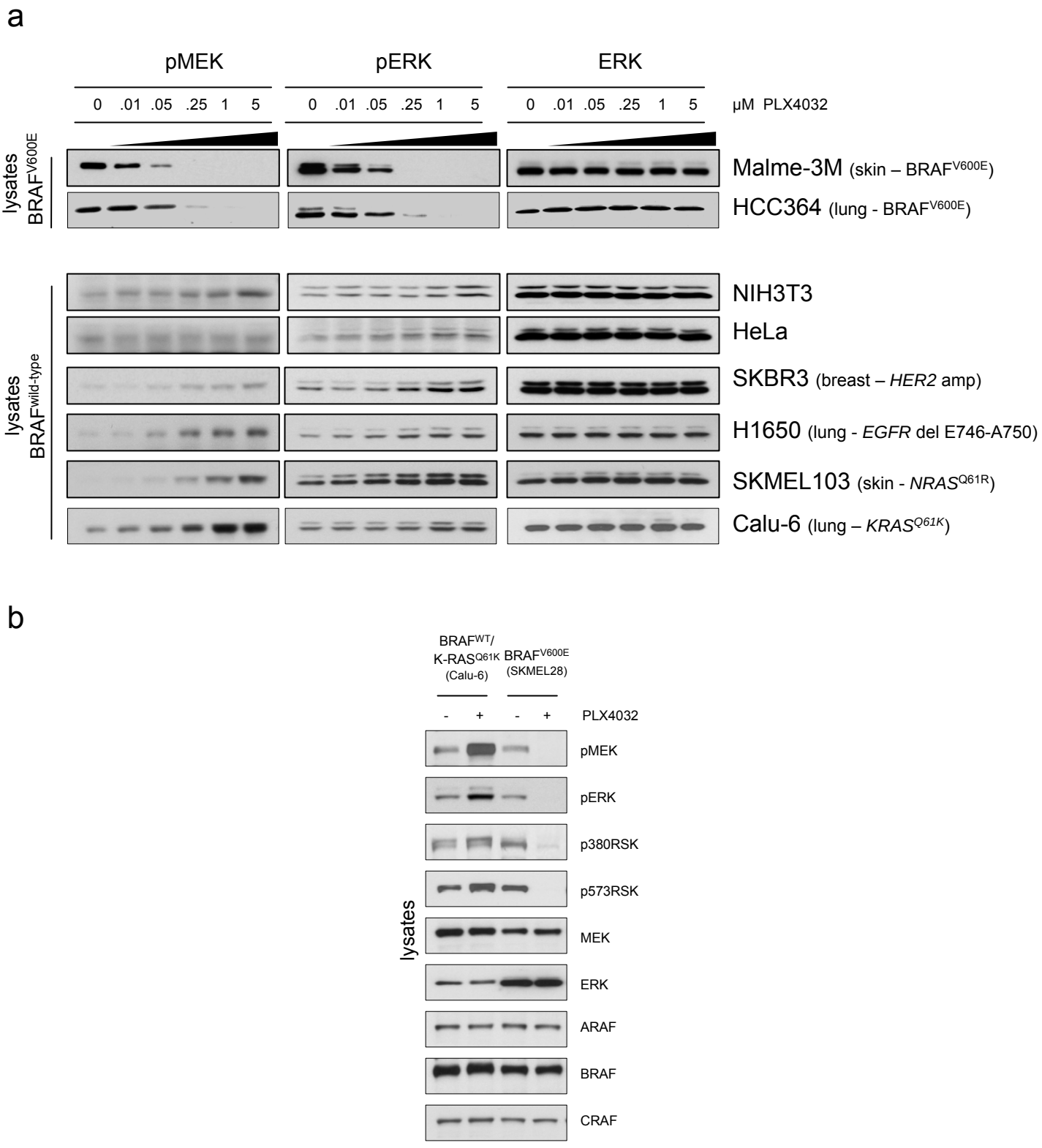
a



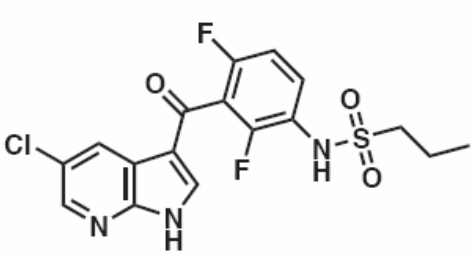
b



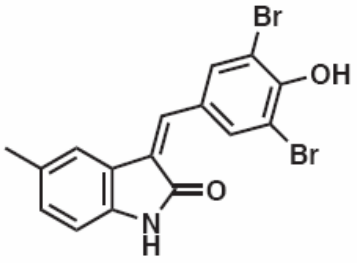
Supplementary Figure 2



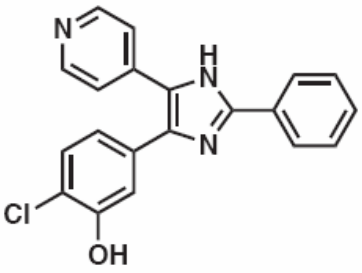
Supplementary Figure 3



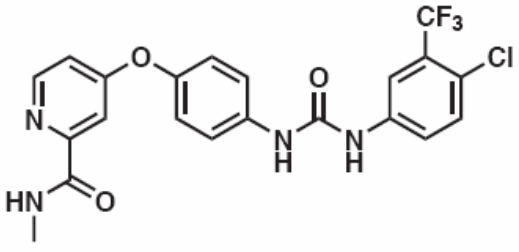
PLX 4720



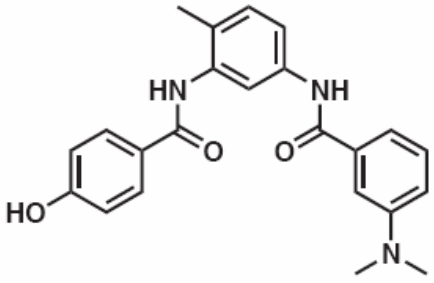
GW 5074



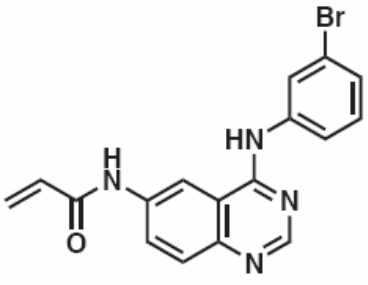
L 779450



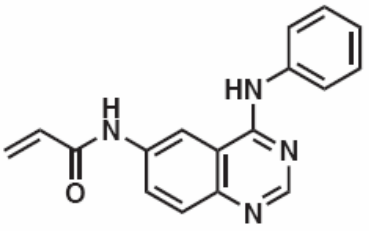
Sorafenib



ZM 336372

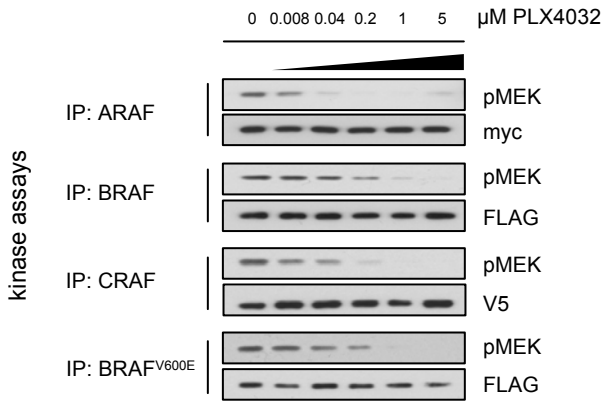


JAB-34

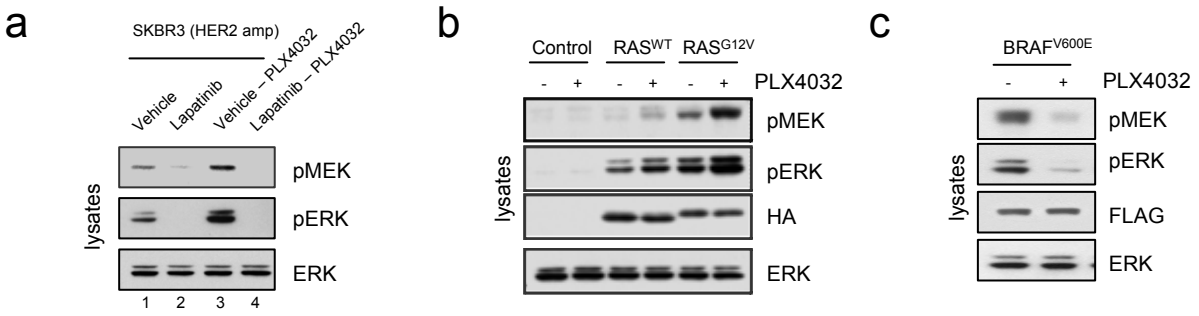


JAB-13

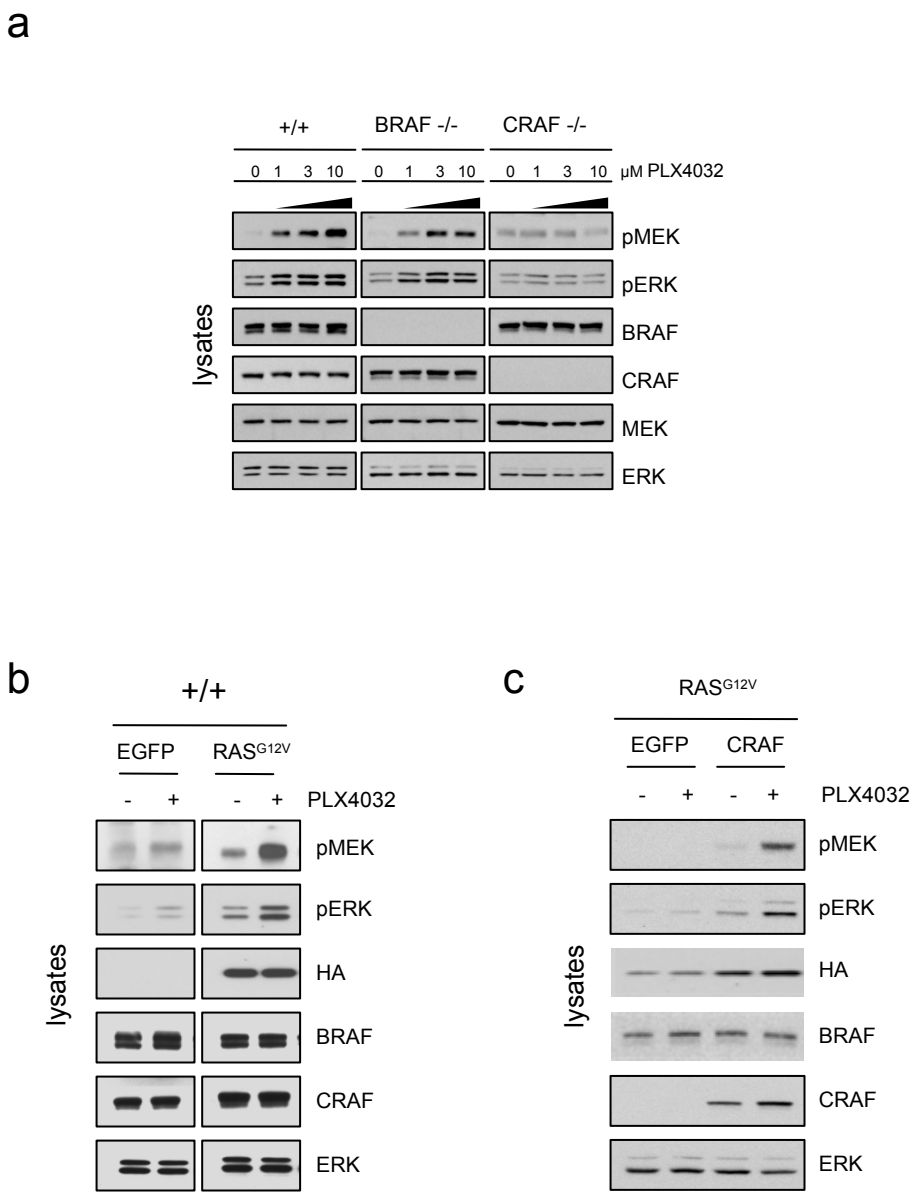
Supplementary Figure 4



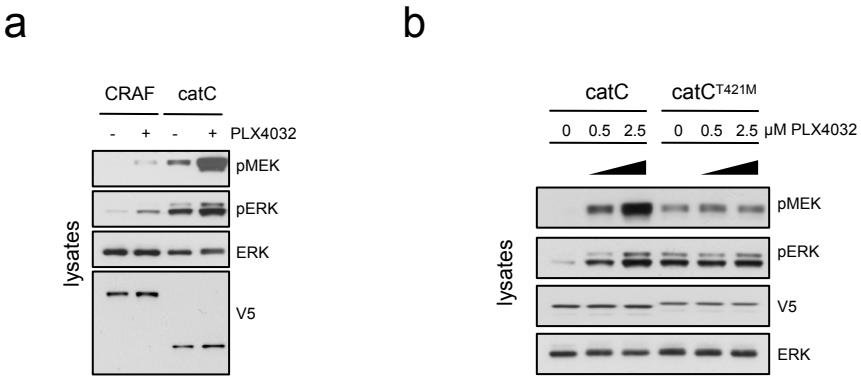
Supplementary Figure 5



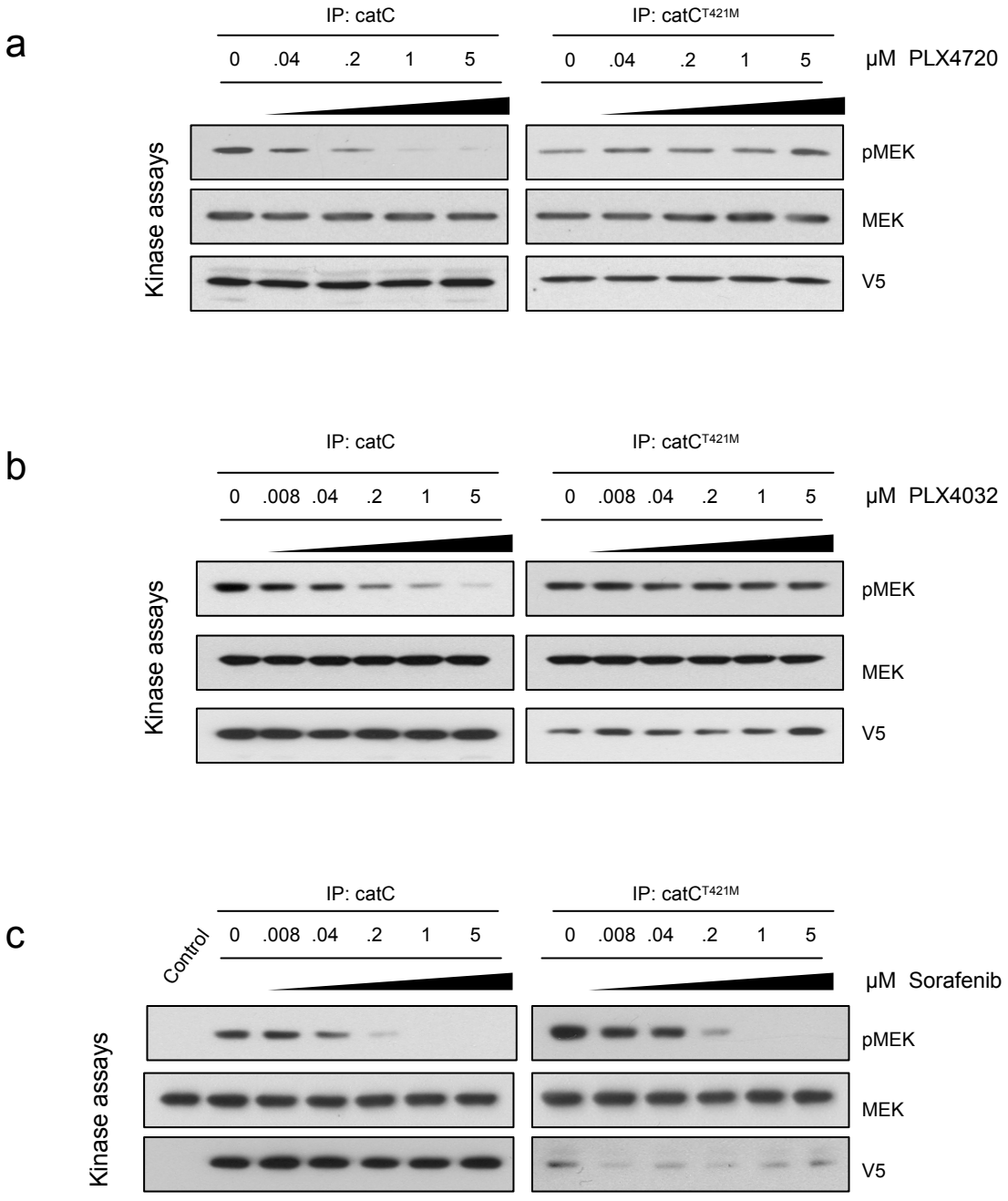
Supplementary Figure 6



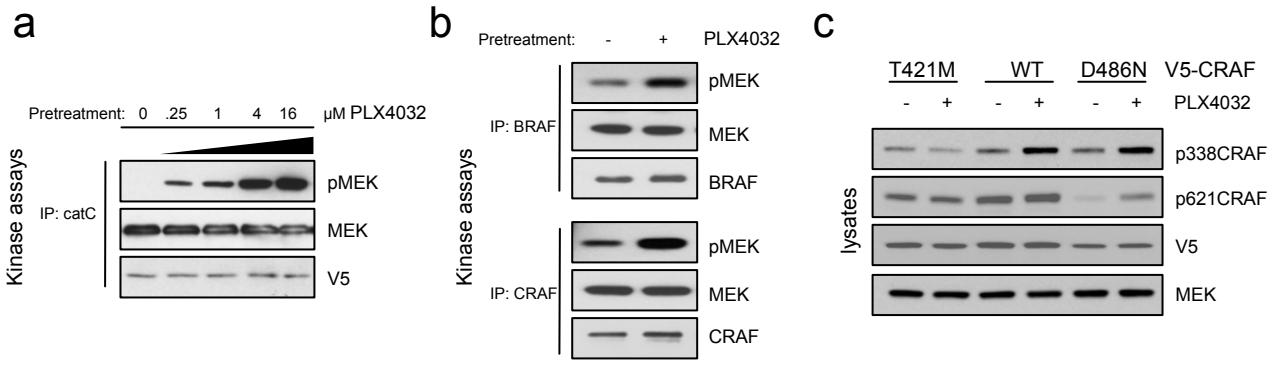
Supplementary Figure 7



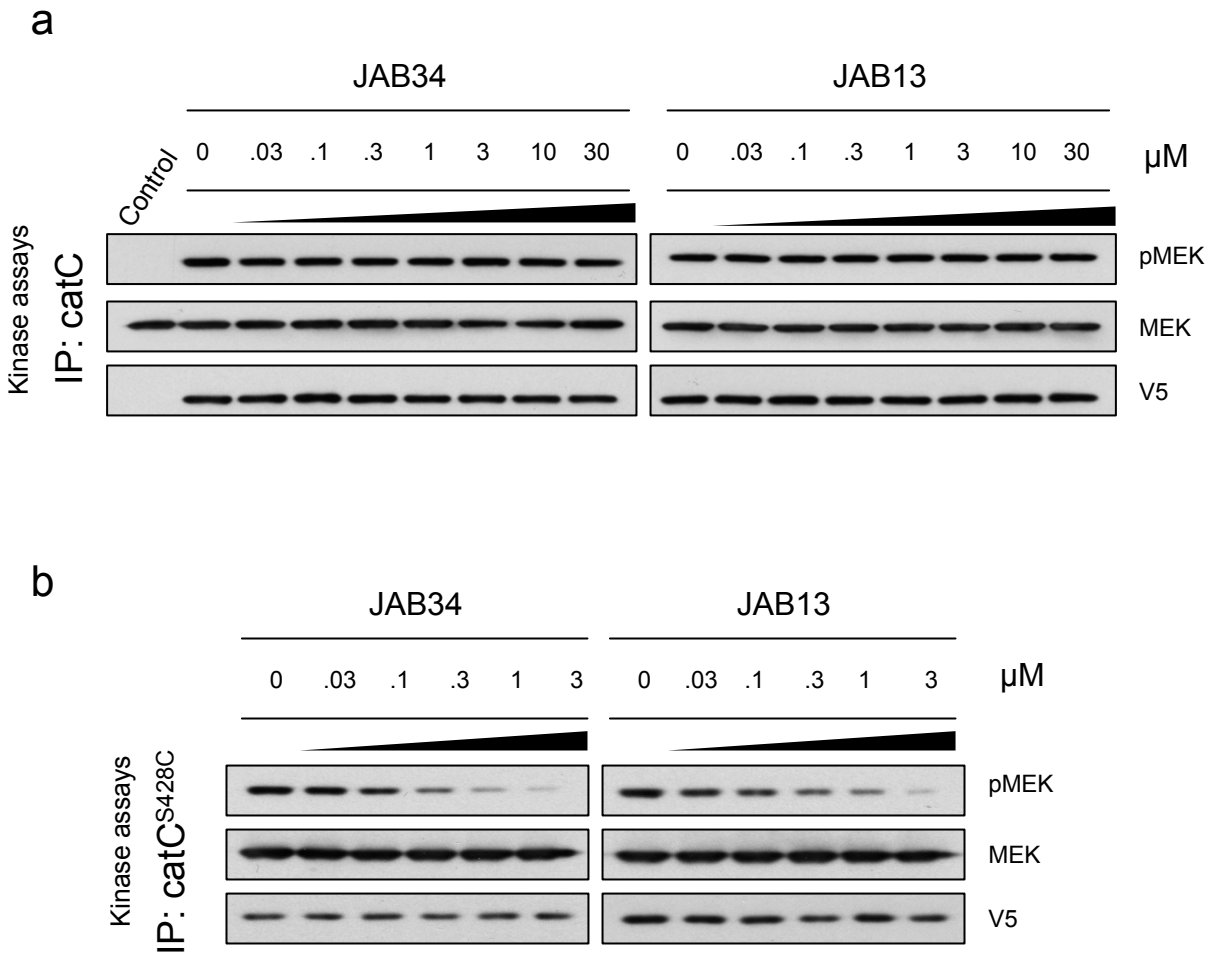
Supplementary Figure 8



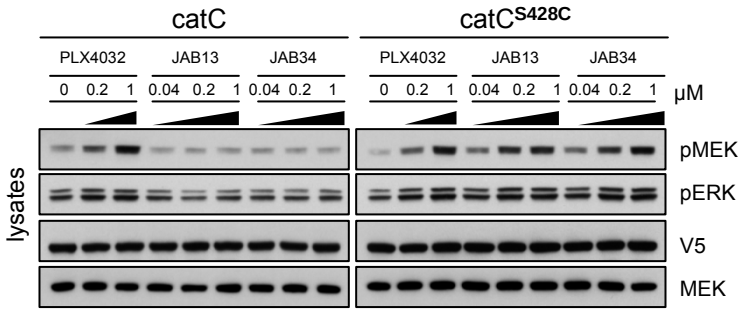
Supplementary Figure 9



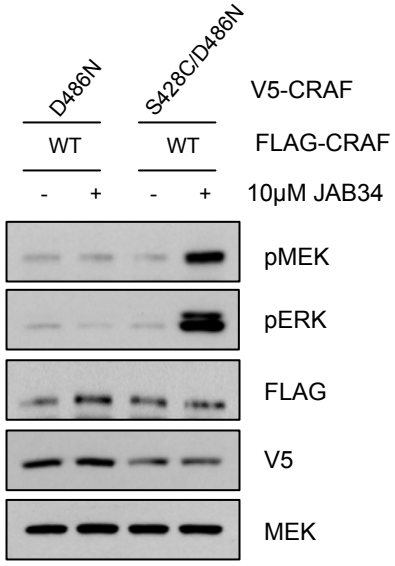
Supplementary Figure 10



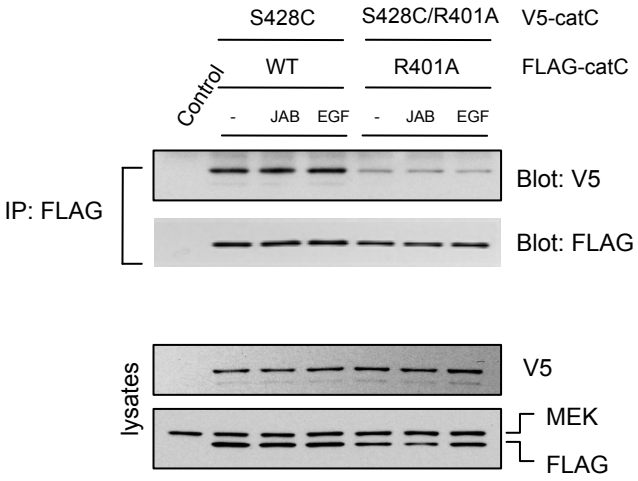
Supplementary Figure 11



Supplementary Figure 12



Supplementary Figure 13



Supplementary Figure 14