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Highlights

- **Grb10 binds to activated Flt3 and activates Akt and STAT5 signaling.**
- **Grb10 increased FLT3-ITD-mediated cell proliferation**
- **Grb10 potentiates downstream signaling**
- **Grb10 promotes transformation of cells by the oncogenic mutant Flt3-ITD**
- **Elevated expression of Grb10 correlates with poor prognosis in acute myeloid leukemia patients**

FLT3 signals via the adapter protein Grb10 and overexpression of Grb10 leads to aberrant cell proliferation in acute myeloid leukemia

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Abstract

The adaptor protein Grb10 plays important roles in mitogenic signaling. However, its roles in acute myeloid leukemia (AML) are predominantly unknown. Here we describe the role of Grb10 in FLT3-ITD-mediated AML. We observed that Grb10 physically associates with FLT3 in response to FLT3-ligand (FL) stimulation through FLT3 phospho-tyrosine 572 and 793 residues and constitutively associates with oncogenic FLT3-ITD. Furthermore endogenous Grb10-FLT3 association was observed in OCI-AML-5 cells. Grb10 expression did not alter FLT3 receptor activation or stability in Ba/F3-FLT3 cells. However, expression of Grb10 enhanced FL-induced Akt phosphorylation without affecting Erk or p38 phosphorylation in Ba/F3-FLT3-WT and Ba/F3-FLT3-ITD. Selective Grb10 depletion reduced Akt phosphorylation in Ba/F3-FLT3-WT and OCI-AML-5 cells. Grb10 transduces signal from FLT3 by direct interaction with p85 and Ba/F3-FLT3-ITD cells expressing Grb10 exhibits higher STAT5 activation. Grb10 regulates cell cycle by increasing cell population in S-phase. Expression of Grb10 furthermore resulted in an increased proliferation and survival of Ba/F3-FLT3-ITD cells as well as increased colony formation in semisolid culture. Grb10 expression was significantly increased in AML patients compared to healthy controls and was also elevated in patients carrying FLT3-ITD mutants. The elevated Grb10 expression partially correlated to relapse as well as to poor prognosis. These results suggest that Grb10 binds to both normal and oncogenic FLT3 and induces PI3K-Akt and STAT5 signaling pathways resulting in an enhanced proliferation, survival and colony formation of hematopoietic cells.

Keywords: FLT3; FLT3-ITD; Grb10; AML.

1. Introduction

In response to the external stimuli cell surface receptors undergo post-translational modifications such as phosphorylation and create docking sites for several classes of proteins for further potentiation of the signal. One group of proteins includes adaptor proteins. These proteins interact with specific receptors and recruit other signaling proteins. The Growth factor receptor-bound protein 10 (Grb10) is a member of Grb7 family adaptor proteins which was first identified as an epidermal growth factor receptor (EGFR) binding protein in a screening with bacterial expression libraries (Ooi et al., 1995). Subsequent studies identified multiple receptor tyrosine kinases including Ret (Pandey et al., 1995), insulin-like growth factor 1 (IGF-1) receptor (Dey et al., 1996; Morrione et al., 1996; O'Neill et al., 1996), insulin receptor (IR) (Frantz et al., 1997; Hansen et al., 1996), platelet-derived growth factor receptor β (PDGFR β) (Wang et al., 1999), ELK (Stein et al., 1996) and c-KIT (Jahn et al., 2002) as Grb10 binding partners. Despite interaction with the receptor tyrosine kinases at the cell membrane, several intracellular kinases also associate with Grb10. For example, Grb10 interacts with Raf1, MEK1 and Akt (Jahn et al., 2002; Nantel et al., 1998). Grb10 has also been shown to be associated with growth hormone receptor (GHR) (Moutoussamy et al., 1998), low-density lipoprotein receptor-related protein 6 (LRP6) (Tezuka et al., 2007), Bim L (Hu et al., 2010), p85 (Deng et al., 2003), Nedd4 (Morrione et al., 1999), 14-3-3 (Urschel et al., 2005), Grb10 interacting GYF protein 1 (GIGYF1) and GIGYF2 (Giovannone et al., 2003). This capability of interaction with various proteins allows Grb10 to play diverse functions in various cellular processes, including the regulation of cellular growth, apoptosis, metabolism and cell migration (Holt and Siddle, 2005). Although Grb10 has been more extensively studied in the context of insulin signaling (Riedel, 2004), recent studies suggest an involvement of this adaptor protein in cancer progression (Deng et al., 2008; Mirmohammadsadegh et al., 2004).

Members of Grb7 family are comprised of Grb7, Grb10 and Grb14. All of these adaptor proteins share a common multi-domain structure including multiple protein and lipid binding

domains. An N-terminal proline-rich region functions in binding to the SH3 domain-containing proteins. This follows by Ras-association (RA)-like, pleckstrin homology (PH) and BPS domains. C-terminal Src homology 2 (SH2) plays the role in interaction with the phospho-tyrosine residues of the activated receptors or intracellular signaling proteins (Holt and Siddle, 2005). Presence of multiple functional domains allows Grb10 to associate with multiple signaling proteins. The human Grb10 gene is located on chromosome 7p11.2-p12 that expresses at least seven alternative splice variants playing differential roles in cell signaling depending on the presence of different functional domains (Dong et al., 1997; Frantz et al., 1997; Laviola et al., 1997; Liu and Roth, 1995; O'Neill et al., 1996; Ooi et al., 1995; Riedel, 2004). Grb10 mRNA is highly expressed in skeletal muscle, pancreas, cardiac muscle and brain (Lim et al., 2004), but its expression is not limited only to those tissues. It is also expressed in a variety of tissues including prostate, lung, liver, kidney, spleen, testis, ovary, placenta, colon and small intestine (Lim et al., 2004). Expression of Grb10 has also been reported in multiple human cancer cell lines such as breast, cervix and liver cancer cell lines (Lim et al., 2004) and elevated levels of Grb10 potentiate mitogenesis in response to growth factors (Deng et al., 2008).

The role of Grb10 in downstream receptor signaling is rather complicated. Overexpression of mouse Grb10 α inhibited IGF-1-mediated growth and colony formation of P6 cells (Morrione et al., 1997), while displayed a positive, stimulatory function in PDGF-BB, IGF-1 and insulin action in mouse fibroblasts (Wang et al., 1999). The human Grb10 β reduced insulin-dependent IR substrate 1 (IRS1) phosphorylation as well as phosphoinositide-3 kinase (PI3K) activation in CHO cells (Liu and Roth, 1995) and overexpression in rat primary adipocytes or CHO cells inhibited insulin induced MAPK pathway activation through Shc (Langlais et al., 2004) suggesting an inhibitory role in insulin signaling. Grb10 γ disrupts insulin and IGF-1 signaling by inhibiting catalytic activity of IR and IGF-1R through its BPS domain that blocks substrate interaction (O'Neill et al., 1996; Stein et al., 2001). Furthermore, human Grb10 γ decreased insulin signaling through IRS-PI3K-Akt by limiting IRS access to IR (Wick et al.,

2003). The human Grb10 ζ has been found to be associated with mitochondrial Raf-1 suggesting that Grb10 might play a role in signaling between receptor and apoptosis inducing machinery (Nantel et al., 1999). Grb10-mediated negative regulation of IR or IGF-1R signaling is not only evident by overexpression studies but also established by siRNA-mediated depletion of endogenous Grb10 (Dufresne and Smith, 2005; Langlais et al., 2004). The Grb10 knockout mice displayed improved glucose tolerance as well as insulin sensitivity further suggesting that Grb10 negatively regulates insulin signaling (Holt et al., 2009; Smith et al., 2007; Wang et al., 2007). Moreover, Grb10 knockout mice showed overgrowth in compared to wild-type animals (Charalambous et al., 2003; Shiura et al., 2005). Recently couple of studies suggests that mTORC1 directly phosphorylates Grb10 enhancing Grb10 stability thus acts as a negative regulator of insulin or IGF-1 signaling (Hsu et al., 2011; Yu et al., 2011).

In response to insulin stimulation Grb10 increases degradation of IR (Ramos et al., 2006) probably recruiting Nedd4 (Morrione et al., 1999). The Grb10-Nedd4 complex increased IGF-1R ubiquitination, internalization and degradation in response to ligand stimulation (Vecchione et al., 2003) indicating that Grb10 negatively regulates IGF-1R signaling through not only blocking substrate access but also limiting receptor stability. Although Grb10 accelerated IGF-1R degradation through Nedd4, it is also evident that Grb10 protected VEGFR2 from Nedd4-mediated degradation (Murdaca et al., 2004). Thus Grb10 might behave differentially to different receptors.

Grb10 has been reported to be deregulated in human osteosarcoma (Li et al., 2008), metastatic malignant melanoma (Mirmohammadsadegh et al., 2004) as well as in cervical squamous carcinoma (Okino et al., 2005) suggesting a role in tumorigenesis. Oncogenic tyrosine kinase Bcr-Abl associates with Grb10 in chronic myelogenous leukemia (CML) and this interaction was kinase activation dependent (Bai et al., 1998). Activated Raf-1 and Grb10 regulates Bad phosphorylation through PI3K-Akt and MAPK pathways and promotes cell survival (Kebache et al., 2007). A study with the mouse model of mammary carcinoma

demonstrated that Grb10 is enhanced in tumor cells inducing a robust immunity (O-Sullivan et al., 2008). These studies indicate that Grb10 is predominantly involved in the growth of numerous rapidly proliferating tumors and further studies are required to define its role in oncogenesis and tumor progression.

The Fms-like tyrosine kinase 3 (FLT3) is an important growth factor receptor in hematopoiesis and gain-of-function mutations of the receptor contribute to the transformation of acute myeloid leukemia (AML) (Gilliland and Griffin, 2002). Most common FLT3 mutation in AML includes the internal tandem duplication (ITD) in the juxtamembrane region and the point mutation at Asp835 in the kinase domain. These mutations cause ligand-independent activation of the receptor as well as uncontrolled activation of downstream signaling. Analysis of patient samples suggests the presence of FLT3 mutations in at least 30% of AML (Gilliland and Griffin, 2002). The ITD mutation varies from three to more than 400 base pairs and the size negatively correlates to the five year overall survival of AML patients (Stirewalt et al., 2006).

FLT3 can activate multiple signaling pathways including PI3K-Akt and Ras-Erk pathways (Masson and Ronnstrand, 2009). FLT3-ITD mutant displays qualitative differences in signal transduction from the wild type FLT3 by activating additional signaling pathways through STAT5 (Masson et al., 2009). The FLT3 signaling might be regulated by protein tyrosine phosphatases and ubiquitin ligases. The protein tyrosine phosphatase DEP-1 interacts with FLT3 as well as dephosphorylates several phospho-tyrosine residues in FLT3 juxtamembrane domain, and shRNA-mediated DEP-1 knockdown stimulates proliferation and clonal growth of FLT3-ITD transfected 32D cells (Arora et al., 2011) suggesting a role of the phosphatase in FLT3 regulation. Ubiquitin ligase Cbl has also been reported to interact with FLT3 initiating covalent association of multiple ubiquitin molecules followed by proteasomal degradation (Sargin et al., 2007). Loss of function mutations in Cbl contributes to oncogenic transformation in AML (Reindl et al., 2009). A number of adaptor proteins also regulate FLT3 signaling by linking receptor to the signaling proteins. For example Grb2 binds to FLT3 and creates docking site for Gab2 resulting

in activation of the downstream signaling (Masson et al., 2009). The suppressor of cytokine signaling 6(SOCS6) associates with FLT3 initiating ubiquitination followed by degradation of receptor resulting in an inhibition of downstream signaling (Kazi et al., 2012).

This study describes the role of Grb10 in FLT3-mediated AML. The Grb10 associates with FLT3 through pY⁵⁷² and pY⁷⁹³ residues. This association leads to Grb10 tyrosine phosphorylation and creates a docking site for p85 resulting in Akt activation through PI3K. The Grb10 regulates cell cycle by increasing S-phase population and also increases FLT3-ITD-mediated cell proliferation, survival and colony formation. Grb10 expression is found to be increased in AML and further elevated in patients carrying FLT3-ITD mutation. Furthermore, pediatric AML patients having higher Grb10 expression failed to enter drug-mediated complete remission as well as relapsed within a short time.

2. Materials and methods

2.1. Reagents and antibodies

Transfection reagents jetPEI and Lipofectamine 2000 were from Polyplus-transfection and Life Technologies respectively. The anti-FLT3 antibody was described previously (Razumovskaya et al., 2009). The phospho-tyrosine antibody 4G10 was from Millipore and anti-phospho-p38 as well as anti-p38 antibodies were from BD Transduction Laboratories. The anti-phospho-Akt antibody was from Epitomics. Polyclonal anti-Akt, anti-STAT5, anti-Grb10, anti-phospho-Erk, anti-Erk and horseradish peroxidase-coupled secondary anti-goat antibodies were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-coupled secondary anti-mouse and anti-rabbit antibodies were from Life Technologies. All inhibitors were from Calbiochem. FLT3 ligand (FL) was from Prospec Tany. The anti-phospho-Gab2 and anti-p85 antibodies were from cell signaling.

2.2. Expression constructs

Expression constructs pcDNA3-FLT3-WT, pMSCV-FLT3-WT and pMSCV-FLT3-ITD were described previously (Heiss et al., 2006; Razumovskaya et al., 2009). The pcDNA3-FLAG-

Grb10-WT construct was constructed by sub-cloning full length open reading frame of human Grb10 β (U66065) into pcDNA3 vector. The pcDNA3-FLT3-K644A and pcDNA3-FLT3-Y572F/Y793F constructs were generated by site-directed mutagenesis using QuikChange mutagenesis XL kit (Stratagene, La Jolla, CA).

2.3. Cell culture and transfection

Ba/F3 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 units/ml penicillin, 100 μ g/ml streptomycin and 10 ng/ml recombinant murine interleukin-3 (IL-3). OCI-AML-5 cells were cultured in α -MEM supplemented with HI-FBS and 10ng/ml human recombinant IL3. COS-1 and EcoPack cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. COS-1 cells were transiently transfected using JetPEI according to manufacturer's instructions. To establish Ba/F3 cells stably expressing the wild-type or mutant form of the human FLT3, packaging EcoPack cells were transfected with the corresponding pMSCV-puro-FLT3 construct. Virus-containing supernatants were collected 72h after transfection. Ba/F3 cells were incubated in virus-containing supernatants for 24h followed by a 2-week selection in 1.2 μ g/ml of puromycin. The surface expression of FLT3 was checked by flow cytometry using a phycoerythrin-conjugated anti-FLT3 antibody. For transfection of siRNA, pcDNA3-FLAG-Grb10-WT or empty vector in Ba/F3 or AML cell line 4D-Nucleofector (Lonza) was used. Cells were selected with 0.8 mg/ml G-418 for around 2 weeks and Grb10 expression was verified by Western blotting.

2.4. Immunoprecipitation and Western blotting

The COS-1 cells were serum-starved overnight 24h after transfection, while Ba/F3 cells were serum-starved for 4h before stimulation. Cells then stimulated at 37°C for the indicated period of time with 100 ng/ml FL. After stimulation cells were washed once with ice-cold PBS, lysed, and processed for immunoprecipitation and Western blotting as described previously (Voytyuk et al., 2003). Immunodetection was done by enhanced chemoluminescence using Immobilon Western

chemoluminescent horseradish peroxidase substrate (Millipore Corporation, Milford, MA, USA) and a CCD camera (LAS-3000, Fujifilm, Tokyo, Japan). Where needed signal intensity was quantified by Multi-Gauge software (Fujifilm).

2.5. Affinity fishing of Grb10 with immobilized FLT3-peptides

The phospho-peptides and unphospho-peptides corresponding to the tyrosine motifs of FLT3 intracellular domain (for sequence see (Kazi et al., 2012; Lin et al., 2012)) were immobilized on UltraLink (Thermo Scientific) according to the manufacturer's instructions. Then immobilized peptides were incubated with Grb10-transfected COS-1 cell lysates at 4°C for 2h. The Grb10 proteins pulled-down by FLT3-peptides were then processed for Western blotting.

2.6. Cell cycle analysis, cell proliferation and apoptosis assay

Ba/F3 cells were washed three times with RPMI-1640 medium and seeded in 24-well plates (60 000 cells/well). Cells were then incubated with or without 100 ng/ml FL or 10 ng/ml IL-3 for 48h. Viable cells were then counted using trypan blue exclusion method. Cells were then stained by Click-iT EdU Alexa Fluor 488 (for cell cycle) or Click-iT EdU Alexa Fluor 647 (cell proliferation) kits (Life Technologies) using manufacturer's protocol and then analyzed by flow cytometry. Apoptosis was measured using an Annexin-V, 7-Amino-actinomycin D (7-AAD) kit (BD Biosciences Pharmingen), according to the manufacturer's instructions; double negative (Annexin-V)/7-AAD)) cells represent viable cells.

2.7. Colony formation in methylcellulose medium

Ba/F3-FLT3-ITD/empty vector or Ba/F3-FLT3-ITD/Grb10 cells were washed three times to remove cytokine and then cultured in semisolid methylcellulose medium (MethoCult M3231, Stem Cell Technologies), in the presence or absence of 100 ng/ml FL, for 5 days.

2.8. Analysis of micro-array data and statistical analysis

Micro-array data of Grb10 expression from patient samples were downloaded from NCBI Geo site. Six representative datasets GSE9476 (AML), GSE5550 (CML), GSE2403 (B-CLL), GSE2550 (APL), GSE2191 (AML) and GSE13280 (ALL) were used for analysis. Data were

normalized and scaled using statistical program 'R' or manual Excel equations. Statistical analyses were done by GraphPad Prism 5. All results were expressed in mean and error bars were calculated using standard error of means (SEM). The unpaired t-test was used for comparisons between groups of data. P-values of less than 0.05 were considered as statistically significant.

3. Results

3.1. Grb10 stably interacts with FLT3 in response to FL

The family of PDGF receptor tyrosine kinases comprises of PDGFR α , PDGFR β , CSF1R, c-KIT and FLT3 (Lemmon and Schlessinger, 2010). The adaptor protein Grb10 associates with PDGFR β and c-KIT in response to the external stimuli (Jahn et al., 2002; Wang et al., 1999). This association is mediated through Grb10 SH2-domain and phospho-tyrosine residues of receptors. To determine whether Grb10 can also interact with other PDGF receptor tyrosine kinases, we transiently expressed FLT3 and FLAG-tagged Grb10 in COS-1 cells. We were able to immunoprecipitate FLT3 from the cell lysates using an anti-FLAG antibody (Fig. 1A, upper panels). Furthermore Grb10 was also immunoprecipitated using an anti-FLT3 antibody (Fig. 1A, lower panels). A constitutive Grb10-FLT3 association was observed in COS-1 cells overexpressing Grb10 and FLT3-ITD (Fig. 1B). In addition we could show Grb10-FLT3 association in OCI-AML-5 cells using an anti-FLT3 antibody (Fig. 1C). The OCI-AML-5 cell line that established from the peripheral blood of a 77-year-old man expresses both FLT3-WT and Grb10 endogenously and responds to G-CSF, GM-CSF, IL-3, and FL (for more information see DSMZ.de, ACC 247). Unfortunately we were unable to detect binding using an anti-Grb10 antibody probably due to the poor specificity of commercial Grb10 antibody. Grb10-FLT3 association was FL-stimulation dependent and probably FLT3-activation is required for the binding. To explore this issue further, we generated a kinase dead mutant of FLT3. Grb10 did not associate to the ligand-stimulated kinase dead mutant of FLT3 (Fig. 1D). The interaction with wild-type FLT3 was fairly stable and did not decrease more than 50% after 60 minutes of ligand

stimulation (Fig. 1E). In addition, the interaction kinetics was also determined from a plot of relative binding against the FL-stimulation time. A linear ($r^2=0.82$) reduction of binding was observed (Fig. 1F). A plot of Grb10-Flt3 relative binding against FLT3 tyrosine phosphorylation showed a linear ($r^2=0.78$) relationship between interaction and FLT3 activation (Fig 1G).

3.2. FLT3 phosphorylates Grb10 on tyrosine residues

The Grb10 tyrosine phosphorylation in intact cells remains debated. In response to external stimuli such as EGF and stem cell factor (SCF), Grb10 undergoes serine/threonine phosphorylation but not tyrosine phosphorylation (Jahn et al., 2002; Ooi et al., 1995). Furthermore, p42/44 MAPK directly phosphorylates Grb10 on Ser150 and Ser476 residues in response to insulin stimulation (Langlais et al., 2005). However, tyrosine kinase Tec, but not Syk or Jak2, strongly induced tyrosine phosphorylation of Grb10 (Mano et al., 1998). These findings suggest that Grb10 shows certain degree of specificity to the upstream tyrosine kinases as well as to different stimuli. Thus we investigated whether Grb10 can be tyrosine-phosphorylated in response to FL. Cells expressing either Grb10 alone or in combination with FLT3 were stimulated with FL. We observed that Grb10 undergoes tyrosine phosphorylation in response to the FL-stimulation only in Flt3 expressing cells (Fig. 2A). Grb10 phosphorylation was stable at least for 10 minutes of FL-stimulation (Fig. 2B). Moreover this phosphorylation shows a linear ($R^2=0.79$) decrease over the stimulation time (Fig. 2C). In addition to that, Grb10 phosphorylation decreased with the decrease of FLT3 tyrosine phosphorylation as well as Grb10-FLT3 interaction (Fig. 2D). One study suggests that insulin-induced Grb10 tyrosine phosphorylation was mediated by Src family kinases (SFKs) but not by the insulin receptor itself (Langlais et al., 2000). Thus we reasoned that FL-induced Grb10 phosphorylation can be also mediated by SFKs which has shown to be activated upon FLT3 activation (Heiss et al., 2006). Then we blocked SFKs by well know inhibitors PP1 and PP2. Both inhibitors were unable to block FL-induced Grb10 tyrosine phosphorylation (Fig. 2E).

3.3. Grb10 interacts with FLT3 tyrosine 572 and 793 residues

Grb10 associates with receptor tyrosine kinase through Grb10-SH2 domain (Jahn et al., 2002). This association might be mediated by tyrosine residues of activated receptor. FLT3 intracellular domain contains at least 12 tyrosine residues which are predicted to be phosphorylated upon activation. The data presented above suggest that Grb10-FLT3 interaction is dependent on FLT3 tyrosine phosphorylation. Thus we speculated that Grb10-FLT3 interaction might also be mediated by FLT3 phospho-tyrosine residues and Grb10 SH2 domain. We screened Grb10 binding sites in FLT3 by using synthetic peptides corresponding to the FLT3 phospho-tyrosine residues. We observed that phospho-tyrosine peptides corresponding to FLT3 tyrosine residues 572 and 793 can pull-down Grb10 protein from cell lysates and that pY793 residue has higher affinity to the Grb10 protein (Fig. 3A). Then we generated FLT3-Y572F/Y793F mutant to further explore the binding specificity. Co-transfection of FLT3 mutant with Grb10 shows a decrease of binding in the cells expressing FLT3-Y572F/Y793F mutant (Fig. 3B).

3.4. Overexpression of Grb10 did not alter FLT3 receptor phosphorylation

Although FLT3 is overexpressed in around 90% of AML patients (Carow et al., 1996), normal FLT3 expression is limited mainly to the myeloid and lymphoid progenitor cells (Rosnet et al., 1996). To study the role of Grb10 on FLT3 downstream signaling we used murine pro-B cell line Ba/F3 cell as a model. Ba/F3 cells do not express either FLT3 or its ligand but a minimum level of Grb10 expression was observed (Fig. 4A). Thus we generated stably transfected Ba/F3-FLT3-WT, Ba/F3-FLT3-ITD, Ba/F3-FLT3-WT/Grb10 and Ba/F3-FLT3-ITD/Grb10 cell lines for this study. Grb10 and FLT3 expression levels were verified by western blotting (Fig. 4A) and flow cytometry (Fig. 4B) respectively. It has been shown that Grb10 binds the E3 ubiquitin ligase Nedd4 (Morrione et al., 1999) and promotes ligand-stimulated internalization, ubiquitination, and degradation of the IGF-I receptor (Monami et al., 2008). To assess the effect of Grb10 expression on FLT3, we measured the activation and the stability of the receptor. Grb10 expression did not

significantly alter ligand-induced receptor phosphorylation as well as ubiquitination (Fig. 4C), internalization (Fig. 4D) or degradation (Fig. 4E).

3.5. Grb10 increased Akt signaling but not Erk or p38 signaling in response to the FLT3 activation

Recent studies suggest that Grb10 is involved in regulation of receptor downstream signaling (Jahn et al., 2002; Yu et al., 2011). FLT3 ligand induces activation of three major pathways leading to phosphorylation of Akt, Erk 1/2 and p38 (Masson and Ronnstrand, 2009). Using phospho-specific antibodies we observed an increased Akt-phosphorylation in Grb10 expressing cells while p-Erk 1/2 and p-p38 levels remained unchanged (Fig. 5A). Selective knockdown of endogenous Grb10 (Fig. 5B) led to a decrease of Akt phosphorylation in Ba/F3-FLT3-WT cells (Fig. 5C). Overexpression of Grb10 in Ba/F3-FLT3-ITD cells also increased Akt phosphorylation (Fig. 5D). Furthermore, siRNA-mediated depletion of Grb10 in OCI-AML-5 cells (Fig. 5E) decreased FL-induced Akt phosphorylation (Fig. 5F). These observations were further verified by quantification of blots from multiple experiments followed by statistical analysis (Fig. 5G).

3.6. Grb10-mediated increased Akt activation is independent to SFKs or Syk activation

Presence of multiple functional domains allows Grb10 to interact with a variety of signaling molecules. Grb10 has shown to be directly associated to the Akt (Jahn et al., 2002). Thus it is possible that Grb10 might activate Akt other than PI3K pathway. To test this hypothesis we blocked PI3K activity by using selective PI3K inhibitor. We observed a complete inhibition of Akt phosphorylation in Ba/F3-FLT3/Grb10 cells (Fig. 6A) indicating that functional PI3K is required for Grb10-mediated Akt activation. The PI3K can be activated through multiple pathways. The SFKs are the major kinases downstream to the receptor required for PI3K activation through Gab2 tyrosine phosphorylation (Masson and Ronnstrand, 2009). The SFKs inhibitor partially blocked Grb10 mediated Akt phosphorylation (Fig. 6A). Although SFKs inhibitor partially blocked FL-induced Akt activation, it was unable to block Grb10-mediated increased Akt activation (Fig. 6B) suggesting that SFKs are not involved in Grb10-initiated Akt

activation. Another cytosolic tyrosine kinase Syk is known to activate Gab2 leading to Akt activation in response to the external stimuli (Yu et al., 2006). However, a specific Syk kinase inhibitor did not block increased Akt activation (Fig. 6C).

3.7. Grb10-mediated increased Akt activation is facilitated by Grb10-p85 interaction

The data presented above suggest that Grb10 mediated increased PI3K-Akt pathway activation is not facilitated by either SFKs or Syk. This observation further supported by the data that FL-induced Gab2 phosphorylation was unchanged in Grb10 expressing cells (Fig. 7A). Another possibility of activation this pathway is through SHP2. The FL induces SHP2 tyrosine phosphorylation that further activates PI3K-Akt pathway (Masson and Ronnstrand, 2009). Expression of Grb10 did not significantly change the tyrosine phosphorylation of SHP2 in response to FL-stimulation (Fig. 7B). PI3K can be directly activated by receptor tyrosine kinases, such as c-Kit and PDGFR, through association of p85 SH2 domain and receptor phospho-tyrosine residue. However there is evidence that human FLT3 does not directly associate with p85. A report described that Grb10 regulates PI3K activity by interacting with the p85 subunit of PI3K and links between PI3K and IR (Deng et al., 2003). This interaction further regulates specific insulin mediators downstream to the PI3K including Akt, p70^{S6K} and p38 (Deng et al., 2003). Thus we hypothesized that Grb10 might play a role in linking FLT3 to PI3K. We immunoprecipitated p85 with an anti-p85 antibody from FL stimulated and unstimulated cell lysates and observed that p85 is associated with Grb10 in response to the FL-stimulation (Fig. 7C and 7D).

3.8. Grb10 regulates cell cycle and increases cell proliferation and survival

Akt activity facilitates cell cycle progression through transcriptional activation of cyclins or phosphorylation-dependent inhibition of regulatory proteins (Franke et al., 2003). As we observed Grb10 expression led to an aberrant Akt activation, we thought that Grb10 might also play a role in cell cycle regulation. Ba/F3-FLT3-ITD cells expressing Grb10 or empty vector were cultured for 46h with or without FL. Then cells were incubated with 20 μ M of EdU for further 2h

followed by azide-coupled Alexa flour 488 and 7AAD staining. Cells were then analyzed by flow cytometry. Grb10 expression resulted in a considerable increase of S-phase population (Fig. 8A). Furthermore, Grb10 expression decreased G2-M population while G1 population remained unchanged (Fig. 8A). Increased S-phase population is related to the increased cell proliferation. To test this theory we further measured cell proliferation in both Ba/F3-FLT3-WT and Ba/F3-FLT3-ITD cells. Expression of Grb10 significantly increased cell proliferation after 48h in trypan blue exclusion assays (Fig. 8B and 8C). Similar result was also observed in the EdU cell proliferation assays (Fig. 8D). Selective knockdown of endogenous Grb10 in OCI-AML-5 cells displayed a significant decrease of FL-dependent cell proliferation (8E). It worth to note that in both Ba/F3-FLT3-WT and OCI-AML-5 cells we did not observe any significant difference in cell proliferation without cytokines might be due to the fact that both cell lines need cytokines for proliferation. Grb10 expression significantly decreased apoptosis of Ba/F3-FLT3-WT cells upon IL-3 withdrawal (Fig. 8F). Expression of FLT3-ITD promotes cytokine independent survival of Ba/F3 cell. We observed around 80% of cell survival of Ba/F3-FLT3-ITD cells after IL-3 withdrawal. Grb10 expression significantly contributed to the Ba/F3-FLT3-ITD cell survival by decreasing apoptosis (Fig. 8G).

3.9. Grb10 increases FLT3-ITD induced STAT5 phosphorylation and colony formation

FLT3-ITD activates STAT5 pathway leading to cellular transformation through adapter protein Gab2 (Masson et al., 2009). Thus we tested whether Grb10 has a role in STAT5 activation. Overexpression of Grb10 in Ba/F3 cells expressing FLT3-ITD increased STAT5 activation (Fig. 9A and 9C) while shRNA-mediated Grb10 depletion resulted in a decreased STAT5 activation (Fig. 9B and 9C). Furthermore cells expressing Grb10 has higher colony formation potential in comparison to empty control vector (Fig. 9D).

3.10. Grb10 expression is increased in AML

The role of Grb10 in cancer has been studied and an increased Grb10 expression was reported in melanoma metastases (Mirmohammadsadegh et al., 2004). To define the role Grb10 in leukemia,

we analyzed microarray data from patient samples. Analysis of a dataset of 20 healthy donors and 26 AML patients showed an increased Grb10 expression ($P < 0.001$) in AML patients (Fig. 10A left). Grb10 expression also was significantly upregulated ($P < 0.0001$) in chronic myelogenous leukemia (CML) patients (Fig. 10A right). To test the hypothesis that Grb10 expression might be regulated by FLT3 mutation, we analyzed a set of microarray data from 18 acute promyelocytic leukemia (APL) patients. APL represents about 10–15% of AMLs. In this dataset, 7 patients had FLT3-ITD mutation while other 7 patients carried FLT3 gene in wild-type configuration. We observed an increased Grb10 expression ($P < 0.01$) in patients carrying FLT3-ITD mutation (Fig. 10B). Most of AML patients enter a complete remission after chemotherapy treatment. However, a large number of patients experience relapse with resistant disease. An analysis of Grb10 expression from 54 pediatric AML patients' microarray data revealed that an elevated Grb10 expression results in relapse with resistant disease (Fig. 10C left). Moreover analysis of a dataset from primary lymphocytes from B-cell chronic lymphocytic leukemia (B-CLL) patients with indolent or progressive disease disclosed a positive correlation of Grb10 expression with progressive disease (Fig. 10C middle). In addition, we analyzed a dataset from pediatric acute lymphoblastic leukemia (ALL) patients. Samples were divided into two groups such as apoptosis-sensitive and resistant phenotype. A significant increase of Grb10 expression was observed in apoptosis-resistant samples (Fig. 10C right). Frequent loss of expression of the tumor suppressor PTEN in cancer is linked to constitutive Akt activation. As we observed Grb10 expression resulted in hyper-activation of Akt, we analyzed PTEN expression in leukemia patients. However, we did not observe any significant change in PTEN expression either in AML or in CML patients (Fig. 10D).

4. Discussion

Grb10 interacts with multiple growth factor receptors and plays differential roles depending on tissues and splice forms. Although Grb10 has been extensively investigated in the context of

insulin signaling, its role in cancer is poorly defined. In this report we put the evidence that Grb10 expression has a prognostic value in leukemia. We show that Grb10 associates with FLT3 in response to the FL stimulation and this association leads to hyperactivation Akt followed by increased cell proliferation.

Growth factor stimulation regulates cellular signaling by initiating post-translational modifications, mainly phosphorylation of receptors as well as downstream signaling proteins. Grb10 undergoes tyrosine phosphorylation following the receptor activation which is reported to be mediated by non-receptor tyrosine kinases Tec, Src and Fyn (Giorgetti-Peraldi et al., 2001; Langlais et al., 2000; Mano et al., 1998). The FL-induced Grb10 tyrosine phosphorylation decreased with the decrease of FLT3 activation as well as Grb10-FLT3 interaction, and Src family inhibitors PP1 and PP2 were unable to block this phosphorylation. These observations indicate that Grb10-FLT3 interaction and also FLT3 activation are indispensable for Grb10 tyrosine phosphorylation and that SFKs are not involved in this process. Grb10 tyrosine phosphorylation might be facilitated by FLT3 and it is of interest to show Grb10 as a FLT3 substrate.

Grb10 associates with FLT3 through pY⁵⁷² and pY⁷⁹³ residues. The FLT3 auto-phosphorylation sites Y⁵⁷² and pY⁷⁹³ are identified by our group in intact cell in two different studies (Heiss et al., 2006; Razumovskaya et al., 2009). However, importance of these two residues in receptor signaling remains unknown. The FLT3-Y⁵⁷² is located in the juxtamembrane domain together with three auto-phosphorylation sites which play important roles by recruiting Src family kinases (Heiss et al., 2006). The FLT3-Y⁷⁹³ is located in the kinase domain. Interaction with two tyrosine residues might be mediated by Grb10 dimers. Grb10 has been reported to form a dimer in solution under physiological condition by creating a non-covalent dimer through its SH2 domain and after dimerization Grb10 SH2 domains are still available for association with phospho-tyrosine residues (Stein et al., 2003). Grb10 displays a degree of binding specificity to the FLT3 tyrosine residues. We observed pY⁷⁹³ is the major binding site in peptide fishing study.

FLT3-pY⁷⁹³ might play crucial roles in cell signaling. We also observed several SH2 domain containing proteins in addition Grb10 associate with this residue (Kazi and Rönstrand, unpublished data).

Grb10 act as an adaptor by linking several signaling molecules to activated receptor. For example Grb10 creates a bridge in between IGF-IR and Nedd4 resulting in ubiquitination and degradation of the receptor (Vecchione et al., 2003). Grb10 expression did not show an effect on either FLT3 receptor stability or receptor phosphorylation. This suggests that FLT3 itself is not the target of Grb10 but rather downstream signal transduction molecules. Activation of mTORC1 in response to insulin or IGF-1 initiates Grb10 phosphorylation leading to feedback inhibition of receptor as well as downstream pathways (Arora et al., 2011; Hsu et al., 2011; Yea and Fruman, 2011). The enzymes involved in insulin signaling pathway including glycogen synthase kinase, glycogen synthase, PI3K and Akt were stimulated by increased level of Grb10 in response to insulin activation, while key enzymes in alternative signaling pathways such as Cbl, p38 and p70^{S6} kinase remained unaffected in similar conditions (Riedel, 2004). The key pathways which have been critically implicated in the survival responses to the growth factors involve phosphorylation of three serine/threonine kinases including Akt, p38 and Erk 1/2 (Masson and Rönstrand, 2009). An increased Grb10 levels related to the aberrant Akt activation in response to FL stimulation. In contrast, p38 and Erk 1/2 activation levels remained unchanged in the same system. Elevated Grb10 expression displayed similar alterations of c-Kit signaling (Jahn et al., 2002). These findings suggest that Grb10 is highly selective in activation of downstream signaling and that Grb10 expression induces increased Akt activation.

Akt is located downstream of PI3K. The observation that selective PI3K inhibitor completely blocked FLT3-Grb10-mediated Akt activation put further the evidence that PI3K is required to relay signal from FLT3 to Akt through Grb10. Several cytosolic kinases, phosphatases and adaptor proteins might be involved in this process. For example, FLT3 activates SFKs leading to phosphorylation of Gab2 followed by Akt activation (Masson and Rönstrand, 2009).

Gab2 has also been shown to be phosphorylated by Syk kinase leading to activation of downstream pathways (Yu et al., 2006). An attempt to block signal to Akt using either Src family kinase or Syk inhibitor failed demonstrating that Grb10 activates alternative pathways other than SFKs or Syk to activate PI3K. The protein tyrosine phosphatase SHP2 associates with FLT3 and plays an important role for PI3K activation (Heiss et al., 2006; Masson and Ronnstrand, 2009). Grb10 expression did not change FL-induced SHP2 phosphorylation suggesting that SHP2 is not involved in Grb10-mediated increased Akt activation. The regulatory subunit of PI3K has been shown to interact with Grb10 providing a link to PI3K and insulin receptor (Deng et al., 2003). A Grb10-p85 complex was detectable only in FL-stimulated cell indicating that Grb10 acts as an adaptor to recruit PI3K to the receptor in response to FL-stimulation resulting in aberrant Akt activation.

The PI3K-Akt signaling network is critical to widely divergent physiological processes including cell proliferation, cell cycle progression, cell survival and differentiation (Martelli et al., 2006). Activation of this signaling network ensues in disturbance of regulation of cell proliferation and apoptosis, resulting in competitive growth advantage for tumor cells. Furthermore, about 70% of AML patients display phosphorylation for both serine/threonine residues of Akt and upregulation of PI3K-Akt pathway significantly reduces overall survival. Although the mechanisms that upregulate PI3K-Akt pathway have been studied extensively in many types of cancer, it has still not been fully understood in AML cells. It is well known that Ras can activate the PI3K-Akt pathway and the oncogenic mutation in Ras has been detected in 15-25% of AML patients (Martelli et al., 2006). Another mechanism of activation is gain of function mutation of receptor tyrosine kinases. A number of AML patients carry an FLT3-ITD that can activate PI3K-Akt through SFKs. The data presented here suggest that Grb10 expression further potentiate FLT3-ITD signaling as well as describe a new mechanism of PI3K-Akt pathway activation.

The serine/threonine kinase Akt regulates the cell cycle by altering p21^{Cip1} and p27^{KIP1} activity (Chang et al., 2003). Deregulation of p21^{Cip1} and p27^{KIP1} activity results in aberrant DNA synthesis and cell proliferation. The observation that Grb10 increased cell proliferation and survival of Ba/F3-FLT3-ITD cells might be due to the hyperactivation of PI3K-Akt pathway. This increased cell proliferation is mediated by probably increased S-phase population. Deletion of PTEN from embryonic stem cells exhibits similar phenotype in cell cycle regulation while inhibition of PI3K in HEK293 cells promotes G2 arrest (Kandel et al., 2002). Thus, the biological outcome of Grb10 expression is similar to that of deregulated Akt activation.

The critical difference in normal and oncogenic FLT3 signaling is FLT3-ITD activates STAT5 pathway leading to cellular transformation. This process is partially mediated through adaptor protein Gab2 (Masson et al., 2009). We also observed Grb10 potentiated STAT5 signaling as well as colony formation in semisolid medium. Thus we suggest that in addition to activation of PI3K-Akt pathway, Grb10 activates STAT5 pathway that also contribute to cell survival.

Uncontrolled gene expression is a common feature in cancer. The expression of tumor suppressor genes is down-regulated in most of cancer, while expression of oncogenes or proto-oncogenes is up-regulated. An analysis of samples from AML patients and healthy donors revealed an abundance of Grb10 expression in many patients suggesting the clinical significance of Grb10 in leukemic transformation. Aberrant Grb10 expression in CML further provided the evidence that elevated Grb10 expression is required for the pathogenesis of leukemia.

Patients carrying FLT3-ITD mutations had significantly increased Grb10 expression suggesting that oncogenic FLT3 mutation initiates Grb10 expression that further contributes to leukemic progression through elevated PI3K-Akt pathway activation. Loss of the tumor suppressor gene PTEN displays similar phenotypes in cancer. However, we did not observe a decrease in PTEN expression in different patients. Thus we suggest that FLT3-ITD induced

leukemic pathogenesis is at least partly mediated by Grb10 through PI3K-Akt pathway activation and Grb10 expression might be sufficient for hyper-activation of PI3K-Akt pathway.

The development of resistance to the treatment with kinase inhibitors in leukemia patients is an increasing area of concern. Multiple factors have been identified as potential reasons of resistance including higher expression of FOXO3A, PIM-1, PIM-2 and survivin as well as parallel activation of PI3K/Akt and Ras/MAPK pathways (Weisberg et al., 2010). Grb10 expression was significantly higher in ALL patient samples displaying resistance to the radiation and also in pediatric AML increased Grb10 expression is correlated with relapse and resistance to therapy. These observations further put the evidence in favor that Grb10 expression partially helps cells to get resistance to the targeted therapy promoting relapse.

Taken together our results suggest that Grb10 is highly expressed in leukemia and that elevated Grb10 expression is associated with ALM cell survival mediated by leukemic FLT3-ITD. This effect is partly mediated through activation of PI3K-Akt pathway by Grb10-FLT3 interaction as well as STAT5 activation and thus inhibition of Grb10 activity could be an alternative drug target in leukemia.

Conflict of interest

Authors declare no conflict of interest.

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Figure legends

Fig. 1: Grb10 interacts with FLT3 in response to FL-stimulation.

(A) COS-1 cells were co-transfected with pcDNA3-FLAG-Grb10 and pcDNA3-FLT3-WT expression plasmids. Six hours after transfection, cells were serum-starved overnight and then stimulated by FL or not for 5 minutes before lysis. Cell lysates were immunoprecipitated with an anti-FLT3 or anti-FLAG antibody followed by Western blotting analysis. (B) COS-1 cells were co-transfected with pcDNA3-FLAG-Grb10 and pcDNA3-FLT3-ITD expression plasmids. Six hours after transfection, cells were serum-starved overnight and then stimulated by FL or not for 5 minutes before lysis. Cell lysates were immunoprecipitated with an anti-FLT3 or anti-FLAG antibody followed by Western blotting analysis. (C) OCI-AML-5 cells were lysed and subjected to the immunoprecipitation analysis with an anti-FLT3 antibody. Then proteins were separated by SDS-PAGE followed by Western blotting analysis. (D) COS-1 cells were transfected with pcDNA3-FLAG-Grb10 and pcDNA3-FLT3-K644A plasmids. Cells were then serum-starved, stimulated with FL before lysis, immunoprecipitated and analyzed by Western blotting. (E) Grb10 and FLT3 transfected COS-1 cells were stimulated for different time points after overnight starvation. Cells were then processed for immunoprecipitation and Western blotting analysis. (F) Amount of FLT3 from three individual experiments was quantified and normalized against Grb10 expression. (G) Levels of Phospho-Y-FLT3 were quantified from the three individual experiments and plotted against relative binding. R^2 values were calculated by GraphPad prism 5.

Fig. 2: Grb10-FLT3 interaction leads to Grb10 tyrosine phosphorylation.

(A) COS-1 cells were co-transfected with pcDNA3-FLAG-Grb10 and/or pcDNA3-FLT3-WT expression plasmids. Six hours after transfection, cells were serum-starved overnight and then

stimulated by FL or not for 5 minutes before lysis. Cell lysates were used for Western blotting analysis. (B) Grb10 and FLT3 transfected COS-1 cells were stimulated for different time points after overnight starvation. Cells were lysed and analyzed by Western blotting. (C) Levels of phospho-Grb10 from three individual experiments were quantified and normalized against Grb10 expression. (D) Levels of Phospho-Y-FLT3 and relative binding from three individual experiments were plotted against average phospho-Grb10 values. (E) Grb10 and FLT3 transfected COS-1 cells were treated with 10 μ M PP1 or 15 μ M PP2 for 60 minutes and stimulated for 5 minutes after overnight starvation. Cells were lysed and analyzed by Western blotting. R² values were calculated by GraphPad prism.

Fig. 3. Grb10 interacts with FLT3 tyrosine 572 and 793 residues

(A) Phospho-peptides corresponding to 12 known tyrosine phosphorylation sites in FLT3 were immobilized on UltraLink. Peptide-bound slurry was incubated with Grb10-transfected COS-1 cell lysates and after pulled-down; proteins were processed for Western blotting using an anti-Grb10 antibody. (B) COS-1 cells were transfected with Grb10 and FLT3 wild type or mutant plasmids. Cells were then serum-starved, stimulated with FL before lysis, immunoprecipitated and analyzed by Western blotting.

Fig. 4. Expression of Grb10 did not alter receptor stability.

(A) Ba/F3 cells were transfected with pcDNA3-FLAG-Grb10-WT or empty vector by 4D-nucleofector. One day after transfection cells were subjected to 0.8 mg/ml G-418 selection. Expression levels were checked by western blotting. (B) Grb10 or empty vector-transfected Ba/F3 cells were further transfected with FLT3-WT or FLT-ITD retroviral plasmids (pMSCVpuro) and selected with 1.2 μ g/ml of puromycin. Surface expression was assessed by flow cytometry with an anti-FLT3 antibody. Dark area shows the FLT3 expression. (C) Cells were serum starved for 4h and then stimulated with FL. Stimulation was stopped with cold PBS followed by lysis. Cell lysates were subjected to immunoprecipitation with an anti-FLT3 antibody and then analyzed by Western blotting. Auto radiograms were quantified with Multi-Gauge

software. Statistical analysis was done using GraphPad Prism. (D) Cells were serum starved and treated with cycloheximide for 4h. After FL-stimulation cells were labeled with a PE-conjugated anti-FLT3 antibody and analyzed by flow cytometry. (E) Cells were serum starved and treated with cycloheximide for 4h before FL-stimulation. After stimulation cells were lysed and immunoprecipitated with an anti-FLT3 antibody followed by Western blotting. Band intensity was measured by Multi-Gauge software.

Fig. 5. Grb10 increases Akt phosphorylation but not Erk phosphorylation

(A) Ba/F3-FLT3-WT cells transfected with Grb10 or empty vector were serum starved for 4h and stimulated with FL for different time points. After lysis total cell lysate was analyzed by Western blotting. (B) Ba/F3-FLT3-WT cells were transfected with Grb10 shRNA or control shRNA by 4D-nucleofector. One day after transfection cells were subjected to 0.8 mg/ml G-418 selection. Expression levels were checked by western blotting. (C) Ba/F3-FLT3-WT cells transfected with Grb10 shRNA or control shRNA were serum starved for 4h and stimulated with FL for different time points. After lysis total cell lysate was analyzed by Western blotting. (D) Ba/F3-FLT3-ITD cells transfected with Grb10 or empty vector were serum starved for 4h and stimulated with FL for different time points. After lysis total cell lysate was analyzed by Western blotting. (E) OCI-AML-5 cells were transfected with Grb10 siRNA or control siRNA by 4D-nucleofector. Two days after transfection cells were lysed and Grb10 expression levels were checked by western blotting. (F) OCI-AML-5 cells transfected with Grb10 siRNA or control siRNA were serum starved for overnight one day after transfection and stimulated with FL for different time points. After lysis total cell lysate was analyzed by Western blotting. (G) Auto radiograms from experiments (A, C, D, F) were quantified with Multi-Gauge software. Statistical analysis was done using GraphPad Prism.

Fig. 6. PI3K activity but not SFKs activity is required for Grb10-mediated Akt activation

(A) Ba/F3-FLT3-WT/Grb10 cells were serum starved for 4h and treated with inhibitor or DMOS for 1h followed by FL stimulation. Total cell lysates after lysis were analyzed by Western blotting.

(B) Ba/F3-FLT3-WT/Grb10 cells were serum starved for 4h and treated with Src inhibitor or DMOS for 1h followed by FL stimulation. Total cell lysates after lysis were analyzed by Western blotting. (C) Ba/F3-FLT3-WT/Grb10 cells were serum starved for 4h and treated with Syk inhibitor or DMOS for 1h followed by FL stimulation. Total cell lysates after lysis were analyzed by Western blotting.

Fig. 7. Grb10 transduces signal through interaction with p85

(A) Ba/F3-FLT3-WT cells transfected with Grb10 or empty vector were serum starved for 4h and stimulated with FL for different time points. After lysis total cell lysate was analyzed by Western blotting. Auto radiograms were quantified with Multi-Gauge software. Statistical analysis was done using GraphPad Prism. (B) Ba/F3-FLT3-WT cells transfected with Grb10 or empty vector were serum starved for 4h and stimulated with FL for different time points. After lysis SHP2 protein was immunoprecipitated with an anti-SHP2 antibody followed by Western blotting analysis. Auto radiograms were quantified with Multi-Gauge software. Statistical analysis was done using GraphPad Prism. (C) COS-1 cells transfected with FLT3 and Grb10 were serum starved for 4h and stimulated with FL for 5 min. After lysis endogenous p85 was immunoprecipitated with an anti-p85 antibody and analyzed by Western blotting. (C) OCI-AML-5 cells were serum starved for 4h and stimulated with FL for 5 min. After lysis endogenous p85 was immunoprecipitated with an anti-p85 antibody and analyzed by Western blotting.

Fig. 8. Grb10 increases S-phase cell population followed by an increased cell proliferation and survival

(A) Ba/F3-FLT3-ITD cells transfected with Grb10 or empty vector were cultured without any cytokine or 100 ng/ml FL for 46h followed by 2h EdU incubation. Cells were then fixed and labelled with Alexa Fluor 488 and 7-AAD and then analysed by flow cytometry. (B) Ba/F3-FLT3-WT cells transfected with Grb10 or empty vector were cultured without any cytokine or 100 ng/ml FL or IL-3 for 48h and then subjected to the trypan blue exclusion assay. (C) Ba/F3-FLT3-ITD cells transfected with Grb10 or empty vector were cultured without any cytokine or

100 ng/ml FL or IL-3 for 48h and then subjected to the trypan blue exclusion assay. (D) Cells were cultured without any cytokine or 100 ng/ml FL for 46h followed by 2h EdU incubation. Cells were then fixed and labelled with Alexa Fluor 647 and then analysed by flow cytometry. (E) OCI-AML-5 cells were transfected with Grb10 siRNA or control siRNA were cultured without any cytokine or 100 ng/ml FL or IL-3 for 72h and then subjected to the trypan blue exclusion assay. (F) Ba/F3-FLT3-WT cells were cultured without any cytokine or 100 ng/ml FL for 48h and then labelled with Annexin-V-PE and 7-AAD followed by flow cytometry analysis. (G) Ba/F3-FLT3-ITD cells were cultured without any cytokine or 100 ng/ml FL for 48h and then labelled with Annexin-V-PE and 7-AAD followed by flow cytometry analysis.

Fig. 9. Grb10 increases FLT3-ITD-induced colony formation as well as STAT5 activation

(A) Ba/F3-FLT3-ITD cells transfected with Grb10 or empty vector were serum starved for 4h. After lysis STAT5 protein was immunoprecipitated with an anti-STAT5 antibody followed by Western blotting analysis. (B) Ba/F3-FLT3-ITD cells transfected with Grb10 shRNA or control shRNA were serum starved for 4h. After lysis STAT5 protein was immunoprecipitated with an anti-STAT5 antibody followed by Western blotting analysis. (C) Auto radiograms from experiments (A, B) were quantified with Multi-Gauge software. Statistical analysis was done using GraphPad Prism. (D) Ba/F3-FLT3-ITD cells were cultured without any cytokine or 100 ng/ml FL for 5 days in semisolid culture medium.

Fig. 10. Grb10 expression leukemia patient samples

(A) Grb10 expression was analyzed from microarray data of 20 healthy donor and 26 AML patient samples. In CML dataset 17 patient samples were analyzed. Dataset from different experiments were normalized and scaled. (B) Grb10 expression was analyzed from the microarray dataset of acute promyelocytic leukemia patients carrying either wild-type FLT3 or FLT3-ITD mutation. (C) Microarray datasets of 54 pediatric AML patients having complete remission after treatment or relapsed, 21 patients having B-cell chronic lymphocytic leukemia (B-CLL) with indolent or progressive disease and 44 pediatric patients having acute lymphoblastic

leukemia (ALL) with responsiveness or resistance to drug induced apoptosis were analyzed for Grb10 expression. (D) PTEN expression was analyzed from microarray data of 20 healthy donor and 26 AML patient samples. In CML dataset 17 patient samples were analyzed. Dataset from different experiments were normalized and scaled. Error bar shows SEM, and t-test was performed to determine significance.

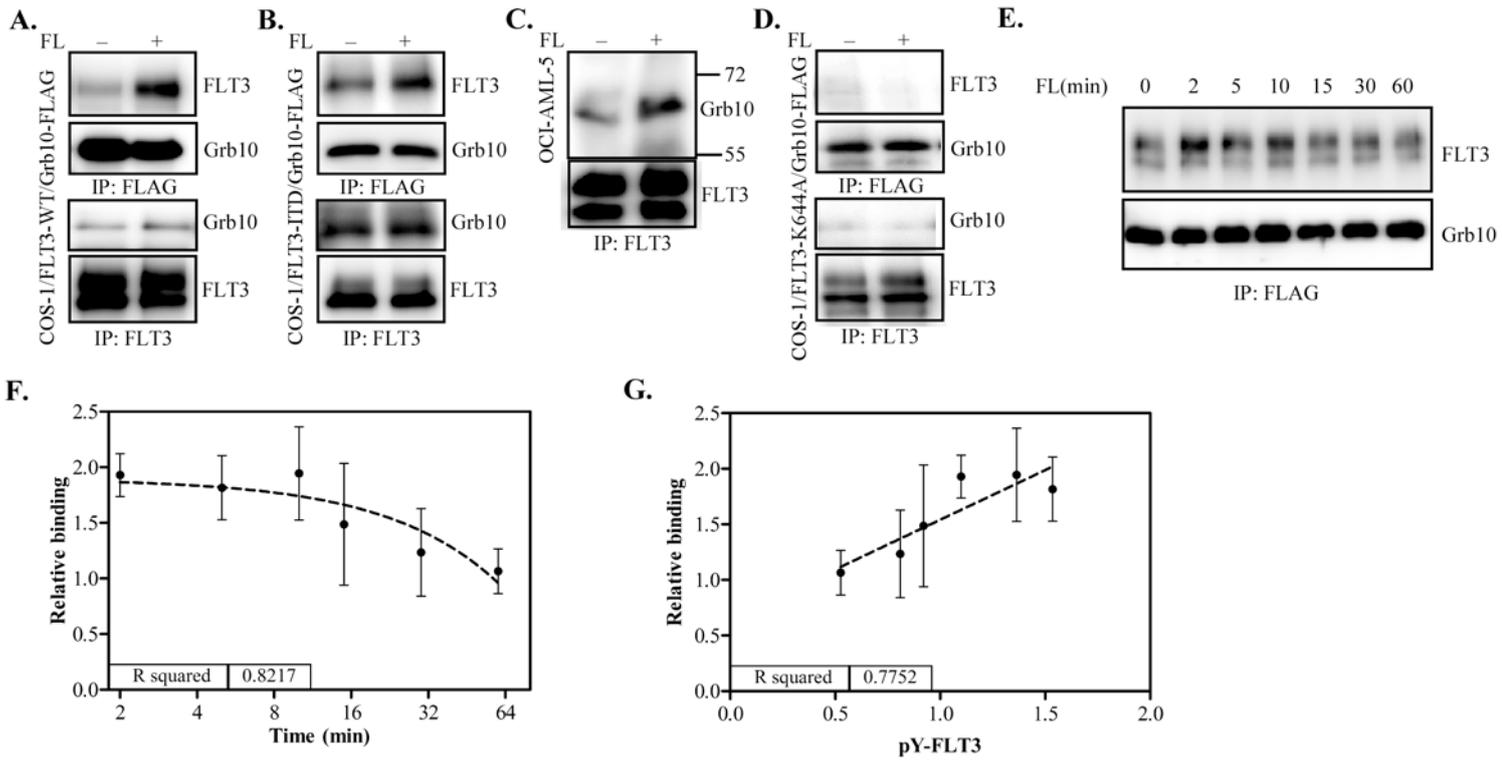


Figure 1

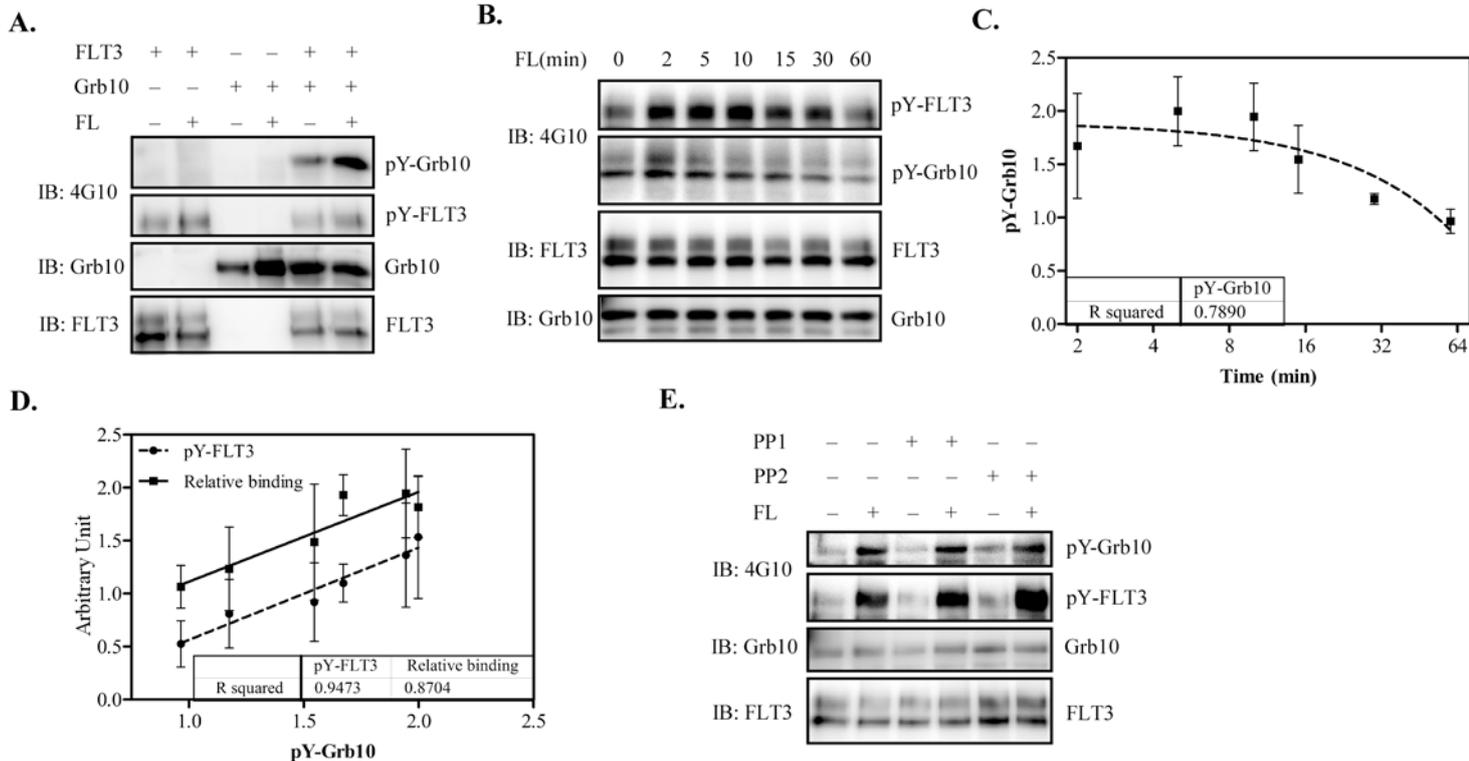


Figure 2

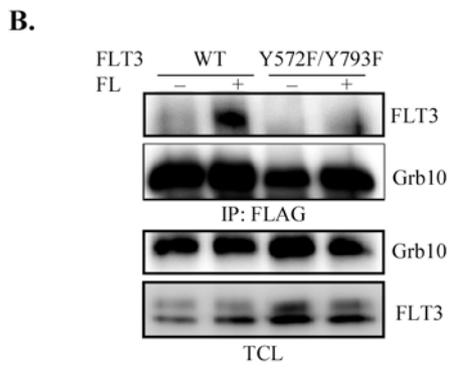
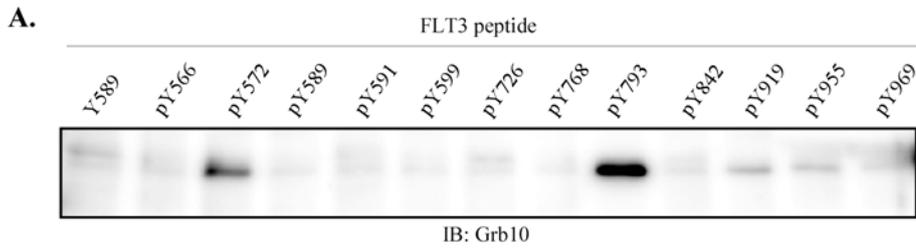


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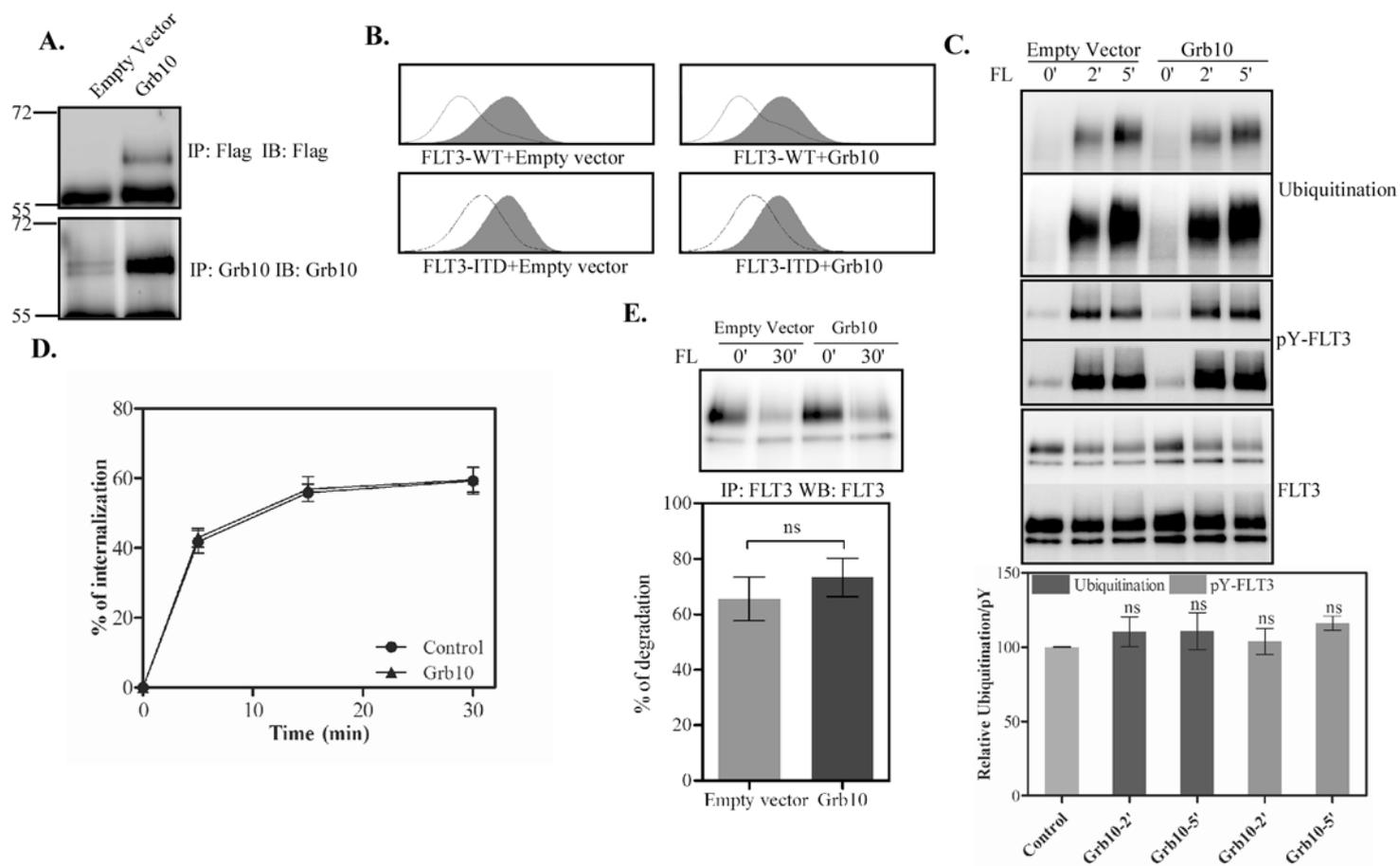


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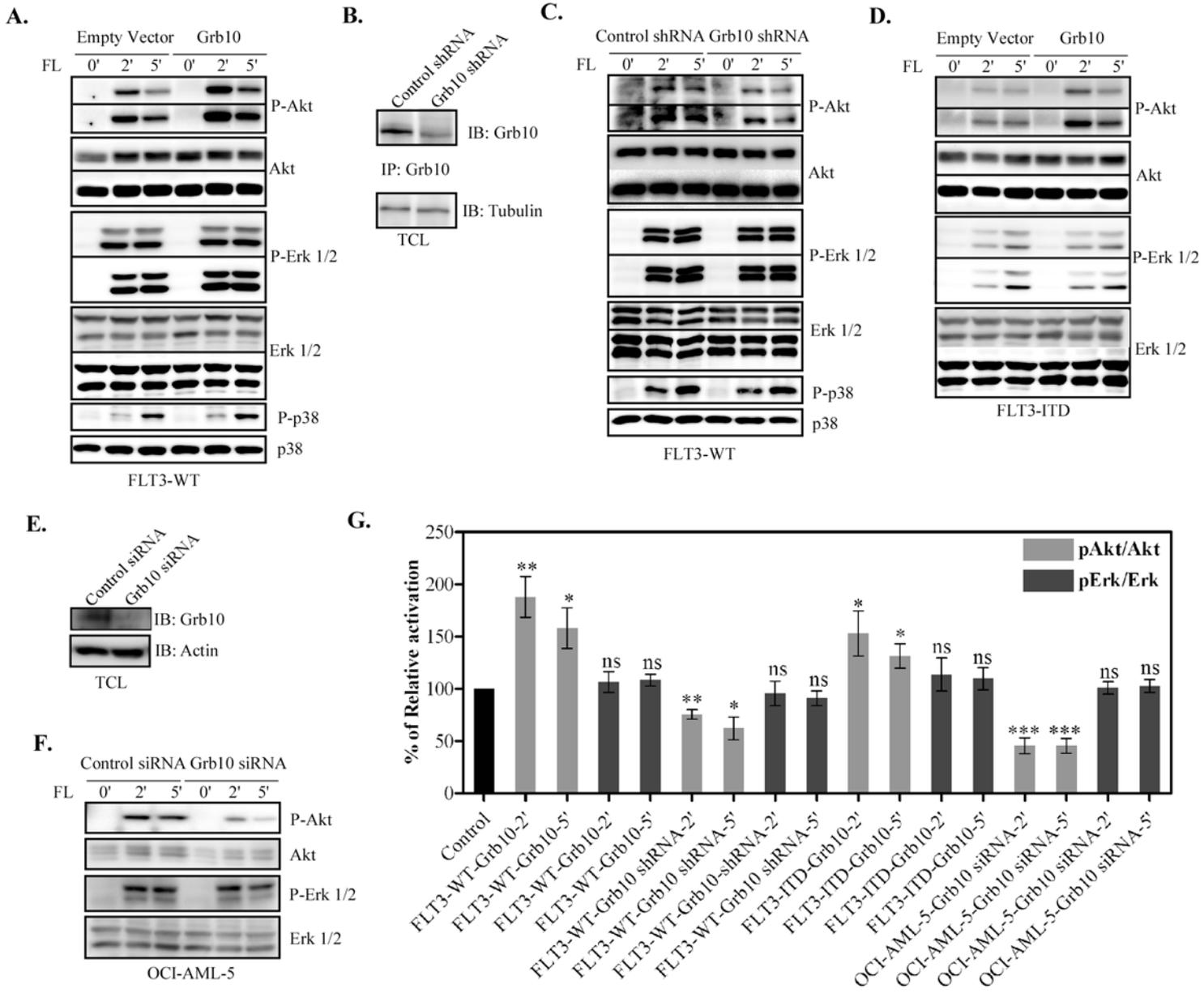


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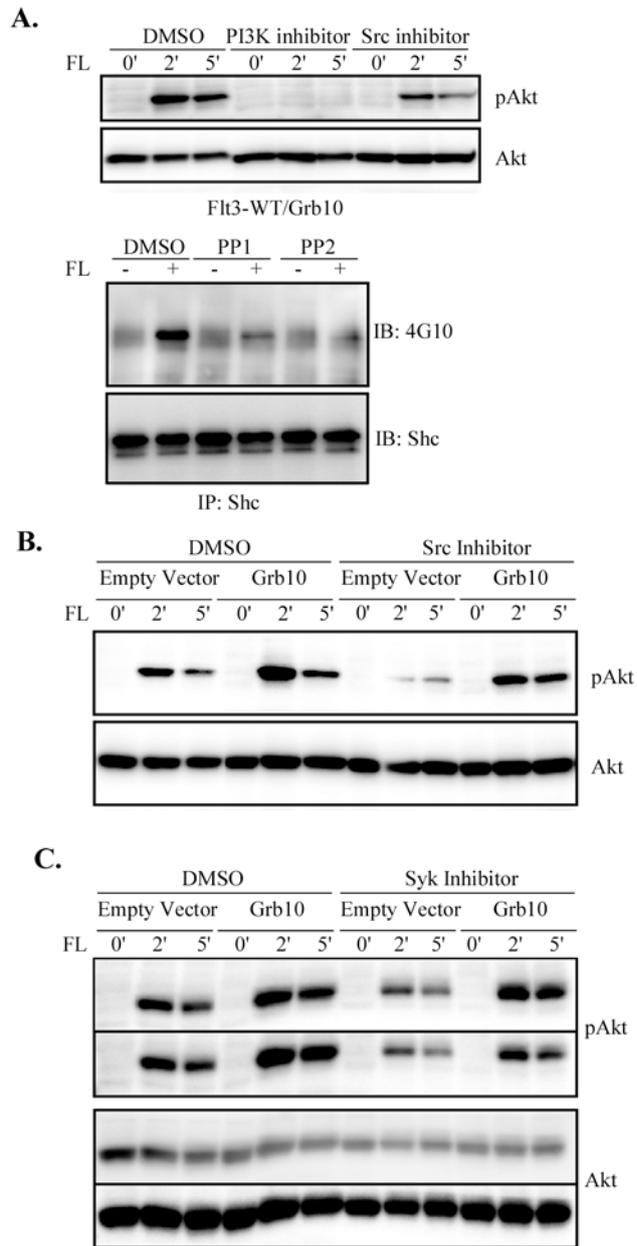


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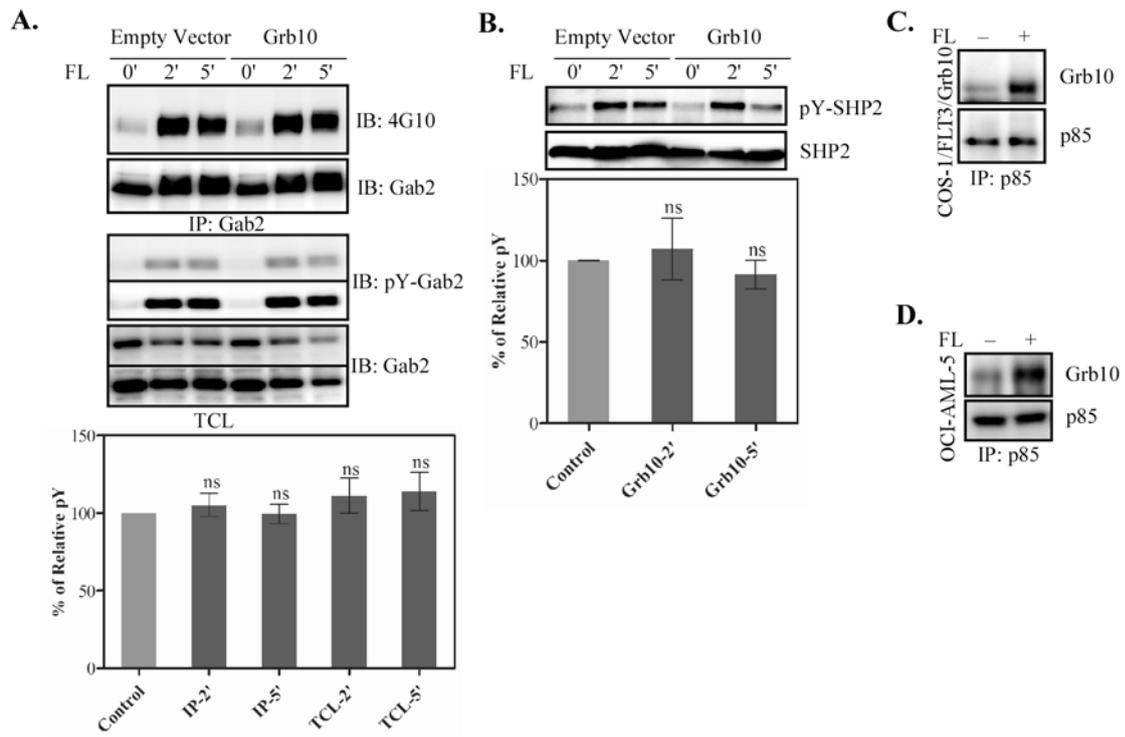
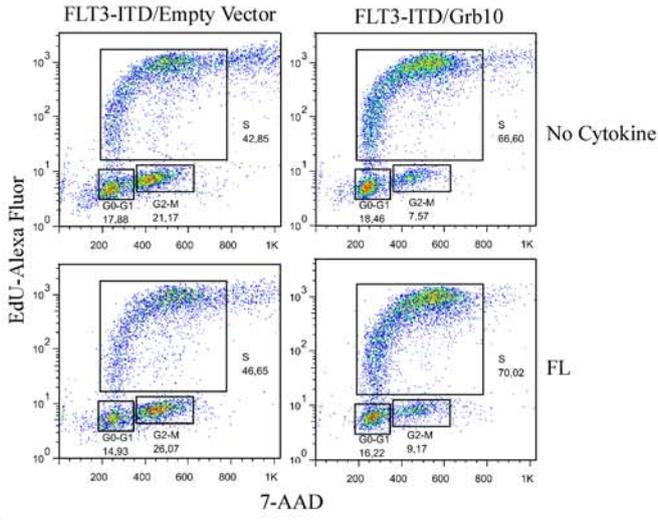
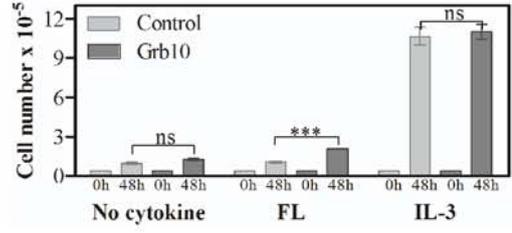
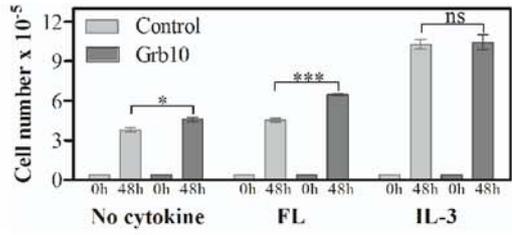
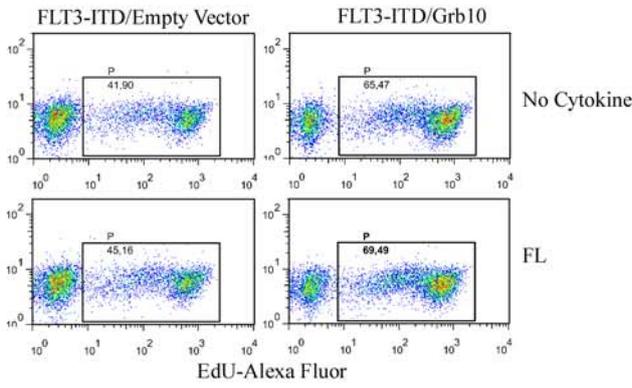
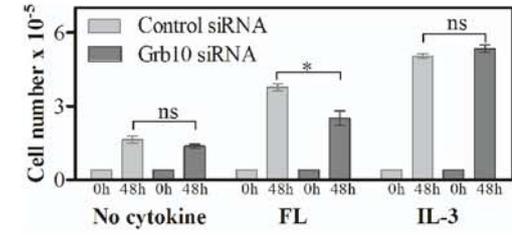
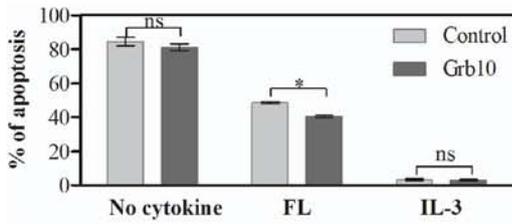
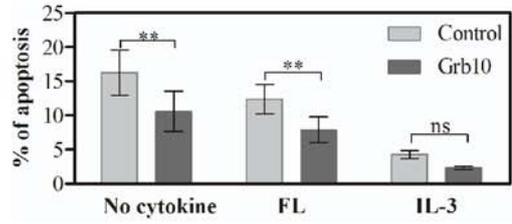


Figure 7

A.**B.****C.****D.****E.****F.****G.****Figure 8**

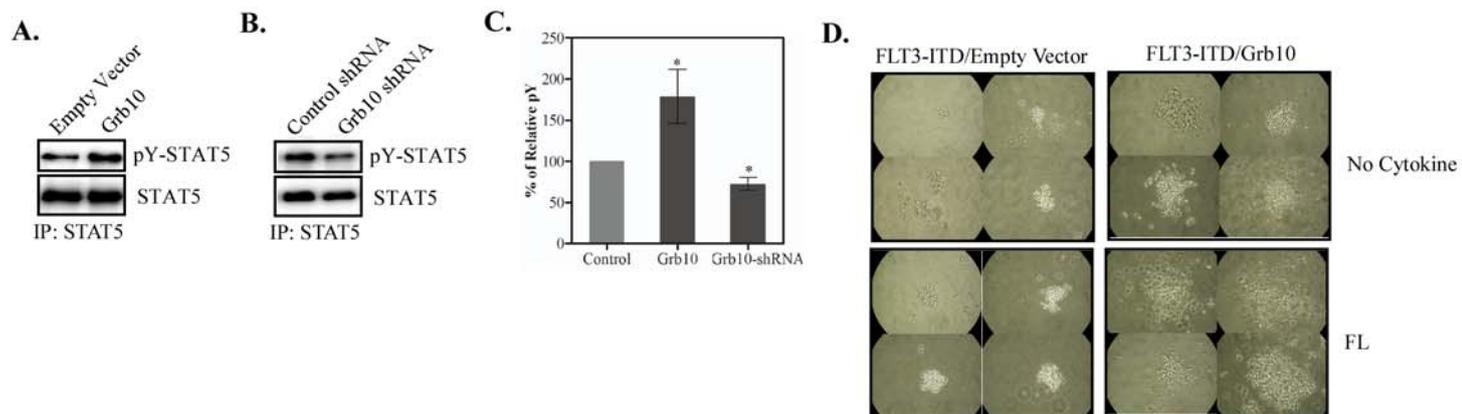


Figure 9

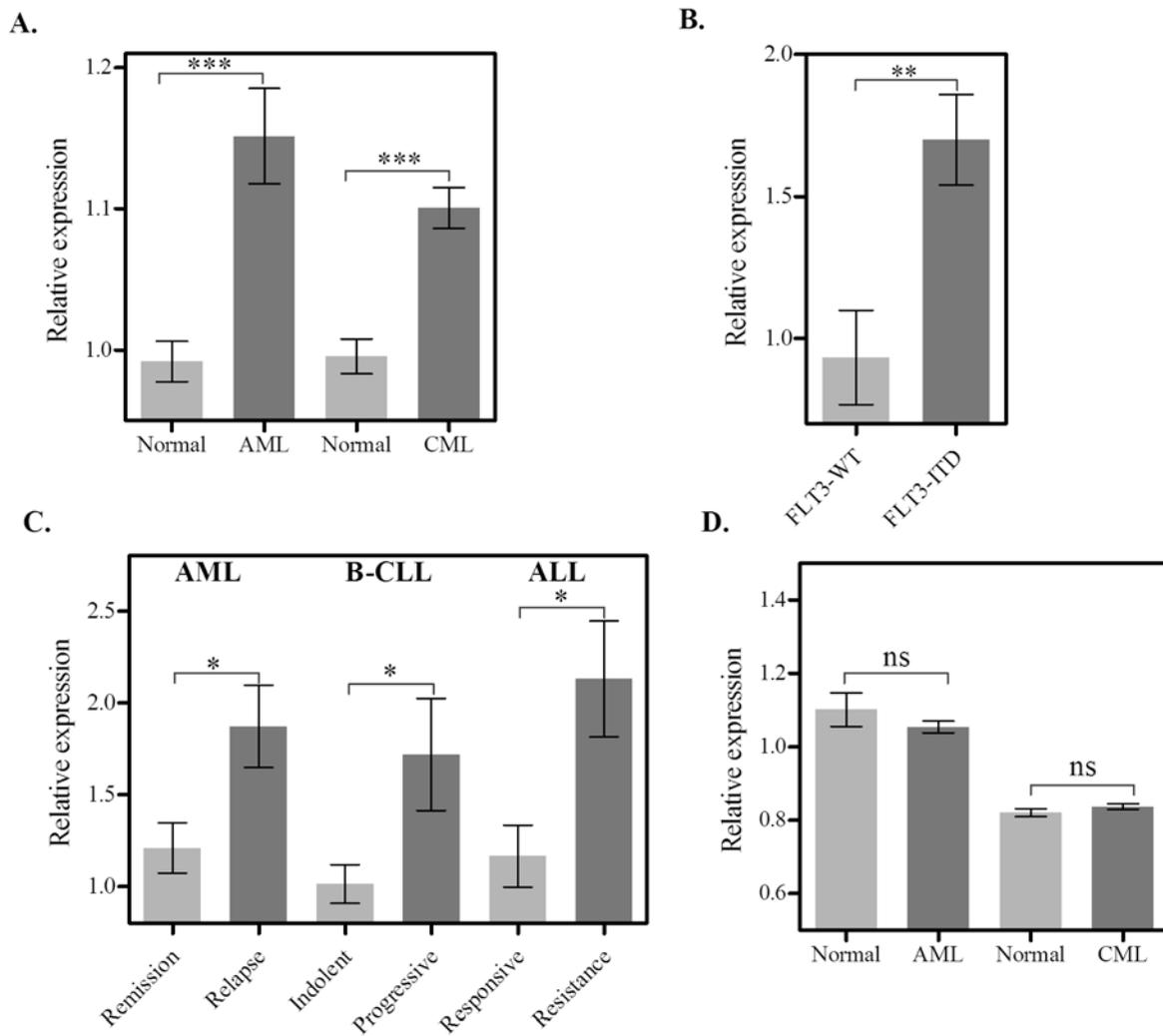


Figure 10