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A role of Gab2 association in Flt3-ITD mediated Stat5 phosphorylation and cell survival

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Running title: Role of Gab2 in Flt3-ITD signaling

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## **Summary**

The hematopoietic growth factor receptor Flt3 has been implicated as major cause of transformation in acute myeloid leukemia. Intracellular signals mediated by wild-type Flt3 are involved in cell differentiation and survival whereas signaling via the mutant Flt3 ITD (internal tandem duplication) promotes enhanced cell growth. In this study, we identified tyrosines 768, 955 and 969 of Flt3 as phosphorylation sites and mediators of Grb2 interaction, leading to the association of Gab2 and contributing to proliferation and survival. We used Ba/F3 cells transfected with either the wild-type Flt3 or the ITD, with or without a triple mutation of the Grb2 binding sites, and characterised the cells in terms of proliferation and viability. Interestingly, we found that the Flt3 ITD promotes increased survival but after introducing the triple mutation, this phenotype is lost. When looking into different downstream pathways we observed that this effect was mainly caused by decreased PI3-kinase and Stat5 signaling, and the Flt3 ITD carrying the Grb2 binding mutations showed less Akt and Stat5 activation compared to the regular Flt3 ITD receptor. These findings not only reveal novel phosphorylation sites in Flt3 but contribute to the understanding of the molecular mechanism by which Flt3 ITD functions in pathological conditions.

**Keywords:** Flt3-ITD, Gab2, Stat5, survival, acute myeloid leukemia

## Introduction

The growth factor receptor Flt3 belongs together with the stem cell factor receptor/c-Kit, the platelet-derived growth factor receptors and the colony stimulating factor-1 receptor, to the type III family of receptor tyrosine kinases and is primarily expressed in hematopoietic progenitor cells (Gilliland and Griffin 2002). Wild-type Flt3 normally functions in hematopoiesis and promotes proliferation and differentiation of stem and progenitor cells through the activation of MAPK as well as phosphoinositide 3 kinase (PI3-kinase) signaling pathways (Maroc, *et al* 1993, Zhang, *et al* 1999). The binding of Flt3 ligand (FL) (Zhang, *et al*) to Flt3 results in dimerisation and autophosphorylation of the receptor, which subsequently creates docking sites for Src homology 2 (SH2) domain containing proteins and activates downstream signaling cascades.

Growth factor receptor binding protein 2 (Grb2) is an adaptor protein known to bind several receptor tyrosine kinases via the binding motif YXN (Songyang, *et al* 1994), and bridges between the receptor and cytosolic proteins in order to transmit signals further downstream. Moreover, Grb2 is part of a complex with Sos1 which activates Ras and ultimately leads to cellular responses such as survival and growth. Grb2 was also found to bind the scaffolding protein Grb2 associated binder 2 (Gab2) which upon phosphorylation associates with PI3-kinase, thus contributes to survival signaling in receptors lacking a direct PI3-kinase binding site such as Flt3 (Zhang and Broxmeyer 1999, Zhang and Broxmeyer 2000). The Gab family of scaffolding/docking/adaptor molecules includes Gab1, Gab2 and Gab3 and is involved in activation of PI3-kinase and MAPK signaling. Gab2 has been implicated in different types of cancer (Zhang, *et al* 2007) and others have shown that Gab2 plays a critical role in *BCR-ABL* mediated transformation where interaction with the receptor complex is required for signaling via the MAPK and the PI3-kinase/Akt pathways (Sattler, *et al* 2002).

Flt3 is often found mutated and constitutively active in acute myeloid leukemia (AML) (Choudhary, *et al* 2005, Yanada, *et al* 2005), commonly caused by an internal tandem duplication (ITD) in the juxtamembrane region of the receptor (Chillon, *et al* 2004, Stirewalt and Radich 2003) which is thought to disturb the inhibitory function this domain normally holds (Griffith, *et al* 2004, Kiyoi, *et al*

2002). The Flt3 ITD signaling pattern differs to some extent from that of the wild-type receptor, not only through the ligand-independent phosphorylation but also in the ability to activate signal transducer and activator of transcription 5 (Stat5) and its downstream targets (Hayakawa, *et al* 2000).

The Janus kinase (JAK)/STAT pathway mediates upon ligand-stimulation, JAK dependent phosphorylation of cytokine receptors, which serve as binding sites for SH2 domain containing STAT proteins. STAT's are transcription factors that cycle between the nucleus and the cytoplasm and once phosphorylated **by** JAKs, form dimers to regulate target gene transcription. In addition, several STAT's have been shown to be phosphorylated by receptor tyrosine kinases. The STAT protein family includes seven members and among those the very closely related Stat5a and Stat5b, which are expressed in hematopoietic stem cells and required for proliferative responses to cytokine signals (Bunting 2007). The constitutively activated form of Stat5 promotes cell survival and growth by regulation of genes involved in the cell cycle and apoptosis, such as *bcl-xL*, *cyclin D*, *p21* and *pim-1* (Nyga, *et al* 2005) and is frequently found over expressed in leukemia (Dumon, *et al* 1999, Moriggl, *et al* 1999, Nosaka, *et al* 1999). Thus, activation of Stat5 by Flt3 ITD is thought to play a crucial role in the transforming capacity of the mutant receptor, although the mechanism of activation is yet to be elucidated.

In the present study, we focused on Flt3 signaling via Grb2 and Gab2 in the wild-type and the mutant ITD receptor, and have identified tyrosines 768, 955 and 969 as phosphorylated Grb2 binding sites in Flt3. The downstream signal transduction involves activation of the MAPK pathway and the PI3-kinase/Akt pathway and when mutating all three tyrosine residues to phenylalanine, the proliferation and survival of murine hematopoietic Ba/F3 cells decreased. Interestingly, this effect was more prominent in the Flt3 ITD cells where the receptor with a triple mutation of the Grb2 binding sites also failed to activate Stat5 to the same extent as the regular Flt3 ITD. Furthermore, siRNA mediated Gab2 knockdown resulted in decreased Stat5 phosphorylation. This suggests that the malignant phenotype observed in Flt3 ITD might be due to Stat5 signaling via Gab2, and proposes a mechanism by which Flt3 ITD activates Stat5.

## **Material and Methods**

### *Plasmids, antibodies, antisera and GST fusion proteins*

pMSCV-puro vector containing human Flt3 cDNA was a kind gift from Dr. D. Gary Gilliland. Cytokines were purchased from Prospec Tany (Rehovot, Israel). The anti-Flt3 antibody has been described (Heiss, *et al* 2006). The phospho-specific antibodies against individual tyrosine phosphorylation sites in Flt3 were raised by immunising rabbits with synthetic peptides (JPT Peptides Technology, Berlin, Germany) corresponding to pY768 (CSEDEIEpYENQKRLEE), pY955 (CDAEEAMpYGNVDGRVS) and pY969 (CSESPHTpYQNRPFPSR) conjugated to keyhole limpet hemocyanin. All antibodies were affinity purified as described (Voytyuk, *et al* 2003). Anti-pErk (p42/p44), anti-pAkt, anti-Akt and anti-pGab2 were from Cell signaling Technology (Beverly, MA) and anti-Grb2, anti-Stat5, anti-Gab2 and anti-p85 $\alpha$  were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Erk2 has been described elsewhere (Voytyuk, *et al* 2003). The pan-phosphotyrosine antibody 4G10 was from Upstate, Temecula, CA. The GST fusion protein of Grb2 was a kind gift from Dr. Joseph Schlessinger.

### *Site-directed mutagenesis*

To mutate specific tyrosines to phenylalanines, the QuikChange mutagenesis XL kit (Stratagene, La Jolla, CA) was used and all mutations were confirmed by sequence analysis.

### *Cell culture*

COS-1 cells were cultured in Dulbecco's modified essential medium (PAA Laboratories GmbH, Pasching, Austria), supplemented with 10% FBS, 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin. The human AML cell line, MV4-11 was maintained in RPMI-1640 medium with 20% FBS, and starved over night in 0.5% FBS prior to experiment. Murine pro-B Ba/F3 cells (Deutsche Sammlung von Mikroorganismen und Zellen, Braunschweig, Germany), were kept in RPMI-1640 medium plus 10% heat-inactivated FBS, 10 ng/mL IL-3, 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin. Ba/F3 cells were starved by withdrawing serum and cytokines for 4 hours before stimulation with 100 ng/mL FL for the indicated periods of time.

### *Transient and stable transfection*

Adherent cells were transfected using JetPEI (PolyPlus-transfection, Illkirch, France) according to manufacturer's instructions. Cells stably expressing wild-type or mutant Flt3 were established as previously described (Sun, *et al* 2007). Silencing of Gab2 in Ba/F3 cells was achieved by electroporation (1500V, 1500  $\mu$ F) in a Gene PulserII (Bio-Rad, Hercules, CA) in the presence of 100 nM SMARTpool Gab2-siRNA or scrambled siRNA (Dharmacon, Lafayette, CO) and incubation for another 48 hours before experiments were performed. Silencing of Gab2 in MV4-11 cells was achieved using Accell Set of 4 siRNA (Dharmacon, Lafayette, CO), according to the manufacturer's instructions. We found that the effect of siRNA was not present until after the incubation period of 72 hours (set as time point zero for the Trypan blue exclusion counting assay). In brief, cells were incubated in Accell delivery media alone or in the presence of 1 $\mu$ M of Accell siRNA (non-targeting or two different targeting sequences) for 72 hours prior to performing the experiments.

### *Flow cytometry*

Flow cytometry was performed using a FACSort instrument (BD Biosciences, San Jose, CA). Apoptosis was measured using either an Annexin-V, 7-AAD kit (BD Biosciences Pharmingen, San Jose, CA), according to the manufacturer's instructions; double negative (Annexin-V<sup>-</sup>/7-AAD<sup>-</sup>) cell represents viable cells, while Annexin-V<sup>+</sup>/7-AAD<sup>-</sup> indicates early apoptotic cells and double-positive (Annexin-V<sup>+</sup>/7-AAD<sup>+</sup>) indicates late apoptotic or dead cells. Measurement of loss of the mitochondrial transmembrane potential was performed using the cationic fluorescent dye tetramethylrhodamine ethyl ester (TMRE, 25nM, Molecular Probes, Leiden, Netherlands) as described elsewhere (Khan, *et al* 2008).

### *Cell proliferation / survival assays*

To assess the number of Ba/F3 cells upon IL-3 withdrawal, in the absence or presence of FL, cells were washed twice in IL-3 free medium, seeded in 96-well plates (30,000 cells /well) and treated with FL for 48 hours. An MTT assay (Sigma-Aldrich, St Louis, MO) was performed according to the protocol. In parallel, viable cells were counted using Trypan blue exclusion method.

### *Immunoprecipitation, GST pulldown and Western blotting*

The experiments were conducted as described elsewhere (Lennartsson, *et al* 1999, Voytyuk, *et al* 2003). Immunodetection was performed by enhanced chemoluminescence using Super Signal Dura reagent (Pierce, Rockford, IL) and a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). Bands were quantified using MultiGauge software (Fujifilm).

## **Results**

### *Tyrosines 768, 955 and 969 are ligand-induced phosphorylation sites in Flt3 and the binding sites for the adaptor protein Grb2*

We have previously demonstrated Y572, Y589, Y591 and Y599 to be autophosphorylation sites of Flt3 involved in the activation of Src family kinases and SHP2, respectively (Heiss, *et al* 2006). Next, we focused our interest on three sites that all share a surrounding sequence characteristic of a binding motif for Grb2, namely YXN. In order to investigate whether these three tyrosine residues in Flt3 are phosphorylated in living cells, we designed phospho-specific antibodies against the peptide sequence surrounding these residues in Flt3. When using the Flt3 pY768, pY955 and pY969 antibodies we could show that these sites are phosphorylated in response to FL and the corresponding Y-to-F single mutation specifically blocks this phosphorylation (fig. 1A). These sites were further confirmed to be phosphorylated in the Flt3 ITD receptor in a ligand-independent manner (fig. 1B), suggesting an important role in the mutant receptor as well. As Grb2 was the primary binding candidate to these tyrosine residues, a GST-Grb2 fusion protein was used in a Far Western experiment where we compared the binding of Grb2 to Flt3 in the wild-type receptor to the Y768F, Y955F and Y969F single, double and triple mutations. Although the binding affinity differs somewhat, all three residues appear to be of importance and mutations of all three tyrosines are required for complete loss of Grb2 binding (fig. 1C). Since the Flt3 ITD is commonly found in AML patients, we were also interested in elucidating the downstream signaling events of Flt3 ITD in more detail. As a model system we chose to use Ba/F3 cells stably expressing the Flt3 wild-type or the ITD receptor, with or without a triple mutation of the Grb2 binding sites. We could

verify interaction of Grb2 with both wild-type Flt3 and Flt3 ITD in a co-immunoprecipitation experiment, which was blocked when introducing the triple mutation (fig. 1D). An equal Flt3 protein expression in the different cells was verified both in a parallel immunoprecipitation with Flt3 (fig. 1D lower panel) and by flow cytometry (data not shown) and equal kinase activity of the receptor mutants was confirmed by an in vitro kinase assay using exogenous substrate (data not shown).

*Mutation of the Grb2 binding sites in wild-type Flt3 and Flt3 ITD results in decreased Erk and Akt activation due to loss of Grb2 mediated Gab2 association*

It is well established that Grb2 when recruited to receptor tyrosine kinases, forms a complex with Sos1 which in turn activates the MAPK pathway. Several studies also indicated a role of Grb2 in activation of the PI3-kinase/Akt pathway by receptor tyrosine kinases through recruitment of Gab proteins (Holgado-Madruga, *et al* 1997, Kong, *et al* 2000). When using whole cell lysate from Ba/F3 cell lines expressing either wild-type or ITD Flt3, with or without the triple mutation of the Grb2 binding sites (tm), we found that the phosphorylation of Erk was marginally decreased while phosphorylation of Akt was to some extent decreased upon mutating the Grb2 binding sites, as compared to the wild-type receptors. The effect was more pronounced in the ITD receptor (fig. 2A). This suggests that both signaling pathways are regulated by the Grb2 binding sites Y768, Y955 and Y969. The normal ITD receptor shows a weak and to some extent ligand-induced activation of Erk and Akt (fig. 2A). We and others have shown that this frequently occurs in mutated receptors like Flt3 and c-Kit, and that the constitutive activation of downstream signaling can be further enhanced by ligand stimulation (data not shown). Since Flt3 lacks a direct binding motif for the p85 subunit of PI3-kinase, a possible mediator of PI3-kinase activation is Gab2. We immunoprecipitated p85 $\alpha$  in the different Ba/F3 cell lines and analysed the phosphorylation levels of the Flt3 receptor in the absence or presence of FL. Although the protein levels of p85 $\alpha$  were equal in all samples, wild-type Flt3 and p85 $\alpha$  co-immunoprecipitated in a ligand-dependent manner. In contrast, Flt3 triple mutant showed a decreased interaction with p85 $\alpha$ . Probing the filter with an antibody against Gab2 demonstrated that Gab2 association was also dependent on the three phosphorylation sites

(fig. 2B). This suggests that Flt3 activates PI3-kinase in a Gab2-dependent fashion, via the Grb2 binding sites on Flt3. An interaction between Flt3 and Gab2 in Ba/F3 cells was further confirmed by co-immunoprecipitation (fig. 2C, upper two panels) and when investigating the levels of phosphorylated Gab2, we could conclude that the activation of Gab2 depends on the Grb2 binding sites (fig. 2C, lower two panels). A constitutive interaction between Grb2 and Gab2 has been shown in previous studies (Sun, *et al* 2008, Zhang and Broxmeyer 2000).

#### *Flt3 ITD mediated proliferation and survival of Ba/F3 is dependent on intact Grb2 binding sites in Flt3*

The Flt3 ITD receptor has been studied extensively during the last years but the details of its transforming capacities are not fully understood. Signaling through the ITD receptor inhibits apoptosis by phosphorylating the Forkhead family member Foxo3 $\alpha$  which in an inhibited state prevents transcription of its pro-apoptotic target genes in the Bcl-2 family (Minami, *et al* 2003). In order to study the biological outcomes of the signaling downstream of Y786, Y955 and Y969, we performed MTT and Trypan blue exclusion assays. Addition of FL did not have any noticeable effect in the wild-type Flt3 cells whereas it to some extent increased the proliferative response in the Flt3 ITD expressing cells. Interestingly, when introducing the triple mutation of the Grb2 binding sites in the Flt3 ITD, cell proliferation was dramatically decreased whereas the effect in the wild-type Flt3 was not as pronounced (fig. 3A). Moreover, the number of viable cells was in the same manner decreased in the Flt3 ITD with the triple mutation as compared to the regular Flt3 ITD (fig. 3B). This finding is of much importance since the typical Flt3 ITD promotes proliferation and cell survival, and the Grb2 binding sites seem to be involved in this process, likely through association with Gab2. This effect may be due to less proliferation, but also to higher degree of apoptosis. To look into this we stained the cells with Annexin V and 7-AAD and analysed the amount of living versus apoptotic or necrotic cells by flow cytometry. As expected, a much larger proportion of cells expressing the wild-type Flt3 were apoptotic or necrotic than those with the Flt3 ITD, regardless of stimulation with FL or not (data not shown). However, in the Flt3 ITD with the triple mutation of Y768, Y955 and Y969 an increased number of cells became apoptotic (fig. 4A). This is

further demonstrated in a diagram where the cells were counted and the different subgroups were presented as a percentage of the total cell number (fig. 4B).

*Stat5 activation by Flt3 ITD is regulated by tyrosines Y768, Y955 and Y969 via recruitment of Gab2*

Several groups have shown that Flt3 ITD activates Stat5 in primary AML cells and that this pathway contributes to the observed malignant phenotype of the mutant receptor (Spiekermann, *et al* 2003) . The activation mechanisms of Stat5 by Flt3 are not established but will be of much relevance concerning its potential as target for future therapies in AML treatment. Recent studies have revealed that Gab2 dependent activation of PI3-kinase and MAPK pathways is required for Stat5 induced proliferation of Ba/F3 cells (Nyga, *et al* 2005). This suggests a signaling pathway from Flt3 ITD to Stat5 via Gab2. To investigate this we looked at Stat5 phosphorylation in cells expressing the Flt3 ITD with mutated Grb2 binding sites in comparison to the regular Flt3 ITD expressing cells. As anticipated, the Flt3 ITD but not the wild-type receptor was able to phosphorylate Stat5, but there was a dramatic decrease in Stat5 phosphorylation in the Grb2 binding mutant Flt3 ITD (fig. 5A). The same outcome was observed after stimulation for longer periods of time (fig. 5B). This indicates an important role of Grb2 and Gab2 in Flt3 ITD induced Stat5 activation, and might explain why the effect of this triple mutation is stronger in the ITD than in the wild-type Flt3. However, we were not able to co-immunoprecipitate Gab2 and Stat5 (data not shown), which may be explained by an indirect and transient interaction. Another possibility is that Stat5 activation is further downstream of Gab2.

*Knock-down of Gab2 in human AML cells affects the activation of Akt, Erk as well as Stat5, indicating an important role for Gab2 in Flt3 ITD driven transformation*

To test our hypothesis in a more relevant physiological context, we used the human AML cell line MV4-11, which expresses Flt3 ITD endogenously. The phosphorylation and expression levels of Flt3 were examined and a weak response to FL stimulation was observed (fig. 6A). By the use of two separate sequences of siRNA we knocked down the expression of Gab2 and analysed the whole cell lysates by

Western blotting. As controls, cells were either transfected with scrambled siRNA or mock transfected. In parallel analyses of the same lysates, we could demonstrate that the phosphorylation levels of Akt, Erk and Stat5 were reduced when blocking Gab2 expression (fig. 6B). To verify that these findings were not specific to MV4-11 cells, a similar experiment where we instead used a SmartPool of siRNA against Gab2 was performed in Ba/F3 cells. The results were equivalent in terms of the effect on Erk and Akt phosphorylation (data not shown), although a more pronounced decrease of Stat5 phosphorylation was seen as a result of silencing Gab2 (fig. 6C). In a Trypan blue cell counting assay, we found that the MV4-11 cells transfected with Gab2 siRNA showed significantly less proliferation and survival than the control cells (fig. 6D).

## **Discussion**

It is well recognised that the Flt3 ITD receptor signaling differs from that of the wild-type Flt3, both in terms of ligand independency but also in the ability to activate Stat5. Previous studies have shown that Flt3 ITD has transforming potential in hematopoietic cells, and the ITD receptor induced a strong activation of Stat5 but not Stat3 in primary AML blasts (Spiekermann, *et al* 2003) (Mizuki, *et al* 2000).

In this study, we identified the three tyrosines 768, 955 and 969 as ligand-induced phosphorylation sites in Flt3 by the use of phospho-specific antibodies. As all three sites contain the adaptor protein Grb2 binding YXN motif, we verified a direct interaction with Grb2 which in turn was found to recruit the scaffolding protein Gab2 to the receptor. This feature has also been shown with some other tyrosine kinases, one example being tyrosine 177 of Bcr-Abl, which recruits Gab2 via a Grb2/Gab2 complex where this interaction is required for full activation of the PI3-kinase and MAPK pathways as well as for optimal proliferation and migration in Ba/F3 cells (Sattler, *et al* 2002). The Gab2 protein is over activated in several malignancies and *Gab2* has been identified as co amplified in 70 % of all patients with the *MLL* gene which is a target of chromosome 11q amplification in AML/MDS (Zatkova, *et al* 2006). This indicates that Gab2 plays an important role in AML signaling pathways and if regulated by

Flt3, might contribute to the poor prognosis observed in AML patients who also carry the Flt3 ITD mutation.

By looking at Erk and Akt phosphorylation, we could conclude that both the MAPK and PI3-kinase signaling pathways were affected by reduced Grb2/Gab2 interaction with Flt3. Serine 623 of Gab2 is a phosphorylation target by Erk which further affects the binding of Shp-2 to Gab2. A Gab2 S623A mutant show not only decreased Erk phosphorylation but also reduced Shp-2 recruitment which further leads to a sustained Stat5 activity and cell proliferation (Arnaud, *et al* 2004). When we mutated the three tyrosine residues to phenylalanine in Flt3 ITD, the phenotype of the Ba/F3 cells resembled more that of the wild-type Flt3 in terms of proliferation and survival. Remarkably, Stat5 phosphorylation was also decreased in these cells leading us to hypothesise that the Stat5 pathway is activated by Flt3 ITD via Gab2. Stat5 has previously been shown to associate with Gab2 (Brockdorff, *et al* 2001), and this process is of importance in Stat5-induced cell proliferation and survival via the PI3-kinase and MAPK signaling pathways (Nyga, *et al* 2005). Interestingly, the authors also found that this interaction was independent of JAK and it is believed that formation of the Gab2/Stat5/p85 complex requires Gab2 phosphorylation but so far, no consensus sequences for binding of Stat5 have been identified within Gab2. One might therefore consider the interaction to be indirect through some other signaling molecule. This remains to be shown. Moreover, a Stat3 binding motif was recently identified in Gab2 and proved to be required for Epo-independent growth of Friend virus-infected erythroid progenitor cells (Ni, *et al* 2007). Taken together, the data provided here add to the conclusion that Stat5 is a downstream target of Gab2.

Ba/F3 cells expressing the Grb2 binding Y-to-F triple mutation have an increased apoptotic response upon IL-3 withdrawal, and the effect was more prominent in the Flt3 ITD than in the wild-type Flt3. Thus, this observation is most likely due to decreased Stat5 activity. Wild-type Flt3 signaling has been associated with anti apoptosis through the activation of Bad. However, dephosphorylation of Bad is insufficient to induce apoptosis where also the down-regulation of Bcl-x is required which is restored by Stat5 signaling, hence only seen in the Flt3 ITD expressing cells (Minami, *et al* 2003). Other studies in Ba/F3 cells have shown that Stat5 might promote cell survival not only as a transcription factor by

regulating expression of Bcl-x, but as a signaling intermediate in PI3-kinase signaling (Santos, *et al* 2001) and that PI3-kinase and Stat5 cooperate to promote IL-3 mediated suppression of apoptosis (Rosa Santos, *et al* 2000). Persistent Stat5 phosphorylation was detected in primary cells from patients with AML or CML and a major fraction of Stat5 molecules turned out to be cytoplasmic in the presence of growth factors. These results suggest that a large part of the oncogenic activity of Stat5 involves a cytoplasmic signaling complex between Stat5, Gab2 and PI3-kinase (Harir, *et al* 2007). This is also in line with our findings and when knocking down Gab2 expression in AML cells, the Akt phosphorylation as well as the Stat5 phosphorylation was lowered. These data support the idea of a Flt3 ITD signaling complex which eventually leads to the activation of Stat5 target genes and contributes to the transforming phenotype.

The mechanism by which Flt3 ITD signaling leads to phosphorylation of Stat5 is not fully characterised. It has been suggested that Flt3 ITD-mediated Stat5 activation is directly mediated by Flt3 itself (Choudhary, *et al* 2007). However, those experiments were mainly performed in mouse embryonal fibroblasts known to express low levels of Gab2 (Liu, *et al* 2001); data not shown) leaving the possibility that other tyrosine kinases might mediate phosphorylation of Stat5 at least in part. One such candidate is the Src family kinases. The erythropoietin receptor associates with Lyn in hematopoietic cells, which in turn has the ability to induce phosphorylation of Stat5 (Chin, *et al* 1998). When mutating the two Src binding sites Y589 and Y591 in Flt3 ITD, the Stat5 phosphorylation was decreased, indicating that Src family kinases or some other protein binding to these residues, might mediate the activation of Stat5 in an indirect manner (Hayakawa and Naoe 2006, Rocnik, *et al* 2006). Future studies will elucidate the details of this mechanism.

In conclusion, the data provided here supports the idea of Gab2 as an important mediator in Flt3 ITD signaling. Tyrosines 768, 955 and 969 of Flt3 are phosphorylated and shown to be Grb2 binding sites of importance for cell proliferation and survival, most likely as a result of Gab2 association. Normally, the Flt3 ITD receptor promotes increased survival and growth as well as strong activation of Stat5 which is a potent contributor to the malignant phenotype, and these characteristics are lost when mutating the Grb2 binding sites in Ba/F3 cells. Moreover, after silencing Gab2 expression in both human AML cells and in

Flt3 ITD expressing Ba/F3 cells, the activation of MAPK and PI3-kinase pathways as well as the phosphorylation of Stat5 is lowered, suggesting an important role for Gab2 in Flt3 ITD driven transformation. We hypothesise that Gab2 regulates the activation of Stat5 in Flt3 ITD receptor signaling, possibly through other signal transduction intermediates, and that tyrosines 768, 955 and 969 of Flt3 are of much importance in this process. Our findings contribute to the overall understanding of Flt3 signaling, both in terms of the wild-type receptor and the oncogenic Flt3 ITD receptor in AML, and introduce a signal transduction pathway as a possible future therapeutic target.

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## **Titles and Legends to Figures**

*Fig. 1. Identification of Y768, Y955 and Y969 as phosphorylation and Grb2 binding sites in COS-1 and Ba/F3 cells.*

(A) COS-1 cells transiently transfected with either the wild-type Flt3 receptor or the respective Y-to-F mutant were stimulated with FL for 5 minutes and lysed. Lysates were immunoprecipitated with an Flt3 antibody and analysed by Western blotting. Membranes were probed with phosphospecific antibodies and after stripping re-probed with 4G10 and anti-Flt3. (B) Lysates from Ba/F3 cells with stable expression of either the wild-type Flt3 or the Flt3 ITD receptor were processed as above. (C) Ba/F3 cells stably expressing either wild-type Flt3, or the various Y-to-F mutants of Flt3 were stimulated with FL for 5 minutes. Cell lysates were immunoprecipitated with anti-Flt3 and analysed by Far Western, using a GST-fusion protein of Grb2 SH2 domain. (D) Grb2 (upper two panels) or Flt3 (lower panel) was immunoprecipitated from cell lysates of the Ba/F3 cells mentioned above and samples were analysed by Western blotting with the indicated antibodies. The Y768F/Y955F/Y969F mutation is denoted *tm* (triple mutation).

*Fig. 2. Binding of Grb2 to Flt3 is required for activation of Gab2 and the MAPK as well as the PI3-kinase pathways*

(A) Whole cell lysates from Ba/F3 cells expressing the different Flt3 mutations were analysed by Western blotting with phospho-Akt and phospho-Erk antibodies. As controls, total levels of Akt and Erk2 as well as actin were confirmed. (B) The regulatory subunit of PI3-kinase, p85 $\alpha$  was immunoprecipitated, and samples analysed by Western blotting. The membrane was probed with anti-phosphotyrosine and Gab2 antibodies, and with anti-p85 $\alpha$  as loading control. (C) Immunoprecipitations of Gab2. The membranes were probed with antibodies against Gab2 and Flt3 (upper two panels) and phospho-Gab2 and Gab2 (lower two panels).

*Fig. 3. Cell proliferation and viability is reduced when mutating the Grb2 binding sites of Flt3*

Ba/F3 cells expressing the wild-type Flt3 or the various mutants of Flt3 were kept in IL-3 free media in the presence or absence of FL (100ng/mL) for 0 hours (dark grey), 24 hours (black) and 48 hours (light grey). (A) Cells were incubated with MTT dye for another 4 hours, where after absorbance of the converted dye was read at 595/630 nm. (B) After the indicated period of time, cells were counted with Trypan blue dye. Error bars indicate the standard deviation in three individual experiments.

*Fig. 4. Analysis of apoptosis by flow cytometry*

Cells were kept in media without IL-3 for 48 hours, in the presence or absence of FL (100ng/mL). This was followed by staining of the cells with markers for early and late apoptotic cells, Annexin V and 7-AAD, respectively. The number of living cells versus apoptotic or necrotic cells was analysed by flow cytometry (as indicated). Data was analysed using Cell Quest Software. (A) Two dimensional dot plots showing the distribution of apoptotic cells. (B) Histogram showing the fractions of living cells (grey bars) and apoptotic or necrotic cells (black bars) as percentage of the total cell amount, where the error bars represent the standard deviation from three individual experiments.

*Fig. 5. Activation of Stat5 is affected by Grb2 and Gab2 binding to Flt3*

(A) Lysates of the indicated cells stimulated with FL for 0 or 5 minutes were immunoprecipitated with anti-Stat5 and analysed by Western blotting as indicated. (B) The experiment was performed as above, with stimulation periods from 0 to 30 minutes.

*Fig. 6. Silencing of Gab2 in the Flt3 ITD expressing human AML cell line MV4-11*

(A) Expression and activation of Flt3 ITD was verified by immunoprecipitation of Flt3 after ligand stimulation for the indicated periods of time. (B) Silencing of Gab2 was achieved by transfecting the cells with two separate sequences of Accell siRNA, and control cells were either transfected with non-targeting siRNA (scrambled) or mock (Accell Delivery medium only). Cells were kept in culture for 72 hours after

transfection. Whole cell lysates as well as immunoprecipitates for Gab2 and Stat5 were analysed in parallel by Western blotting. Membranes were probed with antibodies against Gab2 (I), 4G10 and Stat5 (II), phospho-Erk1/2, phospho-Akt and  $\beta$ -actin (III). (C) A similar experiment was performed using Ba/F3 cells, but silencing of Gab2 was achieved by transfecting the cells with Gab2 SmartPool siRNA by electroporation, and control cells were either transfected with non-targeting siRNA or mock-transfected. Cells were kept in culture for **48** hours after transfection and starved for 4 hours before lysis, where after the samples were immunoprecipitated with Gab2 and Stat5 antibodies and analysed for Gab2 levels and Stat5 activation (D) MV4-11 cells from the same experiment as in B were after transfection kept in culture for 0 (dark grey bars) or 24 hours (light grey bars) and counted for Trypan blue dye exclusion. The figures are presented in a diagram where the error bars indicate the standard deviation from three individual experiments.

Fig.1

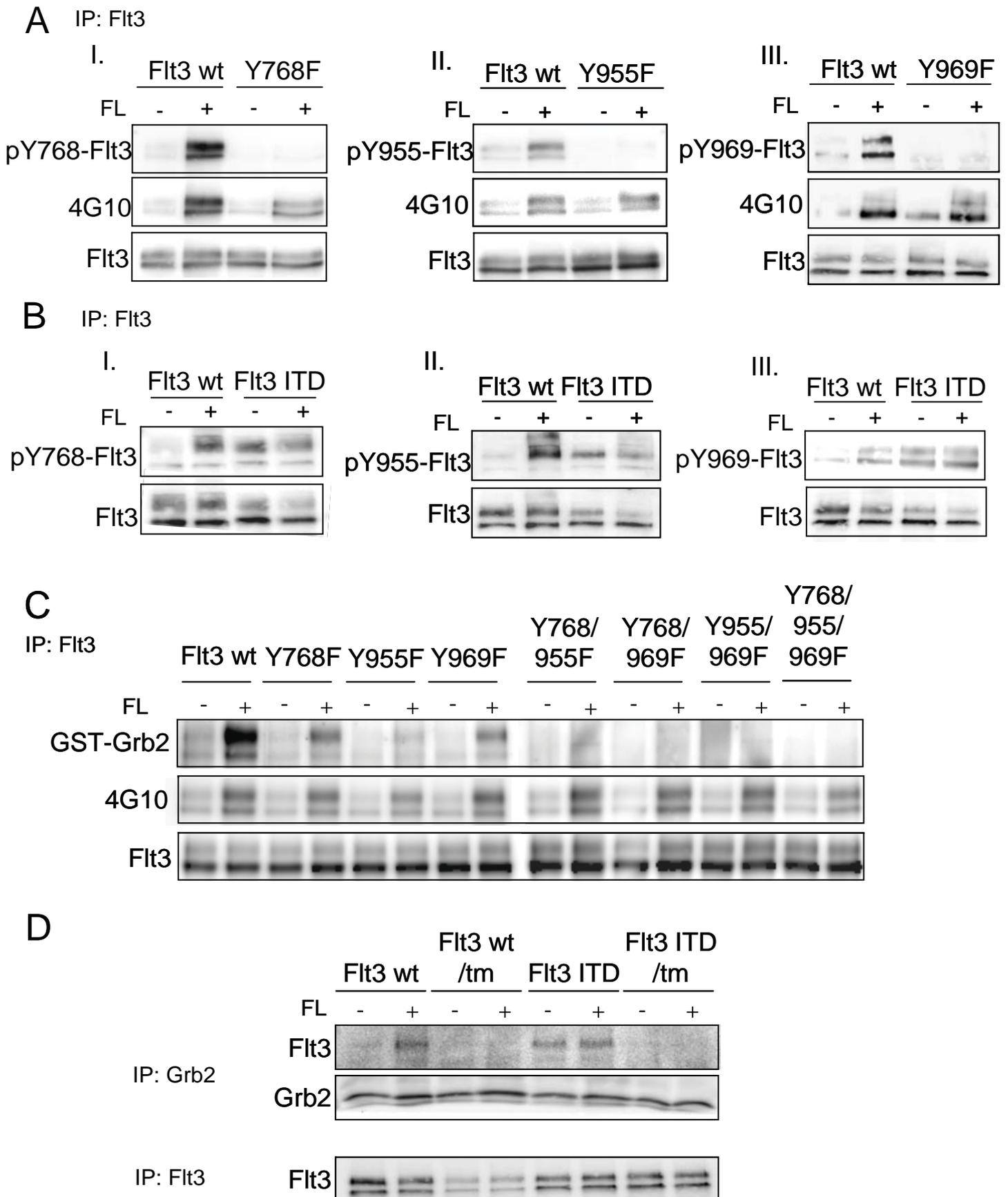


Fig.2

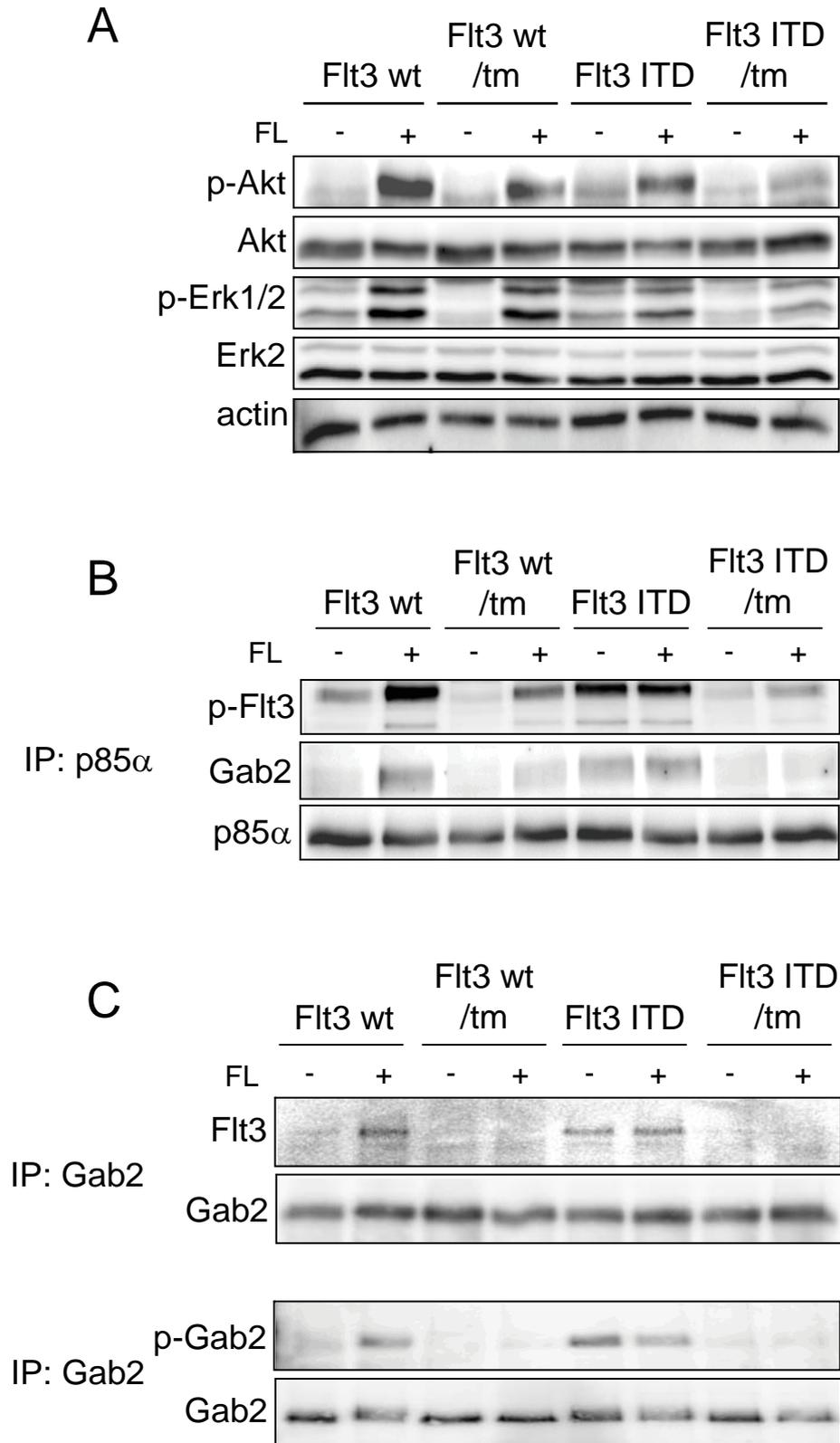
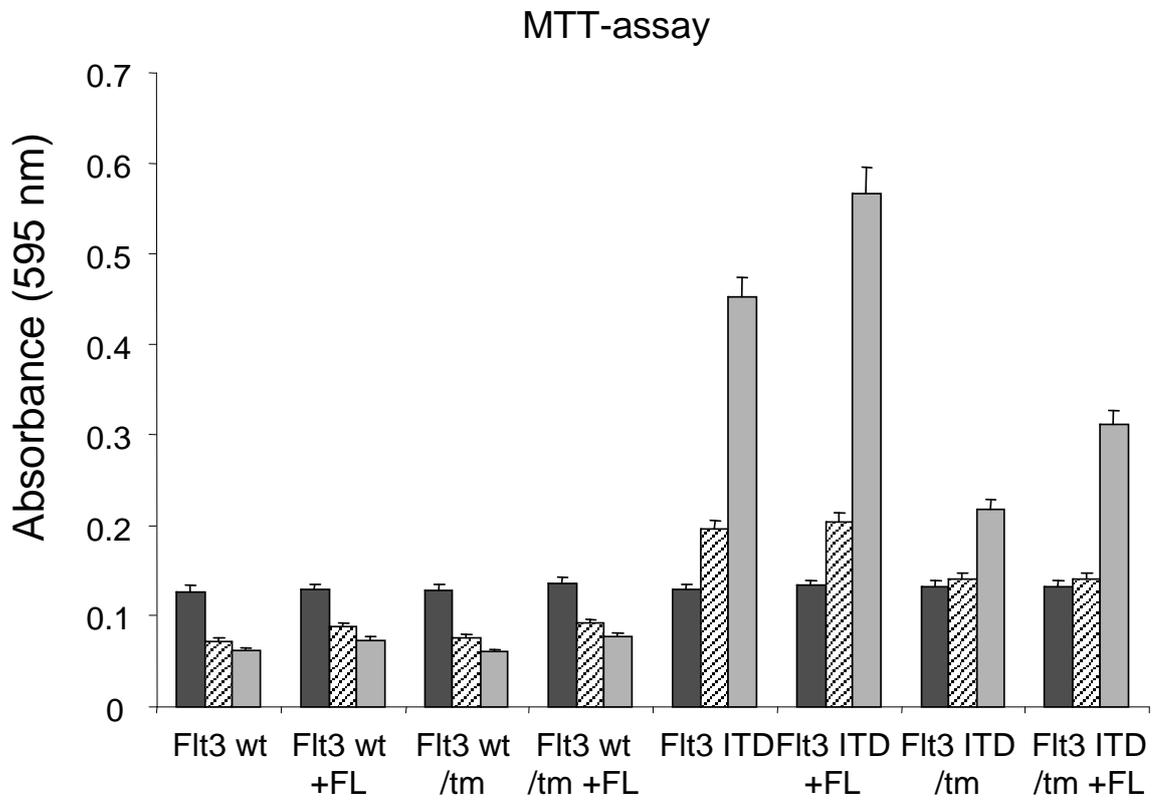


Fig.3

A



B

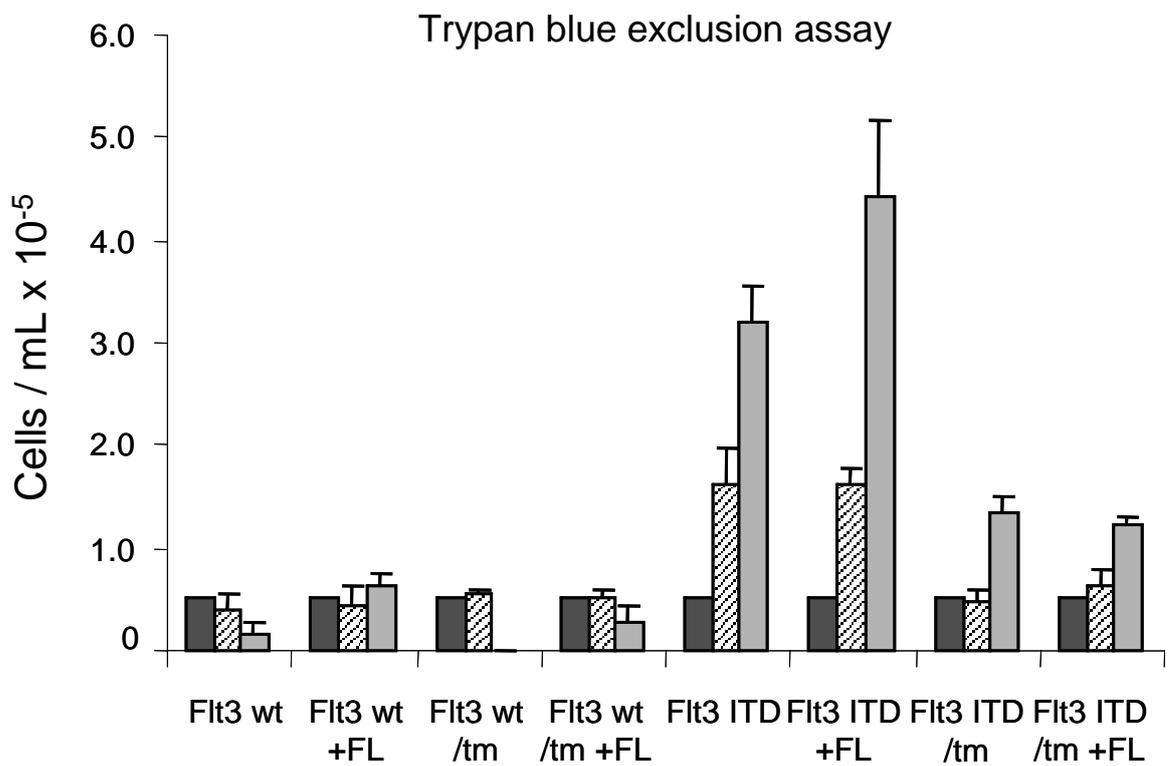


Fig.4

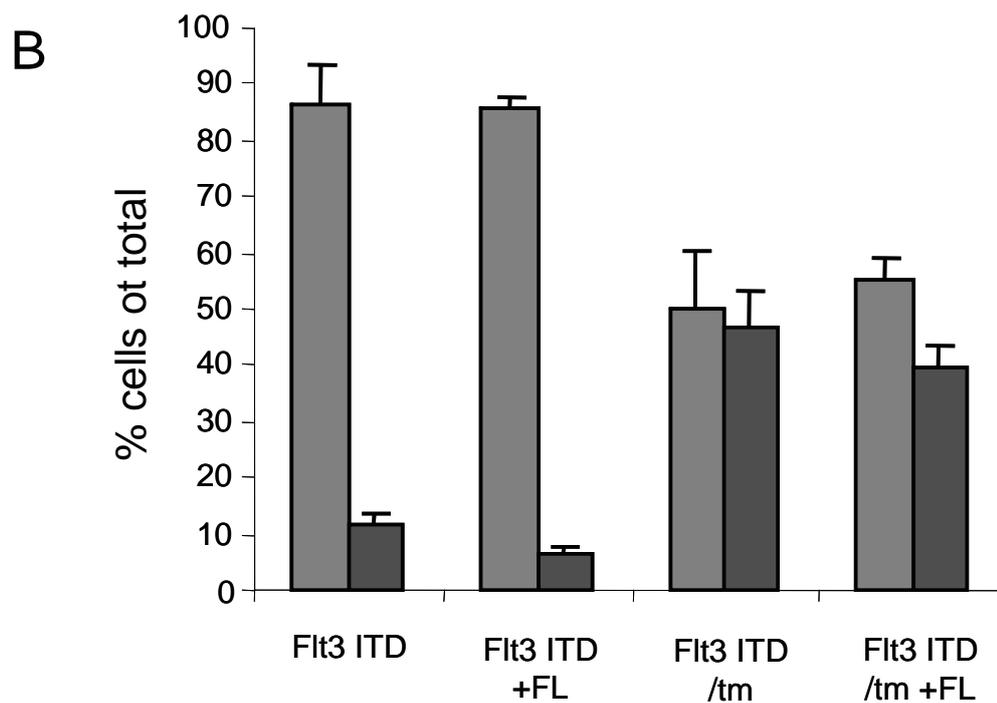
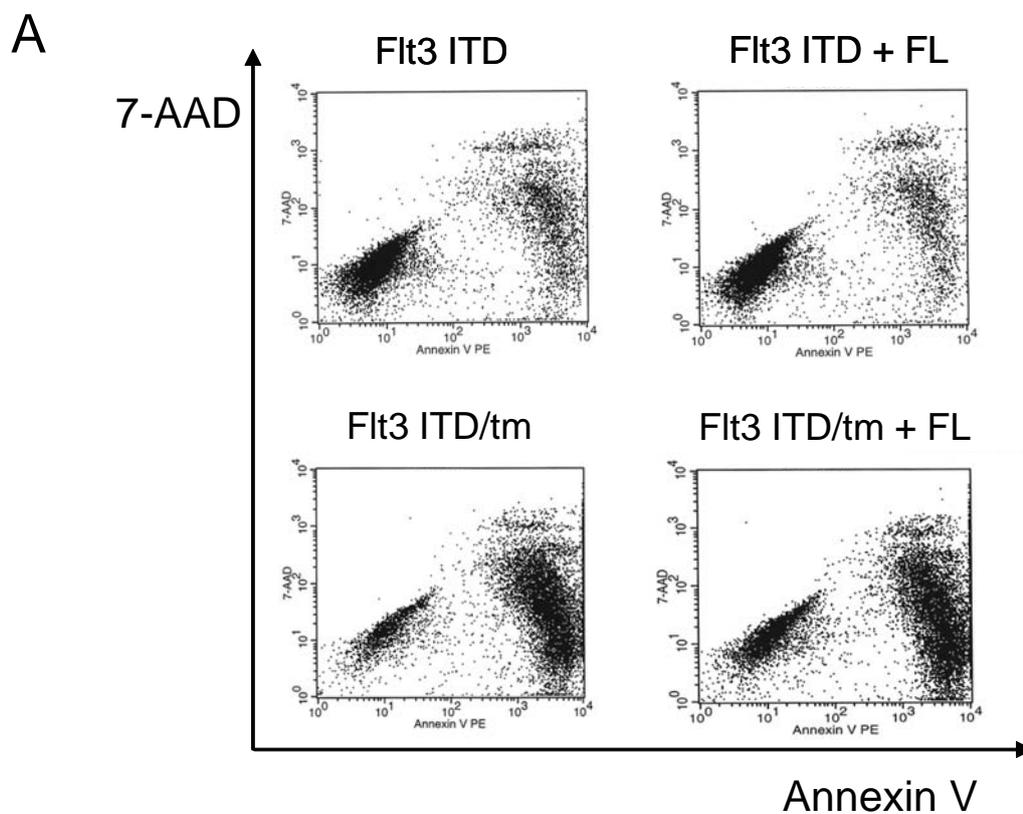
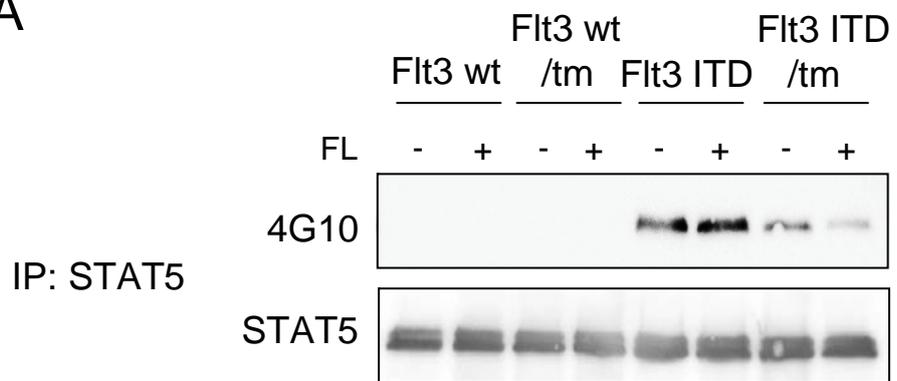


Fig.5

A



B

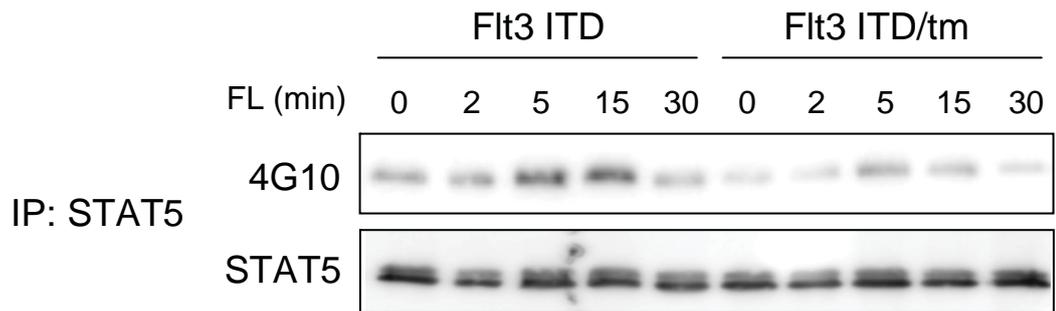
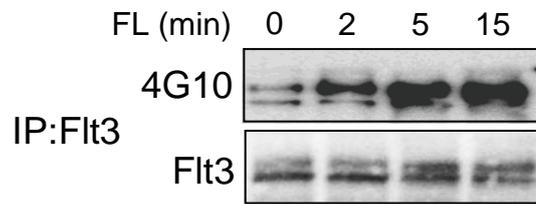
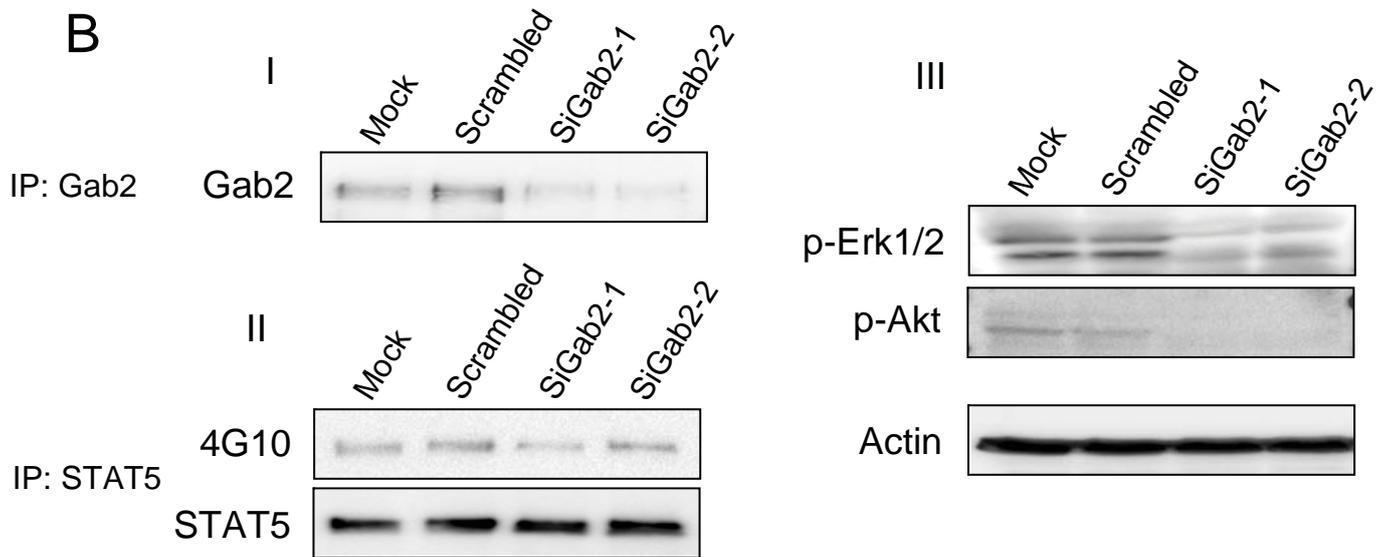


Fig.6

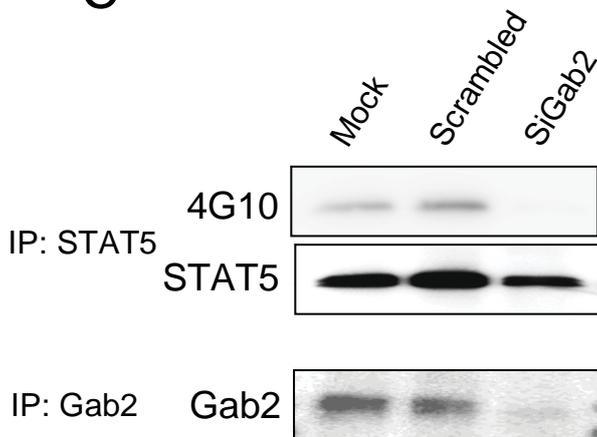
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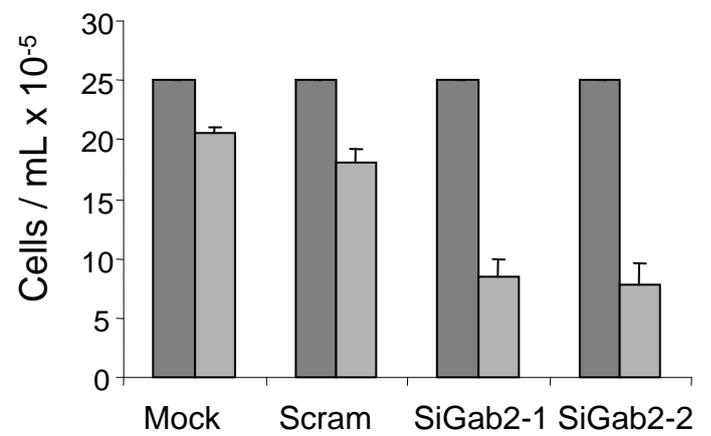
B



C



D



IP: Gab2

