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# **Role of plasma membrane localization of the scaffold protein JSAP1 during differentiation of cerebellar granule cell precursors**

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## **Abstract**

We previously reported that the scaffold protein c-Jun NH<sub>2</sub>-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1) functions in cerebellar granule cell precursors (GCPs) to promote their cell-cycle exit and differentiation. In this study, we used immunocytochemistry to examine the subcellular distribution of JSAP1 in proliferating cultured GCPs. We found that when stimulated with fibroblast growth factor-2 (FGF-2), a factor that promotes GCP differentiation through JNK and ERK signaling, JSAP1 translocated to the plasma membrane and colocalized with activated JNK and extracellular signal-regulated kinase (ERK). In transfected cells expressing a constitutively activated FGF receptor (FGFR), JSAP1 and the activated FGFR colocalized at the plasma membrane with not only activated, but also unphosphorylated and inactive JNK and ERK. These colocalizations did not occur when a mutant JSAP1 lacking the JNK-binding domain was substituted for wild-type JSAP1. Biochemical analyses of transfected cells showed that activated FGFR increased JSAP1's affinity for JNK and ERK, and that JSAP1 enhanced FGFR-induced JNK and ERK activation. Collectively, these results suggest that when stimulated by FGFR, JSAP1 translocates to the plasma membrane, where it recruits JNK and ERK and facilitates their activation, leading to the differentiation of cerebellar GCPs.

## **Introduction**

Mammalian mitogen-activated protein kinase (MAPK) intracellular signaling pathways are activated in response to a variety of stimuli and play key roles in multiple cellular processes, including cell proliferation, differentiation, and apoptosis (Kyriakis and Avruch, 2001; Chang and Karin, 2001). The specificity of MAPK signaling is mediated, at least in part, by scaffold proteins, which are thought to promote the efficient activation of specific MAPK pathways by organizing the signaling components into functional modules (Morrison and Davis, 2003; Yoshioka, 2004; Dhanasekaran, et al. 2007). Some extracellular signal-regulated kinase (ERK) MAPK signaling scaffolds, such as KSR (kinase suppressor of Ras) and MP1 (MEK partner 1), have been shown to coordinate the assembly of multiprotein MAPK complexes through subcellular compartmentalization (Müller et al., 2001; Teis et al., 2002). However, it is largely unknown whether the c-Jun NH<sub>2</sub>-terminal kinase (JNK) MAPK signaling scaffolds possess a similar function.

The JNK/stress-activated protein kinase-associated protein 1 (JSAP1) [also known as JNK-interacting protein 3 (JIP3) or Sunday Driver (Syd)] has been identified as a scaffold protein for mammalian JNK signaling modules (Ito et al., 1999, 2000; Kelkar et al., 2000; Bowman et al., 2000). Although JSAP1 also seems to be involved in ERK signaling, its role in the ERK pathway remains fragmentary (Kuboki et al., 2000; Ha et al., 2005). JSAP1 has been reported to assist axonal transport in injured neurons with the aid of the dynein/dynactin motor complex (Cavalli et al., 2005). In addition, several scaffold proteins, including JSAP1, have been shown to interact with kinesin-1 (Verhey et al., 2001). Collectively, these studies suggest that JSAP1 functions dynamically in intracellular signaling pathways, and interacts with many different intracellular signaling proteins. To date, however, it is not known whether JSAP1 changes its subcellular distribution in response to physiological stimuli, or whether JSAP1 regulates MAPK signaling modules in a spatial manner.

In rodents, granule cell precursors (GCPs) proliferate extensively for 2-3 weeks after birth in the external granular layer, where Purkinje-cell-derived sonic hedgehog (Shh) acts as a potent mitogen (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). The proliferating GCPs eventually stop their proliferation, and the postmitotic granule cells migrate toward their final destination, the internal granular layer (Hatten, 1999). We previously reported that JSAP1 antagonizes the mitogenic effect of Shh on GCPs, and promotes their exit from the cell cycle and their differentiation, by modulating JNK activity (Sato et al., 2008). Fogarty et al. (2007) showed that the fibroblast growth factor-2 (FGF-2) activity, which is mediated by FGF receptor (FGFR)-JNK/ERK signaling, exerts a similar effect on GCPs. In the present study, we found that the subcellular distribution of JSAP1 scaffold protein in proliferating cultured GCPs changes in response to the GCP differentiation-promoting factor FGF-2, and investigated the role of the JSAP1 translocation. Our results suggest that when stimulated by FGF-2/FGFR signaling, JSAP1 regulates the localization of JNK and ERK signaling pathway components, leading to the differentiation of cerebellar GCPs.

## Results

### **FGF-2 stimulation induces JSAP1 to localize to the plasma membrane in proliferating GCPs**

To gain insight into the mechanism by which JSAP1 regulates cellular processes to switch from the cell-cycle exit to the differentiation of cerebellar GCPs, we first examined JSAP1's subcellular distribution, focusing on proliferating GCPs. We used cultured GCPs purified from the cerebellum of postnatal day 4 (P4) mice. First, we characterized the expression of Ki67 (a proliferation marker) and NeuN (a neural differentiation marker) in the primary culture system 24 h and 72 h after plating, in the presence or absence of FGF-2 (Fig. 1). In the absence of FGF-2, the proportion of Ki67-positive cells decreased over time (41% and 25% in cells cultured for 24 h and 72 h, respectively), but the percentage of cells expressing NeuN increased (51% and 71% in cells cultured for 24 h and 72 h, respectively). Incubation with FGF-2 increased GCP differentiation significantly at 24 h (62% NeuN-positive cells) and 72 h (87%), and the proportion of Ki67-positive cells observed was lower (31% at 24 h and 9% at 72 h). These results are essentially consistent with those reported previously (Miyazawa et al., 2000; Fogarty et al., 2007; Sato et al., 2008).

We next cultured GCPs with or without FGF-2 for 5 min, 10 min, 20 min, and 30 min, double-stained the GCPs with antibodies (Abs) against JSAP1 and Ki67, and used confocal microscopy to examine the subcellular distribution of JSAP1 in the Ki67-positive cells (Fig. 2A,B). In most of the vehicle-treated proliferating GCPs, JSAP1 protein was distributed exclusively in the cytoplasm. In contrast, JSAP1 immunosignals were observed at the plasma membrane in a significantly increased proportion of the proliferating GCPs treated with FGF-2 [41% (5 min), 56% (10 min), 41% (20 min) and 45% (30 min)]. These results suggest that JSAP1 translocates to the plasma membrane of Ki67-positive proliferating GCPs in response to FGF-2.

As FGF-2 is known to activate JNK and ERK in GCPs (Fogarty et al., 2007; Fig. S1 in

Supporting Information), we then asked whether phosphorylated and activated JNK (p-JNK) and ERK (p-ERK) also localize to the plasma membrane in proliferating GCPs, and if so, whether p-JNK and/or p-ERK colocalize with the membrane-localized JSAP1 (Fig. 2C,D). GCPs were cultured with or without FGF-2 for 20 min, triple-stained with Abs against JSAP1, Ki67, and p-JNK or p-ERK, and analyzed as described above. While p-JNK and p-ERK did not localize to the plasma membrane in vehicle-treated Ki67-positive GCPs, among FGF-2-treated GCPs, p-JNK and p-ERK colocalized with JSAP1 at the plasma membrane in all of the Ki67-positive GCPs examined.

We also examined whether JSAP1 protein could be phosphorylated at a tyrosine residue when stimulated with FGF-2. To this end, we prepared cell lysates from cultured GCPs after treatment with FGF-2 for 0 min, 10 min, 20 min, and 30 min, immunoprecipitated the samples with an anti-JSAP1 Ab, and then analyzed the precipitates by immunoblotting with an anti-phosphotyrosine-specific Ab (Fig. 3). FGF-2 treatment induced the rapid and marked tyrosine phosphorylation of JSAP1, with the highest level occurring with the 10-min treatment; the signal was attenuated to a level slightly higher than the basal level at 30 min. These results indicated that JSAP1 was phosphorylated on tyrosine following stimulation with FGF-2.

### **JSAP1 colocalizes with myristoylated, activated FGFR1 at the plasma membrane**

We next examined whether the translocation of JSAP1 to the plasma membrane depends on FGFR signaling activation. We transiently coexpressed wild-type JSAP1 or a mutant lacking the JNK-binding domain [JSAP1( $\Delta$ JBD)], along with a constitutively active FGFR1 in which a myristoylation motif was substituted for the extracellular and transmembrane domains of FGFR1 [Myr-FGFR(wt)], thereby targeting the derivative to the plasma membrane (Aronheim et al., 1994; Hart et al., 2000). Both HA-tagged wild-type JSAP1 [HA-JSAP1(wt)] and HA-JSAP1( $\Delta$ JBD) colocalized with the Myc-tagged Myr-FGFR(wt) [Myr-Myc-FGFR(wt)] at the plasma membrane in all of the transfected human embryonic

kidney (HEK) 293T cells examined (Fig. 4). However, when HA-JSAP1(wt) or HA-JSAP1( $\Delta$ JBD) was coexpressed with a kinase-dead version of Myr-Myc-FGFR(wt) [Myr-Myc-FGFR(KD)] in HEK 293T cells, HA-JSAP1 and Myr-Myc-FGFR(KD) did not colocalize at the plasma membrane. Taken together, these results suggest that FGFR signaling is required for JSAP1 to translocate to the plasma membrane, but that JSAP1's interaction with JNK is not required.

We also confirmed that FGFR signaling activation induces the tyrosine phosphorylation of JSAP1 in transfected HEK 293T cells, as was observed in cerebellar GCPs (Fig. 3). We transiently coexpressed HA-JSAP1(wt) with either Myr-Myc-FGFR(wt) or Myr-Myc-FGFR(KD) in HEK 293T cells, immunoprecipitated with an anti-HA Ab, and analyzed the precipitates by immunoblotting with an anti-phosphotyrosine-specific Ab (Fig. S2 in Supplementary Information). Tyrosine phosphorylation of both HA-JSAP1(wt) and HA-JSAP1( $\Delta$ JBD) was observed in cells expressing Myr-Myc-FGFR(wt). In contrast, when Myr-Myc-FGFR(KD) was used instead of Myr-Myc-FGFR(wt), no tyrosine phosphorylation of HA-JSAP1(wt) or HA-JSAP1( $\Delta$ JBD) was detected. These results indicate that JSAP1 can be phosphorylated on a tyrosine residue, and that the phosphorylation is induced by the activation of FGFR signaling.

### **JSAP1 colocalizes with JNK and ERK at the plasma membrane**

To explore the role of JSAP1's translocation to the plasma membrane, we transiently coexpressed HA-JSAP1(wt or  $\Delta$ JBD), Myr-Myc-FGFR(wt), and either Flag-tagged wild-type JNK [Flag-JNK(wt)] or ERK [Flag-ERK(wt)] in HEK 293T cells, and then performed immunocytochemistry with Abs against the HA, Flag, and Myc epitopes. HA-JSAP1(wt), Flag-JNK(wt) or Flag-ERK(wt), and Myr-Myc-FGFR(wt) colocalized at the plasma membrane in almost all of the transfected cells examined; however, these triple colocalizations did not occur when HA-JSAP1( $\Delta$ JBD) was expressed instead of HA-JSAP1(wt) (Fig. 5A). We also analyzed the transfected cells using a different Ab set, in

which we used anti-p-JNK and -p-ERK Abs instead of the anti-Flag Ab, and obtained essentially the same results (Fig. S3 in Supporting Information). These results suggested that JNK and ERK translocate to the plasma membrane through an interaction with the JBD of JSAP1 following FGFR signaling activation. However, it was unclear whether JNK and ERK translocate to the plasma membrane before or after being activated. To address this, we used mutants of JNK and ERK, in which the Thr and Tyr residues of the TXY motif in their activation loops were mutated to Ala and Phe residues, respectively [JNK(APF) and ERK(AEF), respectively]. We transiently coexpressed HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt) with either Flag-JNK(APF) or -ERK(AEF) in HEK 293T cells, and analyzed as described above. Again, triple colocalizations of the HA-, Flag- and Myc-tagged proteins were observed in almost all of the transfected cells examined when HA-JSAP1(wt), but not HA-JSAP1( $\Delta$ JBD), was coexpressed with Myr-Myc-FGFR(wt) and Flag-JNK(APF) or Flag-ERK(AEF) (Fig. 5B). These results indicate that JNK and ERK can translocate to the plasma membrane prior to their activation, through interaction with JSAP1.

### **JSAP1 increases its affinity to JNK and ERK and enhances JNK and ERK activation**

To examine whether FGFR signaling activation affects the interaction of JSAP1 with JNK or ERK, we transiently coexpressed either Flag-JNK(wt) or Flag-ERK(wt) with or without HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt or KD) in HEK 293T cells. Cell lysates were immunoprecipitated using an anti-HA Ab, and subjected to immunoblotting with an anti-Flag Ab (Fig. 6). The interaction between HA-JSAP1(wt) and Flag-JNK(wt) was substantially enhanced by the expression of Myr-Myc-FGFR(wt), but not Myr-Myc-FGFR(KD) (Fig. 6A, compare lanes 2 and 3). In addition, when HA-JSAP1( $\Delta$ JBD) was used instead of HA-JSAP1(wt), no interaction was detected under our conditions (Fig. 6A, lanes 6-8). Concerning ERK, an interaction between JSAP1 and ERK was observed only in cells coexpressing HA-JSAP1(wt), Myr-Myc-FGFR(wt), and

Flag-ERK(wt), but not with other combinations (Fig. 6B).

We next examined whether JSAP1 enhanced the FGFR-induced JNK and/or ERK activation. We transiently coexpressed either Flag-JNK(wt) (Fig. 7A) or Flag-ERK(wt) (Fig. 7B) with or without HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt or KD) in HEK 293T cells, and analyzed by immunoblotting with an anti-phospho-JNK or -ERK Ab after immunoprecipitation with an anti-Flag Ab. HA-JSAP1(wt), but not HA-JSAP1( $\Delta$ JBD), enhanced the activation of Flag-JNK(wt) and -ERK(wt) in cells expressing Myr-Myc-FGFR(wt) (Fig. 7A,B, lanes 2-4). The fold increases for JNK and ERK activation in the presence of JSAP1, compared to those in its absence, were 1.97 and 2.74, respectively. Taken together, these results suggest that when stimulated by FGFR signaling, JSAP1 increases its binding affinity to JNK and ERK, and enhances the activation of JNK and ERK.

## Discussion

We previously reported that JSAP1 signaling pathways regulate cellular programs to switch from proliferation to differentiation in cerebellar GCPs (Sato et al., 2008). In the present study, we found that the scaffold protein JSAP1 changes its subcellular distribution in response to GCP differentiation-promoting signals, and investigated the role of this translocation. We propose that when stimulated by FGFRs, JSAP1 translocates to the plasma membrane where it recruits JNK and ERK and facilitates their activation, leading to the differentiation of cerebellar GCPs. The following lines of evidence support this proposal. In cultured cerebellar GCPs, JSAP1 translocates to the plasma membrane and colocalizes with activated JNK and ERK when stimulated with FGF-2, a GCP differentiation-promoting factor whose activity is mediated by FGFR-JNK/ERK signaling. In addition, in transfected cells expressing a constitutively activated FGFR with a myristoylation signal, JSAP1 and the activated FGFR colocalize at the plasma membrane with not only activated JNK and ERK, but also their unphosphorylated and inactive forms. These triple colocalizations do not occur when a mutant JSAP1, JSAP1( $\Delta$ JBD), is expressed instead of the wild-type JSAP1. Finally, activated FGFR, but not a kinase-dead FGFR, increases the affinity of JSAP1 for JNK and ERK, and JSAP1 enhances the FGFR-induced JNK and ERK activation.

FGFRs, cognate receptors for FGF-2, belong to a receptor tyrosine kinase family. Here we examined cerebellar GCPs stimulated with FGF-2, and found that tyrosine phosphorylation of JSAP1 was induced rapidly (Fig. 3). It is currently unclear whether JSAP1 is directly or indirectly phosphorylated by FGFRs, or whether JSAP1 phosphorylation affects the JNK and ERK signaling pathways. However, the phosphorylation of JSAP1 at tyrosine residue(s) may play a crucial role in the scaffolding activity of JSAP1 in FGFR-JNK/ERK signaling pathways, given that an increased affinity of JSAP1 for JNK and ERK (Fig. 6), JSAP1 tyrosine phosphorylation (Fig. S2 in

Supplementary Information), and JSAP1-enhanced JNK and ERK activation (Fig. 7) were all observed in cells expressing activated FGFR, but not in cells expressing a kinase-dead FGFR.

On the other hand, considering the following observations, it is also possible that the tyrosine phosphorylation of JSAP1 induced by FGF-2 is important for its translocation to the plasma membrane, but not for its scaffolding activity. JSAP1's phosphorylation in GCPs was transient, peaked at 10 min, and returned nearly to baseline by 30 min after FGF-2 stimulation in GCPs (Fig. 3). In contrast, in proliferating cerebellar GCPs, both the plasma membrane localization of JSAP1, and the activation of JNK and ERK by FGF-2 (the latter of which has been reported to be crucial for FGF-mediated inhibition of Shh responses; Fogarty et al., 2007), were sustained for at least 30 min after stimulation with FGF-2 (Fig. 2B and Fig. S1 in Supporting Information). JSAP1's location at the plasma membrane, therefore, may be key for the exertion of its scaffolding activity, and the sustained activation of JNK and ERK may be required for the differentiation of cerebellar GCPs. Further research, including the identification of the phosphorylated tyrosine residue(s) in JSAP1, will be necessary to clarify this issue.

Here we found that JSAP1 colocalized with activated JNK and ERK at the plasma membrane in both FGF-2-stimulated GCPs and transiently transfected cells expressing a constitutively activated FGFR (Fig 2B and Fig. S3 in Supporting Information). In addition, the colocalization of JSAP1 with unphosphorylated and inactive JNK and ERK, as well as with pan-JNK and -ERK, was observed in similar transfection assays (Fig. 5). However, the colocalization profiles at the plasma membrane differed between the GCPs and transfected cells. In the GCPs, the co-localized proteins seemed to be restricted to certain areas, but in the transfected cells, the co-localized proteins were distributed almost uniformly at the plasma membrane. It is likely that the overexpressed constitutively activated FGFR in the transfected cells was distributed differently from the endogenous FGFRs in the GCPs, which resulted in the different distribution profiles of the colocalized proteins.

To date, the role of JSAP1 in ERK signaling remains fragmentary. Coimmunoprecipitation studies have shown that JSAP1 interacts selectively with JNK, but not ERK, among mammalian MAPKs (Ito et al., 1999; Kelkar et al., 2000). In addition, based on the results of cotransfection assays, it has been suggested that JSAP1 inhibits ERK pathways by sequestering Raf-1 and MEK (MAPK and ERK kinase) from ERK signaling modules (Kuboki et al., 2000). On the other hand, biochemical studies of *Jsap1*-deficient mice have suggested a positive role of JSAP1 in ERK signaling (Ha et al., 2005). Here we found that JSAP1 can bind ERK in cells expressing activated FGFR (Fig. 6B), and that JSAP1 enhances the ERK activation induced by activated FGFR (Fig. 7B). In our previously reported cotransfection assays (Kuboki et al., 2000), JSAP1 was unable to exert scaffolding activity, probably because it was unable to bind ERK under the experimental conditions; conversely, JSAP1 might acquire a scaffolding role in ERK modules if it gains the capacity to interact with ERK. Indeed, we observed that JSAP1 interacted with ERK in cells expressing activated FGFR, but not in cells expressing a kinase-dead FGFR (Fig. 6B). Thus, it seems likely that JSAP1 assembles ERK signaling components into functional modules following FGFR activation.

In summary, this study shows for the first time that the scaffold protein JSAP1 regulates the localization of components in the JNK and ERK signaling pathways. Our findings suggest that when stimulated by FGFRs, JSAP1 can convert unscaffolded kinases to scaffolded kinases through its translocation to the cell plasma membrane, and promote activation of the relevant signaling cascades. Furthermore, this study implies that JSAP1 functions as a scaffolding factor to modulate the parallel but distinct JNK and ERK signaling pathways elicited by FGFR stimulation during the differentiation of cerebellar GCPs.

## **Experimental procedures**

### **Animals**

All of the experiments involving animals were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan. C57BL/6J mice were purchased from SLC (Hamamatsu, Japan).

### **Cell cultures and transfection**

HEK 293T cells were cultured as described previously (Bayarsaikhan et al., 2007). Transient transfection into HEK 293T cells was performed using FuGene6 (Roche, Basel, Switzerland), according to the manufacturer's instructions. Cerebellar GCPs were prepared from P4 mice, purified, and cultured as described previously (Sato et al., 2008). The purified GCPs were plated onto eight-well Lab-Tec chamber slides (8 x 10<sup>5</sup> cells/well; Permaxo slide; Nunc Inc., Naperville, IL, USA) coated with 50 µg/ml poly-L-lysine (Sigma, St. Louis, MO, USA). For some experiments, FGF-2 (25 ng/ml; Sigma) dissolved in phosphate-buffered saline (PBS) was added to the culture medium 1 h after plating.

### **Plasmids**

The mammalian expression plasmids, pCL20c CMV-HA-JSAP1(wt), pCL20c CMV-HA-JSAP1(ΔJBD), and pFlag-CMV2-JNK3(wt) were described previously (Ito et al., 1999; Sato et al., 2008). To generate the expression plasmid for Flag-tagged wild-type ERK, the entire coding region of mouse ERK2 (RefSeq accession no. NM\_011949) was amplified by polymerase chain reaction (PCR). The product, which contained a *NotI* site at the 5' end and a stop codon followed by a *BamHI* site at the 3' end of the sense strand, was digested with *NotI* and *BamHI*, and subcloned into *NotI/BamHI*-digested pFlag-CMV2 (Codak,

Rochester, NY, USA) to generate pFlag-CMV2-ERK2(wt). Expression plasmids for Flag-tagged kinase-dead JNK3 and ERK2, pFlag-CMV2-JNK3(APF) and -ERK2(AEF), were constructed by replacing Thr183 and Tyr185 (both JNK3 and ERK2) with alanine and phenylalanine, respectively. To introduce the nucleotide substitutions into the cDNAs, site-directed mutagenesis was carried out by overlapping PCR as described previously (Ito et al., 1999) using the following primers: *Jnk3*, sense strand, 5'-ACAAGCTTCATGATGGCTCCGTTCGTGGTGACGCGATAT-3'; *Erk2*, sense strand, 5'-CACACAGGGTTCTTGGCAGAGTTCGTAGCCACACGTTGG-3'. The underlined letters represent the mutated nucleotides. Annealed oligonucleotides encoding the myristoylation signal of c-Src (GSSKSKPKDPSQR, one-letter code for amino acids) were inserted into the *EcoRV* site of pcDNA3 (Invitrogen, Carlsbad, CA, USA) to generate pcDNA3-Myr: forward, 5'-GCCACCATGGGCAGCTCCAAGAGCAAGCCCAAGGACCCCAGCCAGCGCGAT-3'; reverse, 5'-ATCGCGCTGGCTGGGGTCCTTGGGCTTGCTCTTGGAGCTGCCCATGGTGGC-3'. Annealed oligonucleotides encoding the Myc epitope (EQKLISEEDL, one-letter code for amino acids) were inserted into the *EcoRV* site of pcDNA3-Myr to generate pcDNA3-Myr-Myc: forward, 5'-GAGCAGAAGCTGATCAGCGAGGAGGACCTGGATT-3'; reverse, 5'-ATCCAGGTCCTCCTCGCTGATCAGCTTCTGCTC-3'. The region encoding amino acid residues 398-822 of mouse FGFR1 (RefSeq accession no. NM\_010206) was amplified by PCR. The product, which contains an *EcoRV* site at the 5' end and a stop codon followed by an *SalI* site at the 3' end of the sense strand, was digested with *EcoRV* and *SalI*, and subcloned into *EcoRV/XhoI*-digested pcDNA3-Myr-Myc to generate pcDNA3-Myr-Myc-FGFR1(wt). The expression plasmid for a kinase-dead version of Myr-Myc-FGFR(wt), pcDNA3-Myr-Myc-FGFR1(KD), was constructed by replacing Lys514 with arginine. The corresponding nucleotide substitutions were introduced by overlapping PCR using the following primers: *Fgfr1*, sense strand, 5'-

GACCAAAGTGGCCGTGAGAATGTTGAAGTCCGACG-3'. The underlined letters represent the mutated nucleotides.

Nucleotide sequences of the expression plasmids were confirmed by DNA sequencing.

### **Immunocytochemistry**

HEK 293T cells were plated onto plastic cover slips (Sumitomo Bakelite, Tokyo, Japan) in 24-well plates at a density of  $1 \times 10^4$  cells per well. The cells were transfected with 20 ng pcDNA3-Myr-Myc-FGFR(wt or KD) and 40 ng pCL20c CMV-HA-JSAP1(wt or  $\Delta$ JBD) with or without 40 ng of either pFlag-CMV2-JNK3(wt or APF) or pFlag-CMV2-ERK2(wt or AEF), as indicated in the figures. pCMV $\beta$  (Clontech, Palo Alto, CA, USA) was used to normalize the total quantity of transfected DNA (200 ng/transfection). Twenty-four hours after the transfection of HEK 293T cells or after the treatment of cultured cerebellar GCPs with FGF-2 or vehicle (i.e., PBS) for the times indicated in the figures and/or figure legends, cells were fixed with 4% paraformaldehyde in Tris-buffered saline (TBS) pH 7.4 for 30 min at room temperature. Fluorescence immunocytochemistry was performed by standard protocols using blocking solution (2% BSA, 2% goat serum, 0.4% Triton X-100 in TBS), primary Abs, and secondary Abs.

Primary Abs were as follows: rat monoclonal anti-Ki67 (1:100; Dako, Glostrup, Denmark), mouse monoclonal anti-NeuN (1:300; Chemicon, Temecula, CA, USA), rabbit polyclonal anti-JSAP1 (1  $\mu$ g/ml; Miura et al., 2006), mouse monoclonal anti-phospho-JNK and anti-phospho-ERK (both diluted to 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Flag M2 and rabbit polyclonal anti-Myc (both diluted to 1:1000; Sigma), and rat polyclonal anti-HA (1:1000; Roche) Abs. Secondary Abs were goat Alexa fluor 488-conjugated anti-rat or rabbit IgG, goat Alexa fluor 568-conjugated anti-mouse or rat IgG, and goat Alexa fluor 647-conjugated anti-rat or rabbit IgG Abs (each diluted to 1:1000; Invitrogen). Nuclei were stained with 2  $\mu$ g/ml Hoechst 33258 (Invitrogen).

Images were captured with a confocal laser-scanning microscope (LSM510; Zeiss, Oberkochen, Germany).

### **Immunoblotting and immunoprecipitation**

HEK 293T cells were plated in 35-mm dishes at a density of  $2 \times 10^5$  cells per dish. The cells were transfected with various combinations of pcDNA3-Myr-Myc-FGFR(wt or KD), pCL20c CMV-HA-JSAP1(wt or  $\Delta$ JBD), pFlag-CMV2-JNK3(wt or APF), and pFlag-CMV2-ERK2(wt or AEF) as indicated in the figures, in which the amount of these vectors used for each transfection was 100 ng, 200 ng, 200 ng, and 200 ng, respectively. pCMV $\beta$  (Clontech) was used to normalize the total quantity of transfected DNA (1,000 ng/transfection). Twenty-four hours after the transfection of HEK 293T cells or after the treatment of cultured cerebellar GCPs with FGF-2 for the times indicated in the figures, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.1% sodium deoxycholate) containing 25 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and Protease Inhibitor Cocktail (Sigma). Immunoblotting was carried out as described previously (Sato et al., 2004), using mouse monoclonal anti-phospho-tyrosine (1:1000; Millipore, Bedford, MA, USA), mouse monoclonal horseradish peroxidase (HRP)-conjugated anti-Flag M2 and rabbit polyclonal anti-Myc (both diluted 1:5000; Sigma), rat polyclonal anti-HA (1:5000; Roche), rabbit polyclonal anti-JSAP1 (0.33  $\mu$ g/ml; Miura et al., 2006), rabbit polyclonal anti-JNK, anti-ERK, anti-phospho-JNK and anti-phospho-ERK (each diluted 1:1000; Cell Signaling Technology, Beverly, MA, USA) Abs. Secondary Abs were sheep anti-mouse, donkey anti-rabbit (both from GE Healthcare, Buckinghamshire, UK), and goat anti-rat (Santa Cruz) HRP-conjugated IgG Abs (each diluted 1:5000). Protein bands were visualized by the Immobilon Western Chemiluminescence system (Millipore), and were quantified using ImageJ software.

To examine protein phosphorylation, cell lysates prepared using RIPA buffer were

immunoprecipitated with rabbit polyclonal anti-JSAP1 (1  $\mu$ g/ml; Miura et al., 2006), rat polyclonal anti-HA (1:500; Roche), or mouse monoclonal anti-Flag M2 (1:1000; Sigma) Abs bound to protein G-Sepharose beads (GE Healthcare), and then subjected to immunoblotting. To analyze protein-protein interactions, transfected HEK 293T cells were lysed in Lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Triton X-100) containing 25 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and Protease Inhibitor Cocktail. The cell lysates were immunoprecipitated with rat polyclonal anti-HA (1:500; Roche) Ab bound to protein G-Sepharose beads, and subjected to immunoblotting.

### **Statistical analysis**

Results are represented as the mean  $\pm$  SEM from the number of experiments indicated in the figure legends. Statistical differences were analyzed using the two-tailed unpaired Student's *t*-test. Values of  $P < 0.05$  were considered to be statistically significant.

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## Figure legends

**Figure 1.** Expression of Ki67 and NeuN in cultured cerebellar GCPs. GCPs prepared from P4 mice were cultured with vehicle or FGF-2 for 24 h or 72 h, fixed, and stained with Abs against Ki67 and NeuN. Percentages of Ki67 and NeuN-expressing cells are shown (means  $\pm$  SEM from 3 experiments, \*,  $P < 0.01$ , Student's *t*-test). Over 500 cells were counted per experiment.

**Figure 2.** JSAP1 translocates to the plasma membrane in cultured proliferating cerebellar GCPs in response to FGF-2. **(A)** P4 GCPs were treated with vehicle or FGF-2 for 10 min. The cells were double-stained with Abs to JSAP1 (green) and Ki67 (red) as indicated, and used in confocal analysis. Bright-field images are also shown. FGF-2 induces JSAP1 to translocate from the cytoplasm to the plasma membrane in Ki67-positive GCPs. **(B)** GCPs were treated with vehicle or FGF-2 for the indicated times, and analyzed as in **(A)**. Percentages of plasma membrane-localized JSAP1 in Ki67-positive GCPs are shown (means  $\pm$  SEM from 3 experiments, \*,  $P < 0.001$ , Student's *t*-test). Over 100 cells were examined in each experiment. **(C, D)** GCPs were treated with vehicle or FGF-2 for 20 min. The cells were triple-stained with Abs to JSAP1, Ki67, and p-JNK **(C)** or p-ERK **(D)** as indicated, and used in confocal analysis. FGF-2 induces p-JNK and p-ERK to localize at the plasma membrane in Ki67-positive GCPs, where they colocalize with JSAP1 in all the cells examined. A minimum of 50 cells was examined in each experiment. Scale bars, 5  $\mu$ m in **A**, **C** and **D**.

**Figure 3.** Tyrosine phosphorylation of JSAP1 in cultured cerebellar GCPs when stimulated with FGF-2. P4 GCPs were treated with FGF-2 for the indicated times. The cells were lysed, immunoprecipitated (IP) using anti-JSAP1 Ab, and analyzed by immunoblotting (IB) with anti-p-Tyr Ab. Aliquots of the immunoprecipitates were also analyzed by IB, using

anti-JSAP1 Ab as a control for immunoprecipitation efficiency. JSAP1 expression in total cell lysates is shown (input, bottom panel). The results shown are representative of three independent experiments.

**Figure 4.** JSAP1 colocalizes with myristoylated, activated FGFR at the plasma membrane in HEK 293T cells. HEK 293T cells were transiently cotransfected with expression vectors for HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt or KD) as indicated. The cells were double-stained with anti-Myc (green) and anti-HA (red) Abs, and used in confocal analysis. Both HA-JSAP1(wt) and HA-JSAP1( $\Delta$ JBD) colocalize with Myr-Myc-FGFR(wt), but not with Myr-Myc-FGFR(KD), at the plasma membrane in all the transfected cells examined. A minimum of 100 cells was examined in each experiment. Scale bars, 10  $\mu$ m.

**Figure 5.** JSAP1 colocalizes with JNK and ERK at the plasma membrane in HEK 293T cells expressing activated FGFR. HEK 293T cells were transiently cotransfected with expression vectors for HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt), along with either Flag-JNK(wt) or Flag-ERK(wt) (**A**), or Flag-JNK(APF) or Flag-ERK(AEF) (**B**), as indicated. The cells were triple-stained with anti-HA (green), anti-Flag (red) and anti-Myc (blue) Abs, and used in confocal studies. HA-JSAP1(wt) and Myr-Myc-FGFR(wt) colocalizes with not only Flag-JNK(wt) and Flag-ERK(wt) (**A**), but also their mutants Flag-JNK(APF) and Flag-ERK(AEF) (**B**) at the plasma membrane in almost all the transfected cells examined. These triple colocalizations were absent in cells expressing HA-JSAP1( $\Delta$ JBD) instead of HA-JSAP1(wt). Over 100 cells were examined in each experiment. Scale bars, 10  $\mu$ m.

**Figure 6.** Activated FGFR enhances the affinity of JSAP1 to JNK and ERK in HEK 293T cells. HEK 293T cells were transiently cotransfected with expression vectors for HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt or KD), along with either Flag-JNK(wt)

(**A**) or Flag-ERK(wt) (**B**) as indicated. The cells were lysed, immunoprecipitated (IP) using anti-HA Ab, and analyzed by immunoblotting (IB) with anti-Flag Ab. Aliquots of the immunoprecipitates were also analyzed by IB, using anti-HA Ab as a control for immunoprecipitation efficiency. The expression of HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt or KD) along with either Flag-JNK(wt) (**A**) or Flag-ERK(wt) (**B**) in total cell lysates is shown (input, bottom three panels in **A** and **B**). The results shown are representative of three independent experiments.

**Figure 7.** JSAP1 enhances FGFR-induced JNK and ERK activation. HEK 293T cells were transiently cotransfected with expression vectors for HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt or KD), with either Flag-JNK(wt) (**A**) or Flag-ERK(wt) (**B**), as indicated. The cells were lysed, immunoprecipitated (IP) using anti-Flag Ab, and analyzed by immunoblotting (IB) with anti-p-JNK (**A**) or anti-p-ERK (**B**) Abs. Aliquots of the immunoprecipitates were also analyzed by IB with anti-Flag Ab as a control for immunoprecipitation efficiency. The expression of HA-JSAP1(wt or  $\Delta$ JBD), Myr-Myc-FGFR(wt or KD) and Flag-JNK(wt) (**A**) or Flag-ERK(wt) (**B**) in total cell lysates is shown (input, bottom three panels in **A** and **B**). The results shown are representative of three independent experiments.

## Supporting Information

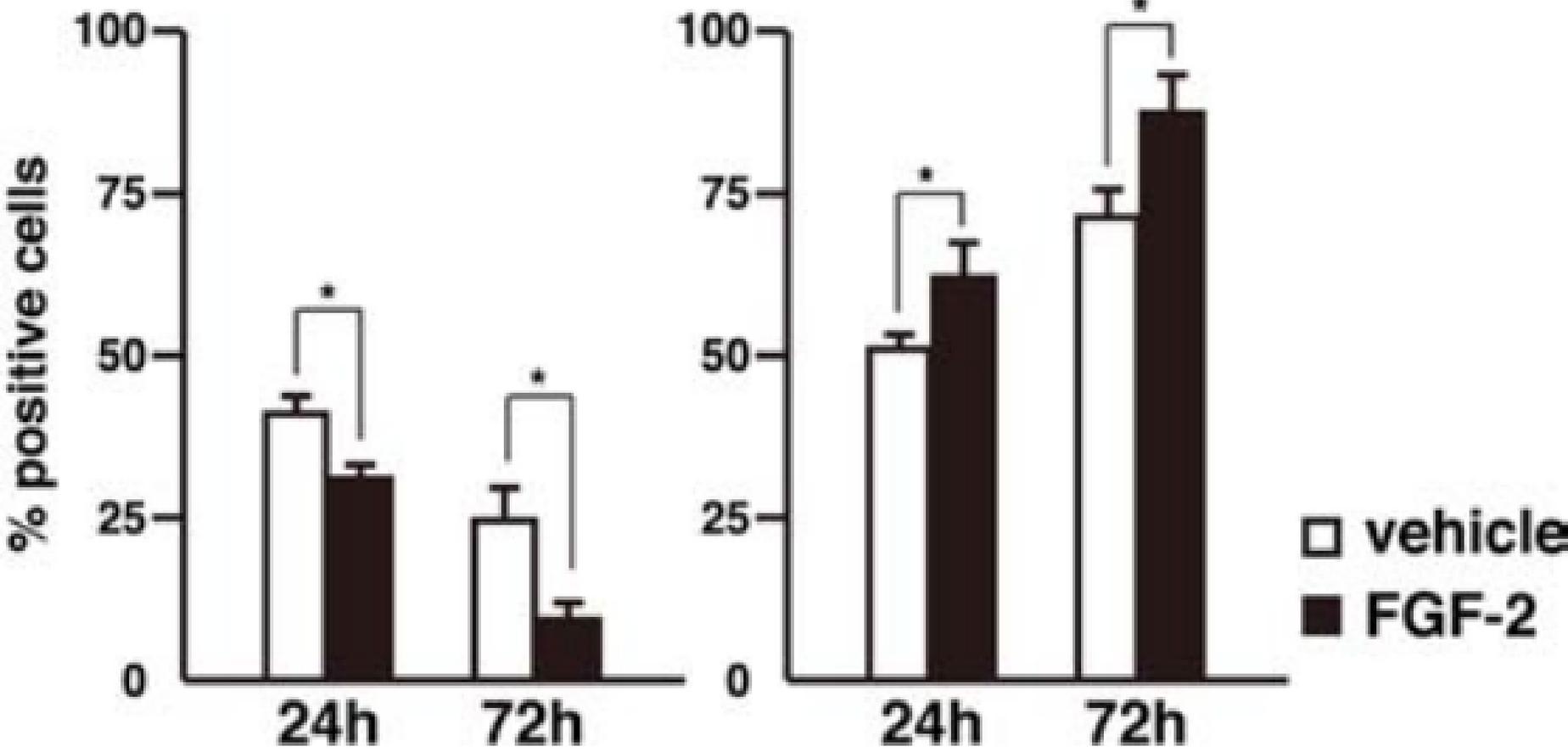
**Figure S1.** FGF-2 activates JNK and ERK in cultured cerebellar GCPs. P4 GCPs were treated with FGF-2 for the indicated times. The cells were lysed and analyzed by immunoblotting (IB) with Ab against p-JNK, JNK, p-ERK and ERK as indicated. The results shown are representative of three independent experiments.

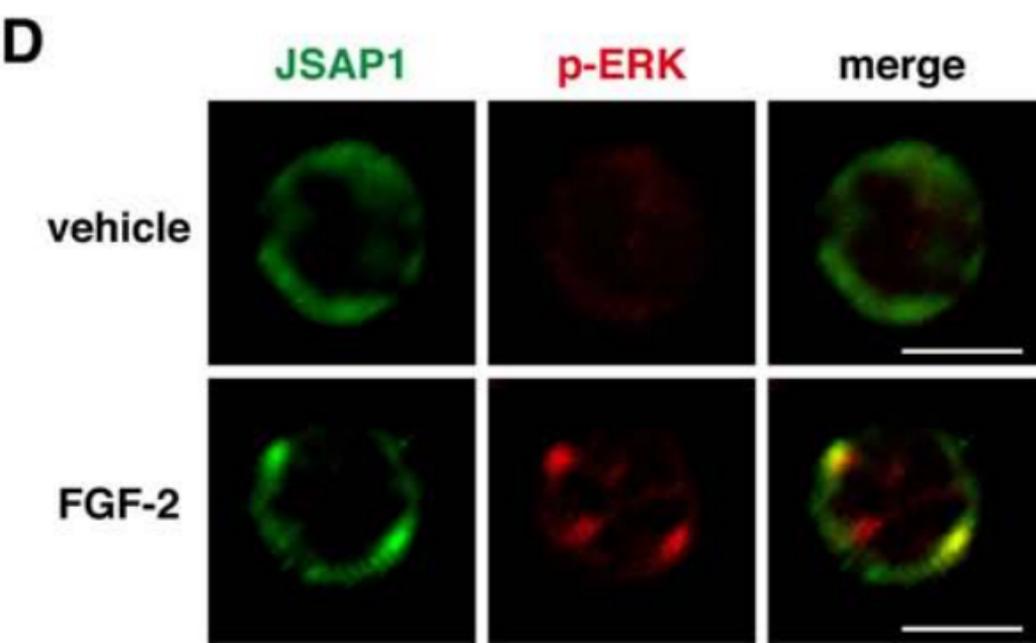
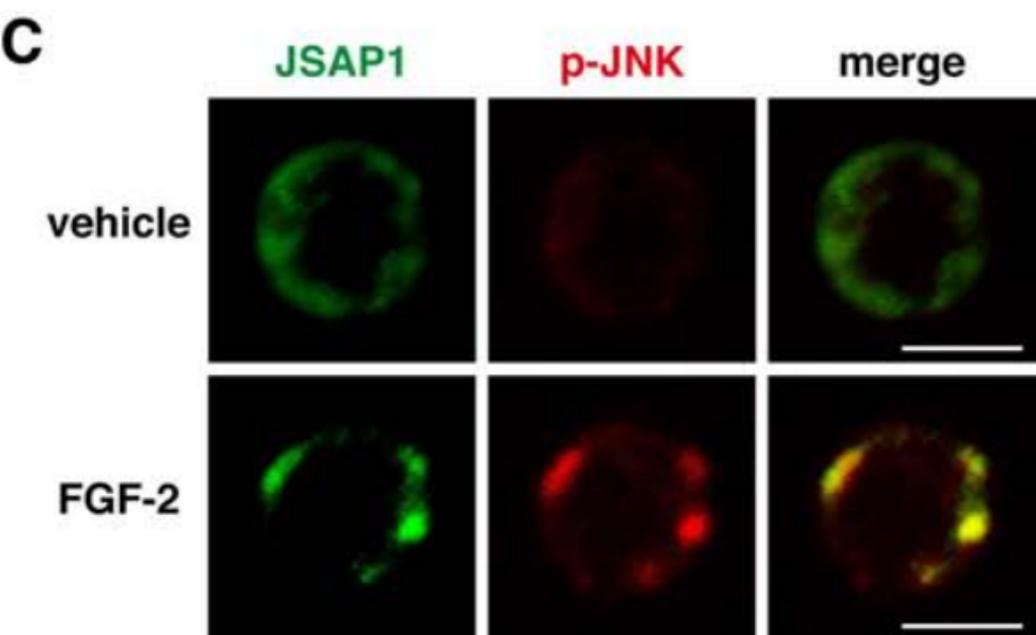
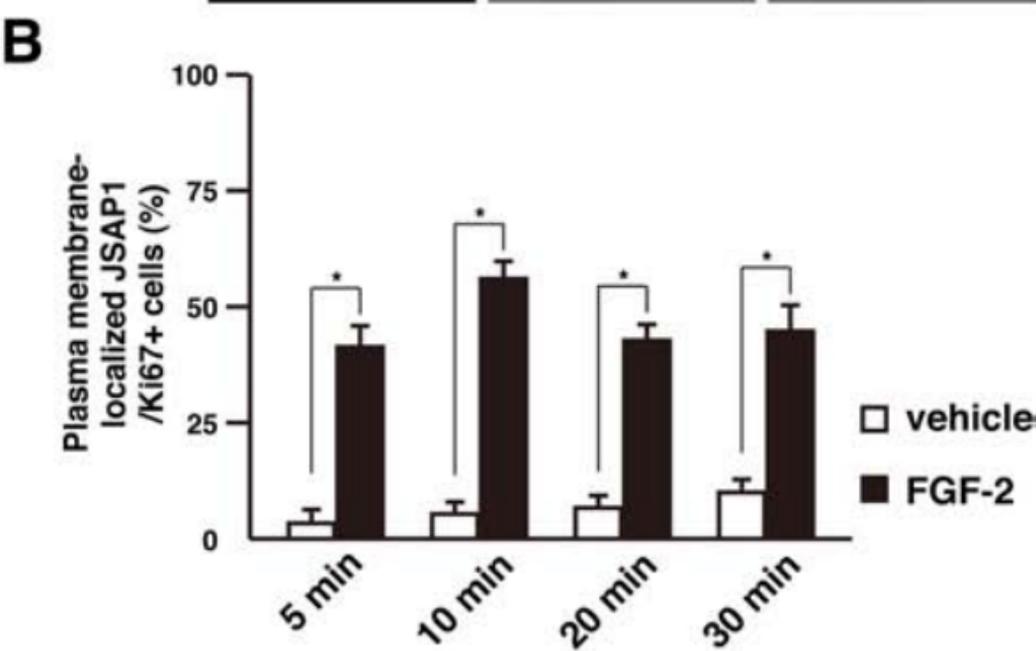
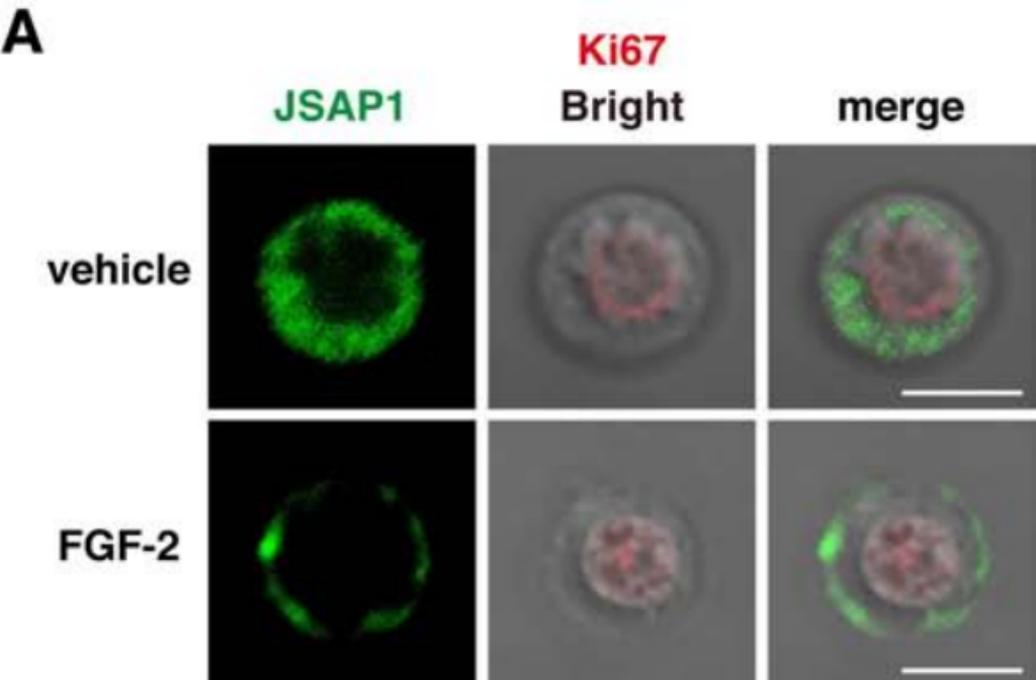
**Figure S2.** JSAP1 tyrosine phosphorylation in HEK 293T cells expressing activated FGFR. HEK 293T cells were transiently cotransfected with expression vectors for HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt or KD), as indicated. The cells were lysed, immunoprecipitated (IP) using anti-HA Ab, and analyzed by immunoblotting (IB) with anti-p-Tyr Ab. Aliquots of the immunoprecipitates were also analyzed by IB, using anti-HA Ab as a control for immunoprecipitation efficiency. HA-JSAP1 and Myr-Myc-FGFR expression in total cell lysates is shown (input, bottom two panels). The results shown are representative of three independent experiments.

**Figure S3.** JSAP1 colocalizes with p-JNK and p-ERK at the plasma membrane in HEK 293T cells expressing the activated FGFR. HEK 293T cells were transiently cotransfected with expression vectors for HA-JSAP1(wt or  $\Delta$ JBD) and Myr-Myc-FGFR(wt) together with Flag-JNK(wt) (**A**) or Flag-ERK(wt) (**B**), as indicated. The cells were triple-stained with anti-HA (green), anti-Myc (blue) and anti-pJNK or anti-p-ERK (red) Abs, and used in confocal studies. HA-JSAP1(wt) and Myr-Myc-FGFR(wt) colocalizes with p-JNK and p-ERK at the plasma membrane in all the transfected cells examined. This triple colocalization does not occur in cells expressing HA-JSAP1( $\Delta$ JBD) instead of HA-JSAP1(wt). Over 100 cells were examined in each experiment. Scale bars, 10  $\mu$ m.

# Ki67

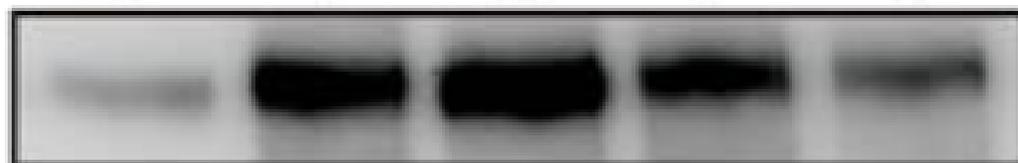
# NeuN





**FGF-2**

0 min    5 min    10 min    20 min    30 min

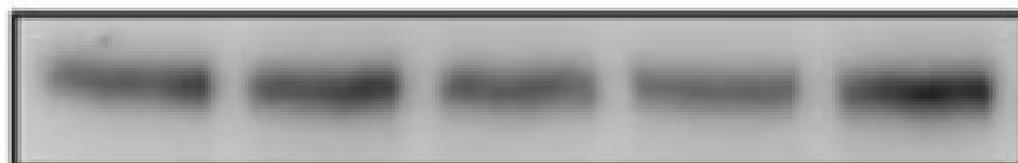


IP:  $\alpha$ -JSAP1  
IB:  $\alpha$ -p-Tyr



IP:  $\alpha$ -JSAP1  
IB:  $\alpha$ -JSAP1

input



IB:  $\alpha$ -JSAP1

1    2    3    4    5

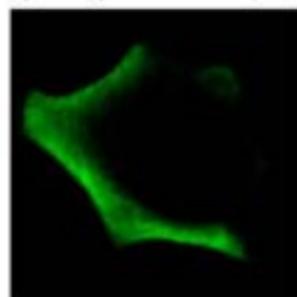
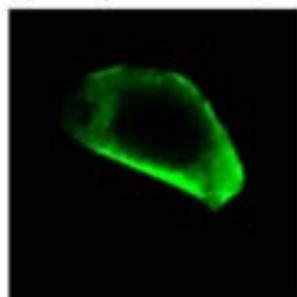
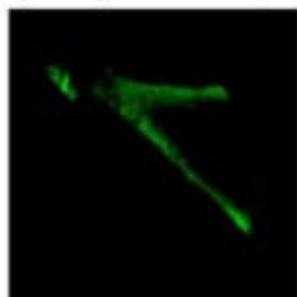
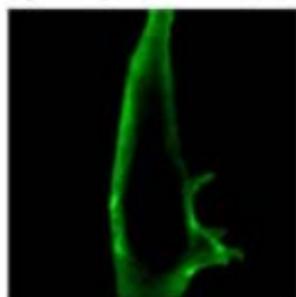
HA-JSAP1(wt)

HA-JSAP1( $\Delta$ JBD)

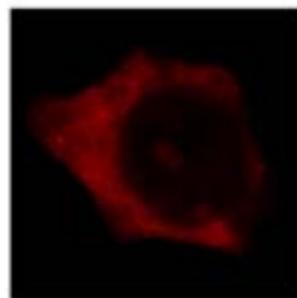
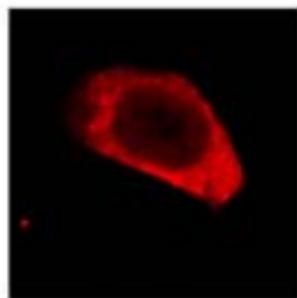
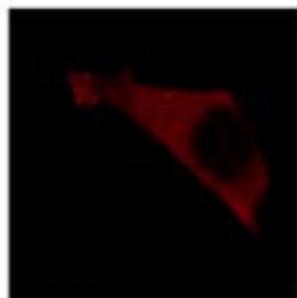
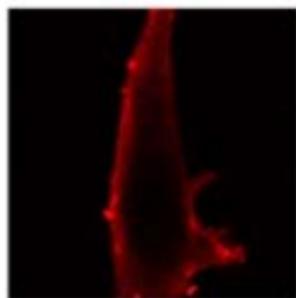
Myr-Myc-FGFR(wt) Myr-Myc-FGFR(KD)

Myr-Myc-FGFR(wt) Myr-Myc-FGFR(KD)

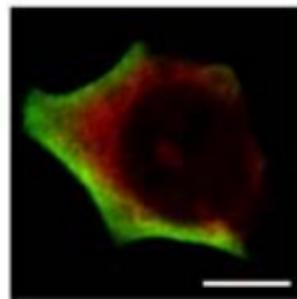
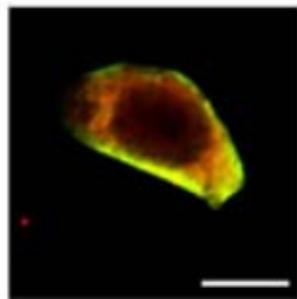
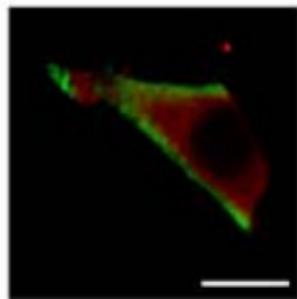
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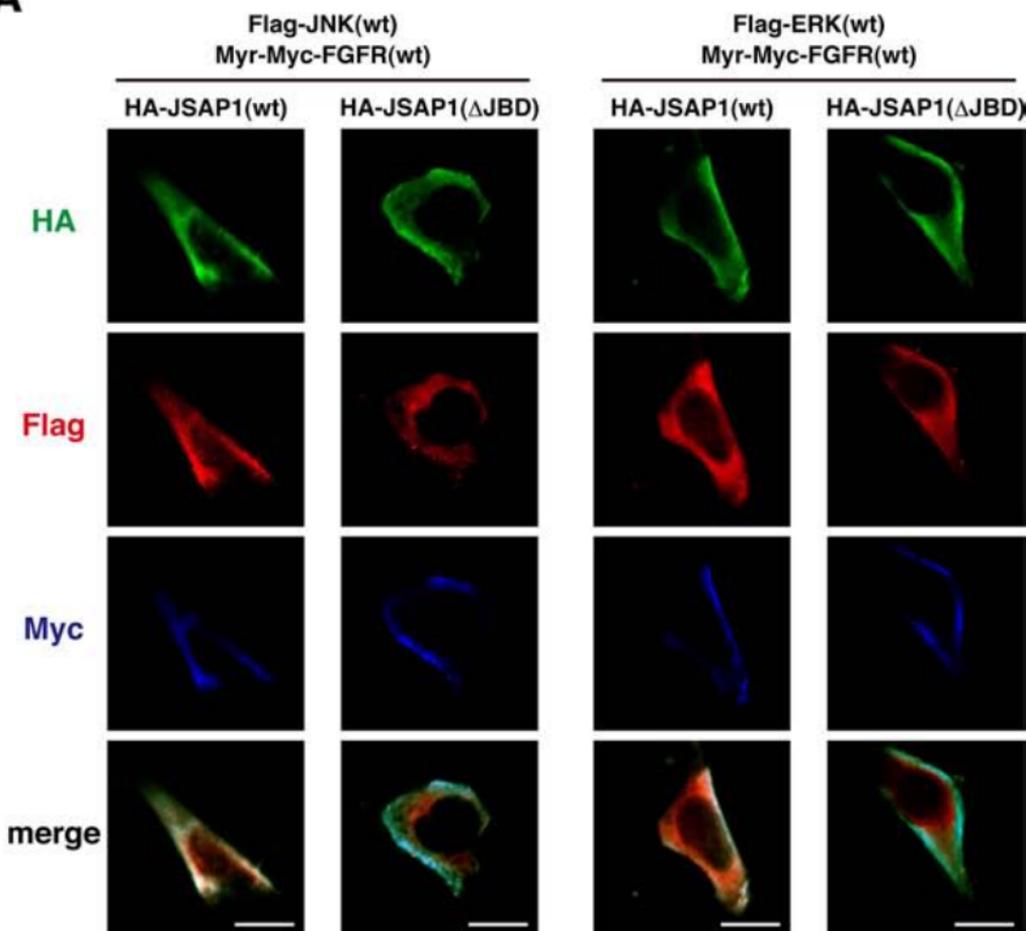
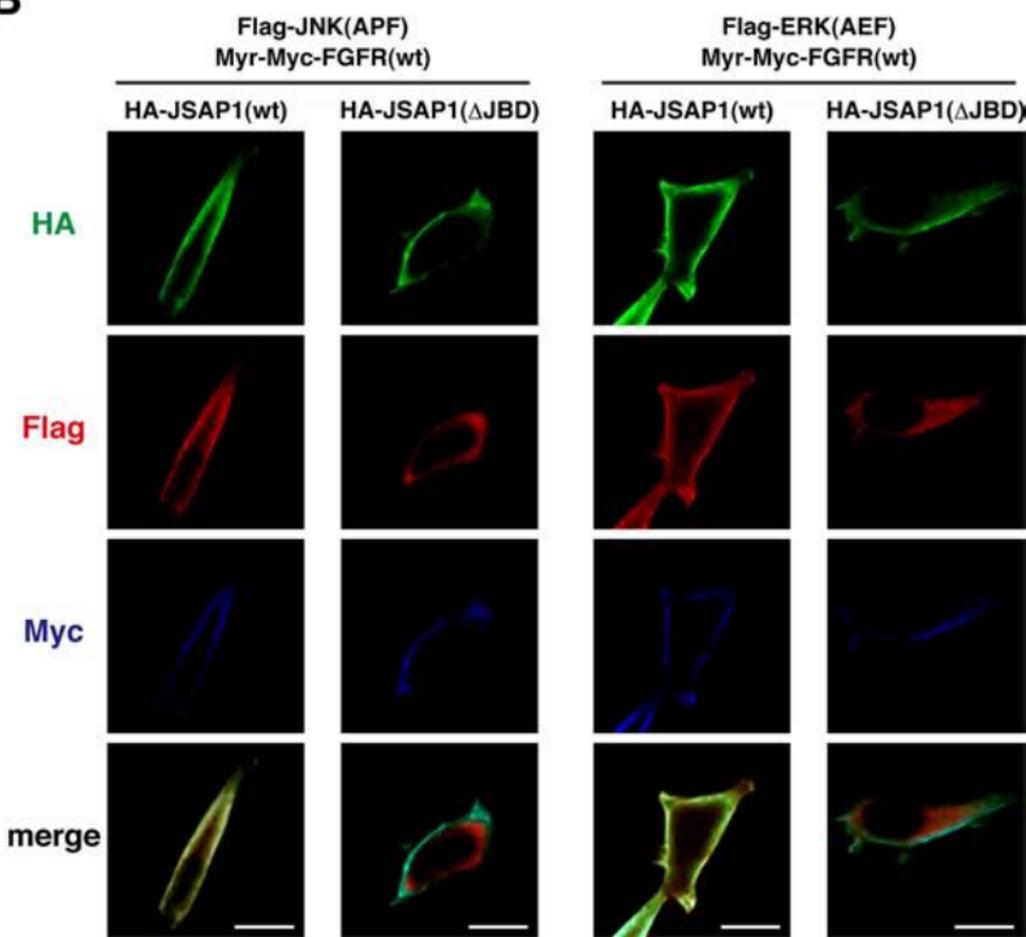


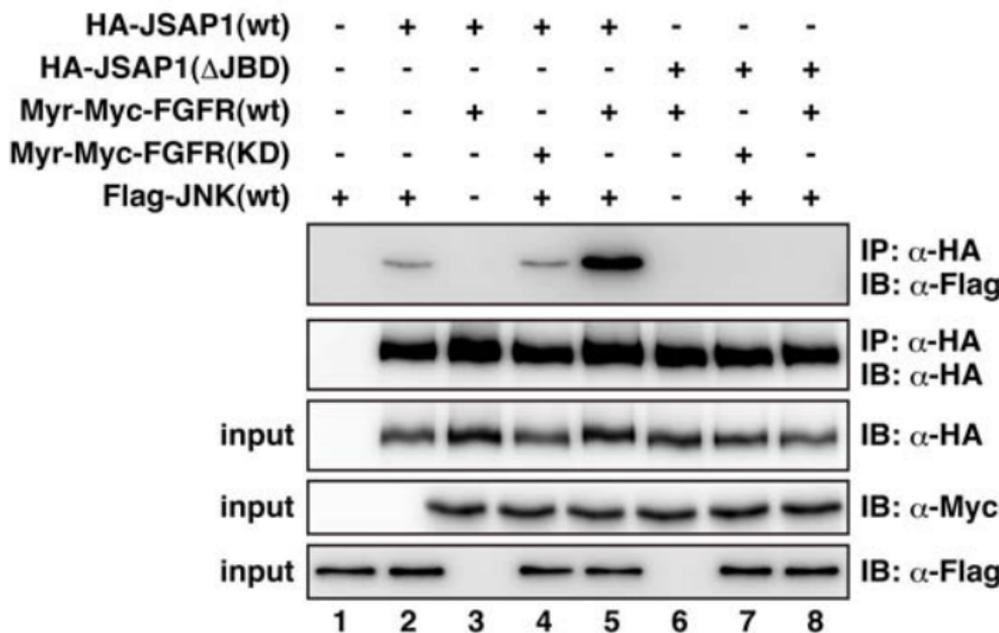
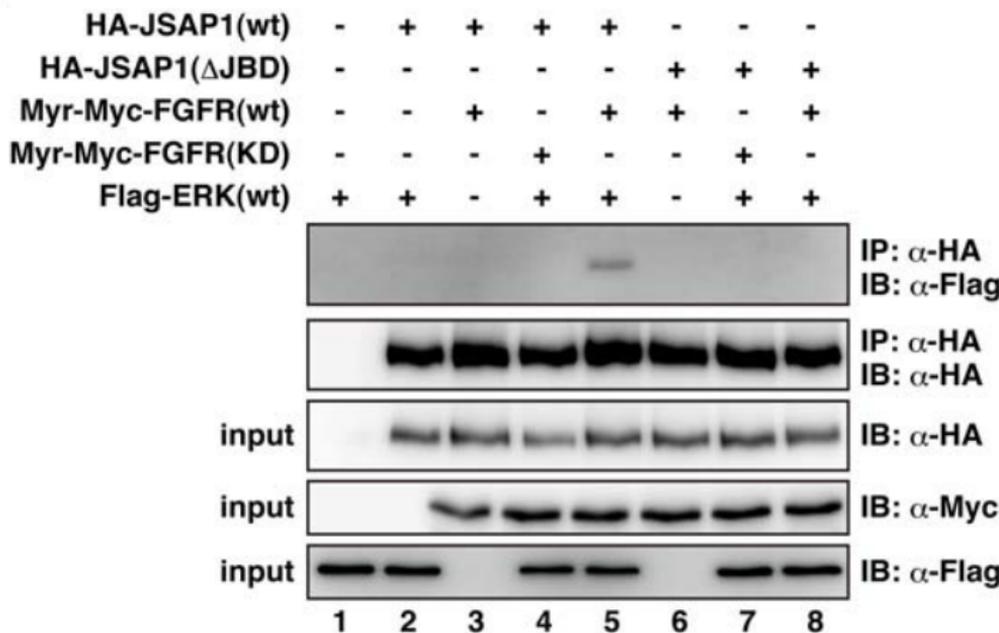
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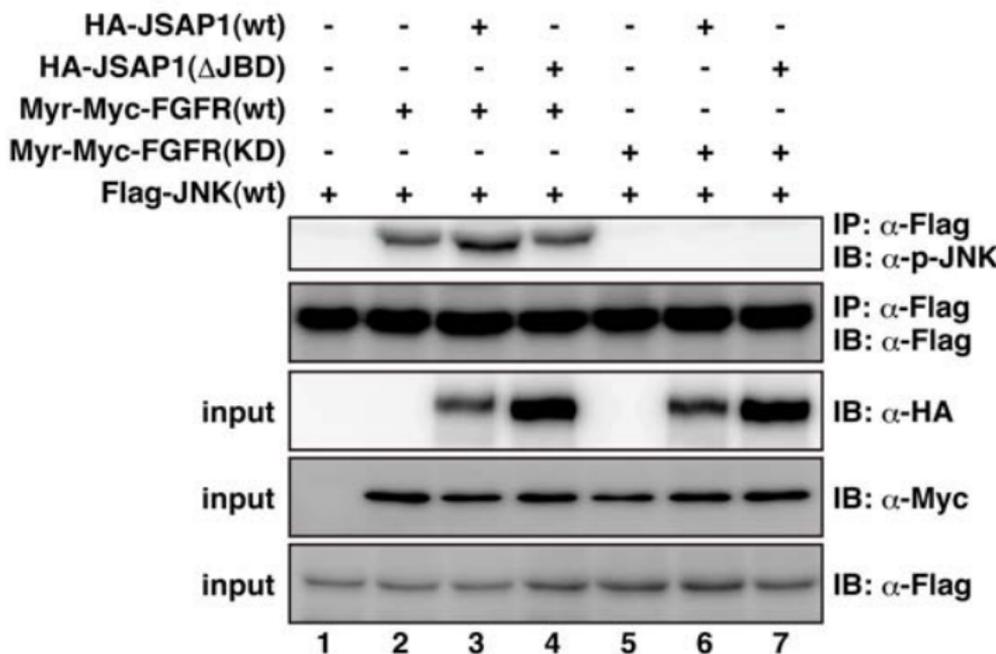
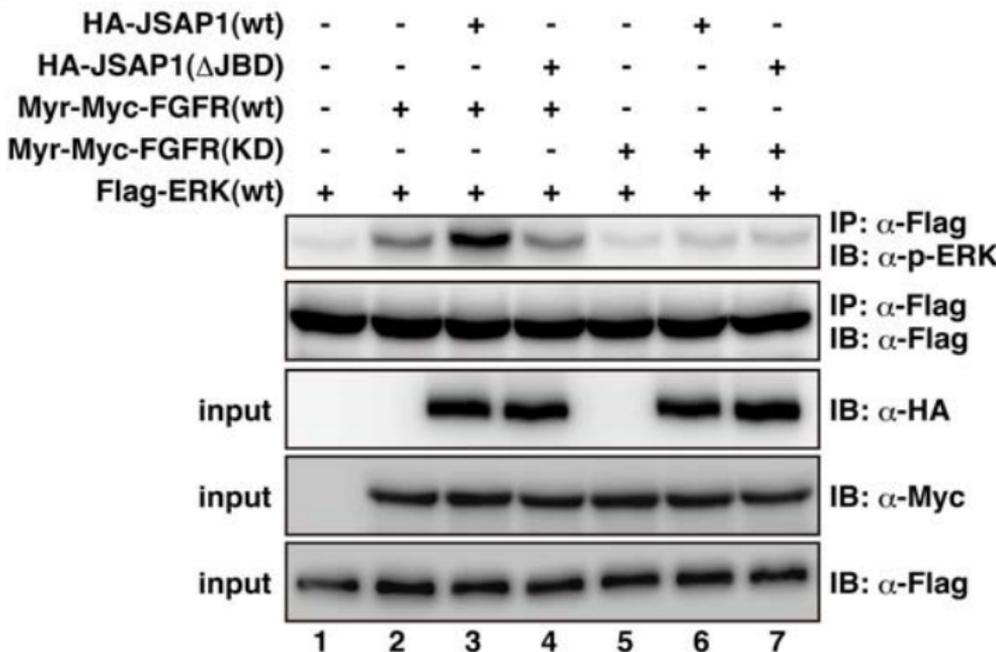


merge



**A****B**

**A****B**

**A****B**

FGF-2

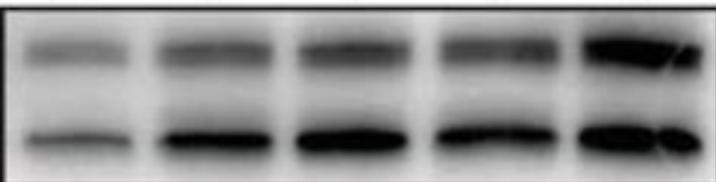
0 min

5 min

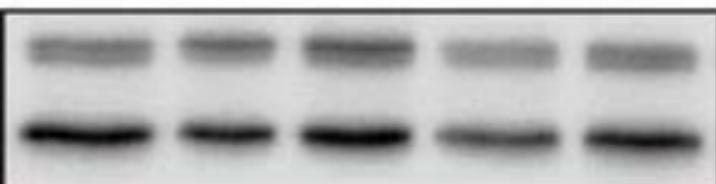
10 min

20 min

30 min



IB:  $\alpha$ -p-JNK



IB:  $\alpha$ -JNK



IB:  $\alpha$ -p-ERK



IB:  $\alpha$ -ERK

1

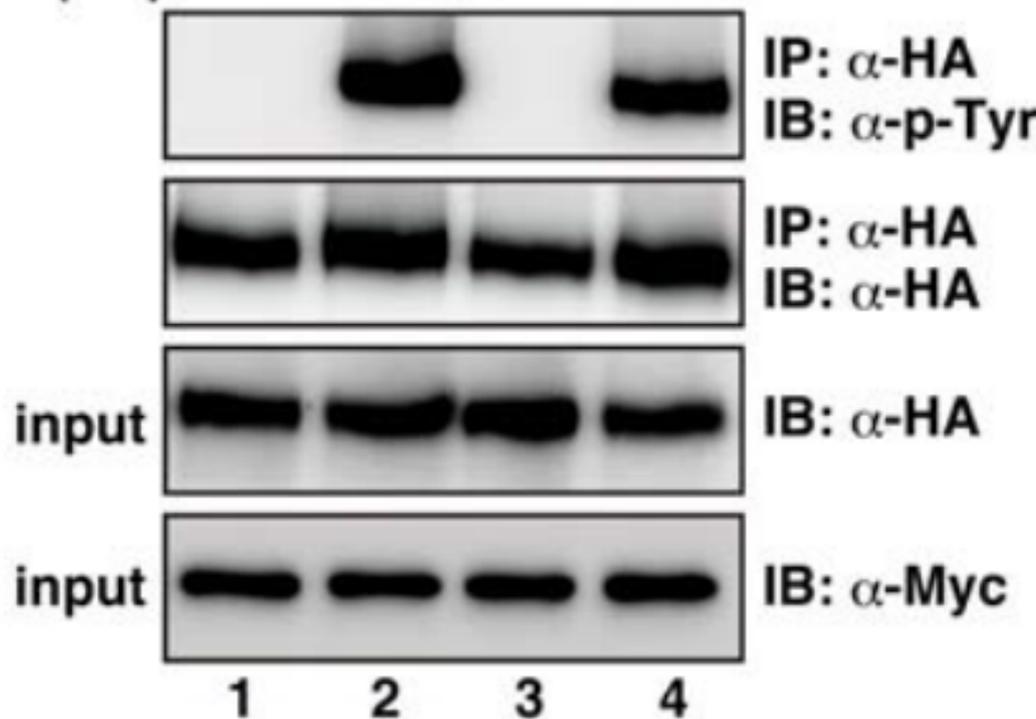
2

3

4

5

|                         |   |   |   |   |
|-------------------------|---|---|---|---|
| HA-JSAP1(wt)            | + | + | - | - |
| HA-JSAP1( $\Delta$ JBD) | - | - | + | + |
| Myr-Myc-FGFR(wt)        | - | + | - | + |
| Myr-Myc-FGFR(KD)        | + | - | + | - |

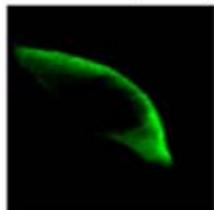


**A**Flag-JNK(wt)  
Myr-Myc-FGFR(wt)

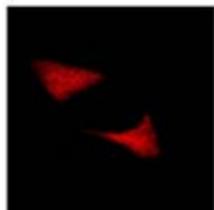
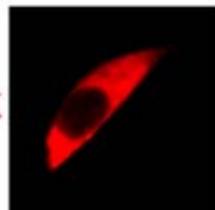
HA-JSAP1(wt)

HA-JSAP1( $\Delta$ JBD)

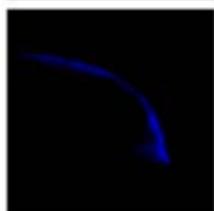
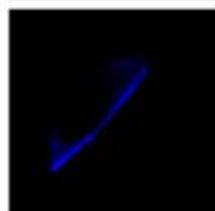
HA



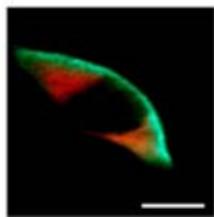
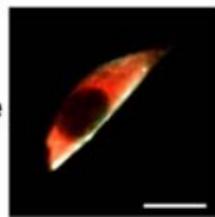
p-JNK



Myc



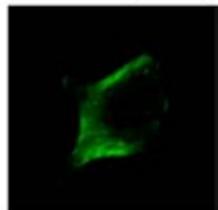
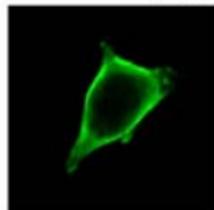
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**B**Flag-ERK(wt)  
Myr-Myc-FGFR(wt)

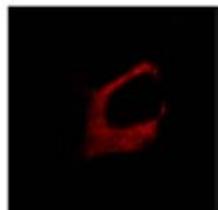
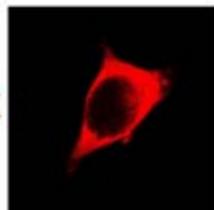
HA-JSAP1(wt)

HA-JSAP1( $\Delta$ JBD)

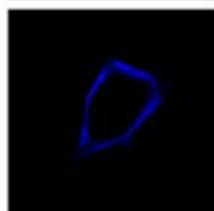
HA



p-ERK



Myc



merge

