Cell Biol Toxicol
DOI 10.1007/s10565-012-9216-z

ORIGINAL RESEARCH

Ectopic expression of Flt3 kinase inhibits proliferation and promotes cell death in different human cancer cell lines

Eystein Oveland · Line Wergeland · Randi Hovland · James B. Lorens · Bjørn Tore Gjertsen · Kari E. Fladmark

Received: 7 December 2011 / Accepted: 27 February 2012

Abstract Stable ectopic expression of Flt3 receptor tyrosine kinase is usually performed in interleukin 3 (IL-3)-dependent murine cell lines like Ba/F3, resulting in loss of IL-3 dependence. Such high-level Flt3 expression has to date not been reported in human acute myeloid leukemia (AML) cell lines, despite the fact that oncogenic Flt3 aberrancies are frequent in AML patients. We show here that ectopic Flt3 expression in different human cancer cell lines might reduce proliferation and induce apoptotic cell death, involving Bax/Bcl2 modulation. Selective depletion of Flt3-expressing cells occurred in human AML cell lines transduced with retroviral Flt3 constructs, shown here using the HL-60 leukemic cell line. Flt3 expression was investigated in two cellular model systems, the SAOS-2 osteosarcoma cell line and the human embryonic kidney HEK293 cell line, and proliferation was reduced in both systems. HEK293 cells underwent apoptosis upon ectopic Flt3 expression and cell death could be rescued by overexpression of Bcl-2. Furthermore, we observed that the Flt3-induced inhibition of proliferation in HL-60 cells appeared to be Bax-dependent. Our results thus suggest that excessive Flt3 expression has growth-suppressive properties in several human cancer cell lines.

Keywords Acute myeloid leukemia · Cell death · Flt3 · Receptor tyrosine kinase

Authors Eystein Oveland and Line Wergeland contributed equally to this study and should share the first authorship.

E. Oveland
Proteomics Unit at University of Bergen (PROBE),
University of Bergen,
Bergen, Norway

E. Oveland · J. B. Lorens
Department of Biomedicine, University of Bergen,
Bergen, Norway

L. Wergeland · B. T. Gjertsen
Department of Medicine, Hematology Section,
Haukeland University Hospital,
Bergen, Norway

R. Hovland
Center for Medical Genetics and Molecular Medicine,
Haukeland University Hospital,
Bergen, Norway

L. Wergeland · B. T. Gjertsen
Institute of Medicine, Hematology Section,
University of Bergen,
Bergen, Norway

R. Hovland · K. E. Fladmark (✉)
Department of Molecular Biology, University of Bergen,
Thormøhlensgt. 55,
5020 Bergen, Norway
e-mail: mankf@mbi.uib.no

Published online: 16 March 2012

Abbreviations

AML	Acute myeloid leukemia
Flt3ITD	Flt3 with internal tandem duplication
IL-3	Interleukin 3
RTK	Receptor tyrosine kinase
wt	Wild type

Introduction

Receptor tyrosine kinases (RTKs) are associated with cell proliferation and survival that are engendered by a wide range of activating mutations found in numerous cancers. Clinical results obtained using various selective inhibitors of oncogenic kinases have generally been disappointing, with the notable exception of chronic myeloid leukemia treated with imatinib (STI571) (Blume-Jensen and Hunter 2001). Recent reports indicate that multiple RTKs are co-activated in more aggressive tumors (Stommel et al. 2007). Conversely, RTK stimulation may in fact sensitize the patients' cells to chemotherapy without accelerating growth of the cancer (Lowenberg et al. 2003). Indeed, both epidermal growth factor (EGF) receptor and platelet-derived growth factor (PDGF) receptor activation induce apoptotic cell death in selected experimental models (Huang et al. 2002; Okura et al. 1998). Thus, RTK signaling may be anti-proliferative when exceeding a certain threshold (Koonin et al. 1996).

The class III RTKs (CSF1R, Kit, PDGFRα and PDGFRβ, and Flt3) are transmembrane growth factor receptors prevalent in myeloid malignancies (Oveland et al. 2006). Flt3 is expressed in bone marrow cells during early stages of hematopoiesis contributing to proliferation and maintenance of progenitor cells (Small et al. 1994). Mutations in Flt3 are associated with aggressive forms of acute myeloid leukemia (AML) and hyper-responsive signaling through RTKs (Irish et al. 2004). Internal tandem duplication in the juxtamembrane region (Flt3ITD) and point mutations in the tyrosine kinase domain (e.g., Flt3D835Y) result in a ligand-independent Flt3 activation and an altered and potentiated signaling compared to Flt3 wild type (Flt3wt) (Yamamoto et al. 2001). A high-level expression of Flt3wt in AML is also thought to induce auto-oligomerization of the receptors, and thus lead to constitutive activation and signaling (Ozeki et al. 2004).

Studies on Flt3 signaling have primarily been conducted using immortalized cell lines with high endogenous Flt3, or in stably transfected interleukin-3 (IL-3)-dependent cell lines. The IL-3-dependent mouse hematopoietic cell lines Ba/F3 (lymphoid) and 32D (myeloid) that lack endogenous Flt3 expression have been used for transient and stable expression of Flt3, generating IL-3 independence (Hayakawa et al. 2000). When stably expressed in Ba/F3 cells, Flt3ITD mimicked a complete IL-3 proliferative response, while the FL-stimulated Flt3wt proliferative response was weaker. To our knowledge, successfully enforced stable Flt3 expression in human IL-3-independent AML cell lines has not been reported to date, neither with wild-type nor with mutated Flt3. In addition, complications with stable expression of Flt3 in kidney fibroblast-like COS-7 cells have been reported (Koch et al. 2008).

We have examined the effects of sustained and transient Flt3 expression more thoroughly using various human cell lines. Here, we report that sustained stable expression of Flt3wt in the human AML cell line HL-60 (Flt3wt) induces decreased proliferation giving rise to cell loss. Several attempts to transduce the human leukemic cell lines; HL-60, NB4 (Flt3wt), Kg1a (Flt3wt), MV4-11 (Flt3ITD), and Molm-13 (Flt3ITD/Flt3wt) with Flt3wt and auto-activated Flt3 mutants resulted in loss of the transfected cells. Flt3wt also reduced cell proliferation in the human osteosarcoma cell line SAOS-2. In the HEK293 cell line, Flt3-dependent cell death occurred after enhanced global tyrosine phosphorylation, deregulation of Bcl-2 family members, nucleoli-cytoplasmic translocation of nucleophosmin, nuclear condensation, and cell detachment.

Materials and methods

Reagents

The primary antibodies are the following: anti-Bax 2D2, anti-Flt3 S-18, anti-p53 BP53-12, anti-Mcl-1 22 (Santa Cruz Biotechnology), anti-β-Actin (Abcam), anti-GFP, anti-nucleophosmin (Zymed); anti-phosphotyrosine 4G10 (Upstate Biotechnology); anti-Bad and anti-Phospho-p53 Ser15 16 G8 (Cell Signaling). The secondary antibodies are as follows:

anti-mouse-HRP, anti-rabbit-HRP, anti-rabbit-FITC, and anti-mouse-Texas Red (Jackson ImmunoResearch). Flow cytometry antibody was Flt3 PE-conjugated antibody SF1.340 (Beckman Coulter).

Cell lines and cultivation

The HEK293 cell line was from American Type Culture Collection. HEK293 Phoenix cells, originally adapted from HEK293T, had retrovirus packing capabilities (Swift et al. 1999). HEK293 cell lines were cultivated in DMEM supplied with GlutaMAX™ (Invitrogen), 10% fetal calf serum (FCS; Gibco), and 50 IU penicillin/50 μg streptomycin ml^{-1}. The human leukemia cell lines NB4, HL-60, Molm-13, and MV4-11 were obtained and cultivated as previously described (Bredholt et al. 2009). Ba/F3 suspension cells (DSMZ) were cultivated as for the human leukemia cells, but the medium contained in addition 10 % vol interleukin-containing conditioned medium derived from the WEHI-3B cell line (DSMZ). The osteosarcoma cell line SAOS-2 (DSMZ) was cultured in McCoy's 5A medium (Sigma-Aldrich) with 15 % FCS.

Constructs and transfection

Human Flt3 cDNA from pCDHF3 (kind gift from Dr. O. Rosnet, Inserm) was extended with to *Bstx*I restriction sites by PCR (Forward primer 5′GTTCCCACCACCACCATGGGCC AGGAGTTCTGCTG; Reverse primer 5′GTTCCCAATTTAATGGTTTACGAATCTTCGACC TGAGCCTG) and ligated into complementary restriction sites in the retroviral expression vector CRU5-IRES-GFP (Swift et al. 1999) generating CRU5-Flt3-IRES-GFP. The following constructs were generated (Fig. 1) using Quick Change II XL site-directed mutagenesis as described by the provider (Stratagene): CRU5-IRES-Flt3D835Y-GFP (kinase activation (Yamamoto et al. 2001), forward mutagenesis primer 5′GTGACTTTGGATTGGCTCGATATATCATGAGTGATTCCAA CTATG), CRU5-IRES-Flt3Y599F-GFP (docking site for adaptor proteins (Heiss et al. 2006), forward mutagenesis primer 5′ GATTTCAGAGAATATGAATTTGATC TCAAATGGGAG) and CRU5-IRES-Flt3K644A-GFP (kinase inactivation (Sargin et al. 2007), forward mutagenesis primer 5′TCTCAATCCAGGTT GCCGTCCGAATGCTGAAAGAAAAAGCAG).

Four Flt3-ITD constructs were used in transfection of HEK293 cells in the cell survival experiment. Two Flt3-ITDs were generated with cDNA from patient 1 both at diagnosis and at relapse (Hovland et al. 2002) and cloned into CRU5-IRES-GFP. The other two Flt3-ITD constructs contain the W51 mutation (Lee et al. 2005) in two different expression vectors (pcDNA 3.1 and pMSCV) and were kindly provided by DH Gilliland (Harvard Medical School, Boston, MA).

The Bcl-2 and Bax plasmids used have previously been described by Beham et al. (1997).

siRNA targeting BAX and the random siRNA were purchased from Dharmacon. HL-60 cells were transiently transfected with either siRNA or plasmids (2 μg plasmid for 2×10^6 cells) using the Cell Line Nucleofector™ Kit V and the Nucleofector™ Device (Amaxa GmbH).

Transient transfection of HEK293wt was performed using FuGENE 6 (Roche, 1-μg plasmid per well in a six-well plate, 650,000 cells) and SAOS-2 (100 ng plasmid for per well in a 96-well plate, 10,000 cells per well) using MATra (Westburg, Leusden, The Netherlands) according to the manufacturer's instructions.

Stable retroviral transduction using HIV-derived VSV-G pseudotyped virus particles was performed as described in Swift et al. (1999). Four micrograms of plasmid was used for one 10-cm dish of HEK293 Phoenix cells.

Flow cytometry

The flow cytometric analysis of cells was performed using a FACSCalibur with the CellQuest Pro software, and sorting was performed using a FACSAria SORP with the FACS DiVa software (BD Biosciences). Flow data were analyzed with FlowJo (Tree Star Inc.).

Immunostaining and confocal microscopy

HEK293wt cells were transfected with FuGENE 6 and 24 h thereafter fixed in 2 % formaldehyde, permeabilized with 0.1 % Triton X-100, and immunolabeled using dilutions of 1:50 and 1:150 for primary antibodies and secondary antibodies, respectively. Cells were viewed using a Zeiss LSM 510 Meta confocal microscope.

Determination of cell viability and apoptosis

Cell death was determined based on flow cytometry (above), cell morphology, and nucleophosmin

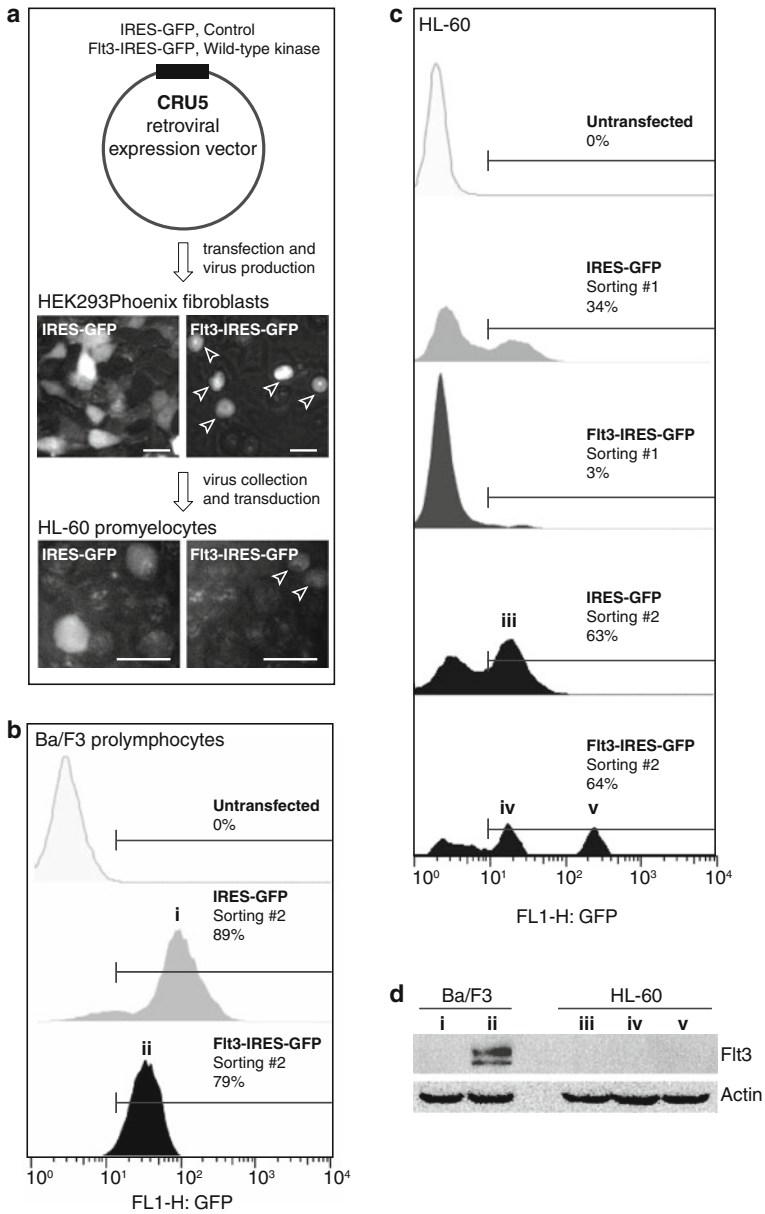


Fig. 1 A retroviral expression system for Flt3 transduction of leukemia cell lines. **a** Flt3 constructs were made in the retroviral expression vector CRU5 to produce virus in HEK293 Phoenix cells for transduction of leukemic cells. The HEK293 Phoenix cells express the SV-40 large T antigen that inhibits the tumor suppressors p53 and pRb, providing resistance to cellular stress (Swift et al. 1999). The CRU5 vector allows simultaneous expression of Flt3 and GFP as two independent proteins, Flt3 by Cap-dependent translation and GFP controlled by an IRES element. Both genes were enclosed by 5′LTR/3′LTR in addition to the presence of a packing signal (ψ) that enables virus production. GFP was used to monitor transfection/transduction efficiency and to select transfected/transduced cells based on fluorescence. A representative experiment ($n>3$) shows fluorescence microscopy of 293Phoenix cells and HL-60 cells 48 h after Flt3 introduction. Rounding up of Flt3-expressing cells is evident in both cell lines (*white arrowheads*). *Scale bar*, 20 μm. **b** Percentage of GFP-positive Ba/F3 cells after transduction with IRES-GFP and Flt3-IRES-GFP after second FACS sorting. **c** Percentage of GFP-positive HL-60 cells after transduction with IRES-GFP and Flt3-IRES-GFP after first and second FACS sorting. **d** Western blot of Flt3 using lysates from the transduced subpopulations of Ba/F3 (*i–ii*) and HL-60 (*iii–v*) as indicated in **b** and **c**, respectively. Actin, loading control

translocation/immunolabeling. Samples for transmission electron microscopy were prepared as previously described (Brustugun et al. 1998) and examined in a Jeol JEM-1230. For studies on nuclear morphology, cells were fixed in 2 % formaldehyde containing Hoechst 33342 (10 μg/ml).

Proliferation rate was determined by the incorporation of ^{3}H-thymidine (TRA 310, Amersham Biosciences) using 37 kBq/well, as previously described (Bruserud et al. 2003), and nuclear radioactivity was assayed after 8 h. Cellular metabolic activity and viability were assayed by the Alamar blue assay as described by the manufacturer (BioSource International).

Cell lysis and Western blotting

Cells were harvested at 500 g for 5 min at 4°C and washed twice in cold PBS. The pellets were resuspended in modified RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 7.4, 1 mM EGTA pH 7.4, 1% Triton X-100, 10 mM NaF, 10 μl/ml phosphatase inhibitor cocktail II) and incubated on ice for 20 min. Cell debris was removed by centrifugation at 17,000×*g* for 20 min, and protein concentrations were determined using the Bradford method. Western blot analysis was performed as previously described (Oveland et al. 2009), and ImageJ 1.37v (National Institutes of Health, MD) was used for quantitation. β-Actin was used as a loading control for normalization of proteins investigated by densitometry-based measurements.

Results

Sustained Flt3 expression in hematopoietic cell lines causes cell death

In order to establish human AML cell lines demonstrating stable Flt3 expression, we used a retroviral expression system (Lorens et al. 2000; Swift et al. 1999) (Fig. 1). HEK293 Phoenix, HL-60 (low endogenous Flt3 expression), and Ba/F3 [successfully used in Flt3-expression studies (Hayakawa et al. 2000)] cells showed GFP expression and Flt3 cell surface expression 24 h after introduction of the construct Flt3-IRES-GFP, as evidenced by flow cytometry (results not shown). Both HEK293 Phoenix and HL-60 Flt3-expressing cells were rounded up and had shrunk about 48 h after Flt3 was introduced (Fig. 1a); however, this was not observed in Ba/F3 cells. Flt3-expressing HL-60 cells were selectively lost within 10 days of in vitro culture, an occurrence also observed with the human AML cell lines NB4, Molm-13, MV4-11, and Kg1a when transduced with Flt3 (n=3, results not shown).

Flt3-transduced Ba/F3 and human AML cell lines were enriched by sequential fluorescence-assisted cell sorting (FACS) based on GFP reporter co-expression. Flt3-IRES-GFP expressing NB4 cells were consequently lost or they died during transduction (results not shown). Results of a representative experiment of Flt3-IRES-GFP transduced Ba/F3 cells after the second FACS sorting are shown in Fig. 1b, and those of the first/second FACS sorting of HL-60 cells are shown in Fig. 1c. The Ba/F3 and HL-60 cell populations indicated in the figure were prepared for Western blotting against Flt3 (Fig. 1d). Vector-expressed Flt3 protein with expected post-translational modifications (Lyman et al. 1993) was evident in Flt3-IRES-GFP transduced Ba/F3 cells, but not seen in HL-60 cells. This indicates that long-term selection favors subpopulations of cells with the constructs IRES-GFP (iv) and GFP only (v) (Fig. 1d).

Flt3 expression in HL-60 cells and in SAOS-2 osteosarcoma cells decreases cell proliferation and metabolic activity

Since stable Flt3 expression was not tolerated by HL-60 cells, transient Flt3 transfections were performed in order to investigate effects on cell proliferation. Flt3 significantly reduced the HL-60 cell proliferation rate (Fig. 2a). In contrast to the high-level p53 HEK293 Phoenix cells, we examined the effect of transient Flt3 expression in the p53-deficient and slow-growing human SAOS-2 osteosarcoma cell line. The SAOS-2 cells were assessed in cell growth assays 72 h after transfection; enforced Flt3 expression significantly reduced cell proliferation and net metabolic activity compared to the control (Fig. 2bc). Expression of authentic Flt3 protein was confirmed by Western blots.

Flt3 expression in HEK293wt induces cell death by apoptosis

To obtain insight into the mechanisms governing cell death induced by Flt3, we used a gain-of-function

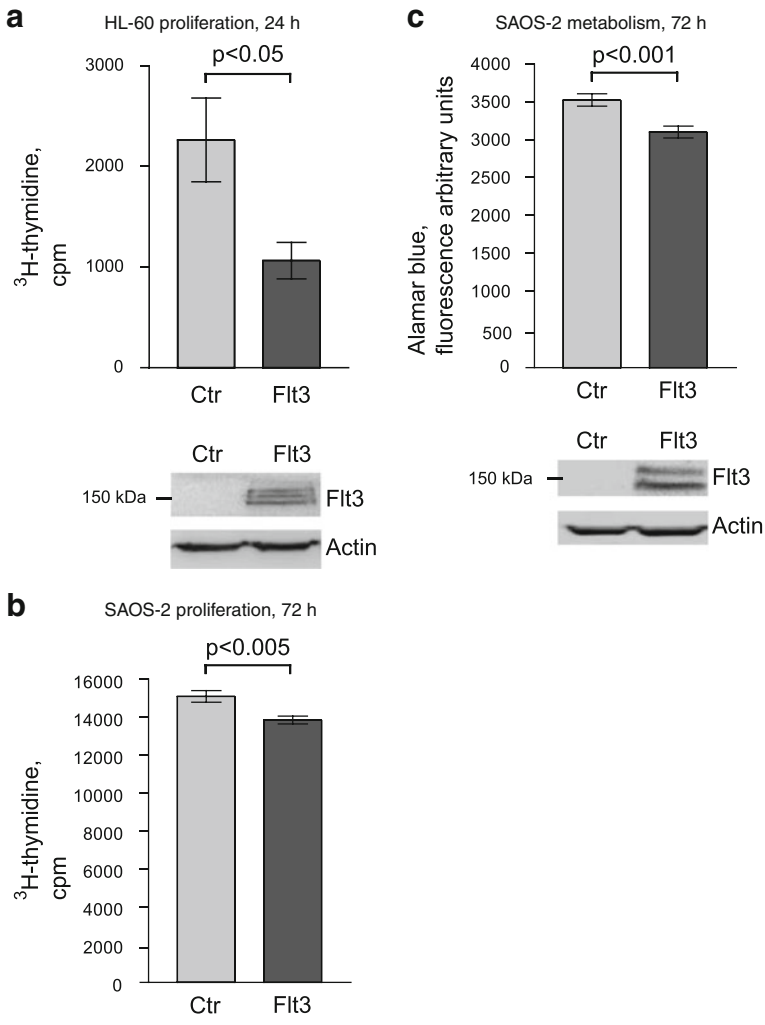


Fig. 2 Enforced Flt3 expression in HL-60 and SAOS-2 cells reduces proliferation and metabolic activity. **a** HL-60 cells were used in a ^{3}H-thymidine incorporation assay (18-h incubation) 24 h after Amaxa transfection with control vector (Ctr, IRES-GFP) or Flt3 (Flt3-IRES-GFP). SAOS-2 cells were subjected to **b** ^{3}H-thymidine incorporation assay (18-h incubation) and **c** Alamar Blue assay (6-h incubation) 72 h after MATra transfection with control vector (Ctr, pcDNA3.1) or Flt3 (pcDHF3). Statistical significance (p) was calculated using two-tailed unpaired t test (n=11 for **a**; n=16 for **b** and **c**) and *bars* represent SEM of three individual experiments. Representative Western blots of corresponding transfections are shown

Flt3D835Y construct representing a constitutively active Flt3 mutation in AML (Yamamoto et al. 2001) and a loss-of-function Flt3Y599F construct where the juxtamembrane Src-SH2 interaction domain is disrupted (Heiss et al. 2006).

HEK293wt cells were transfected with Flt3, Flt3Y599F, and Flt3D835Y and assessed for induction of apoptosis. Initiator procaspase-8 and -9 and effector procaspase-3 were not activated by cleavage when investigated on Western blots 24 or 48 h after transfection with Flt3 constructs (data not shown). This caspase-independent apoptosis reflects an anti-apoptotic resistance phenotype of HEK293wt (Fearnhead et al. 1997). Since annexin V-PI flow cytometry requires cell detachment of adherent cells by either trypsination or mechanical force, we examined an alternative, more gentle method to determine apoptosis. It has been demonstrated that nucleophosmin binds to Bax, a promoter of apoptotic cell death (Thompson et al. 2008), and translocation of nucleophosmin is a marker of UV-induced apoptosis in HEK293 cells (Kerr et al. 2007; Thompson et al. 2008). Immunofluorescence analysis of cells 48 h after transfection showed nucleophosmin (red) in the nucleoli of non-transfected cells and in Flt3Y599F-expressing cells (Fig. 3a). Intriguingly, in cells transfected with Flt3wt or Flt3D835Y (green), translocation of nucleophosmin from nucleoli to the cytoplasm was observed (Fig. 3a, right and middle panel). The translocation of nucleophosmin was most prominent in Flt3D835Y-expressing cells, and chromatin condensation in late apoptotic cells was apparent in cells harboring either of the constructs.

Organelle morphology in Flt3wt-transfected HEK293wt cells was examined by transmission electron microscopy. Severe nuclear and mitochondrial shrinkage was apparent in Flt3-transfected HEK293wt cells, but was not observed in cells transfected with the control vector (Fig. 3b). Chromatin marginalization was observed in the condensed nuclei, and mitochondrial cisternae were disrupted in Flt3-transfected cells. Taken together, our data indicate that enforced Flt3 expression induces apoptotic cell death.

Flt3 functional mutants influence tyrosine phosphorylation and pro-/anti-apoptotic proteins in HEK293wt

The Src-SH2 interaction domain loss-of-function mutant, Flt3Y599F, was not lethal to the HEK293wt cells. To determine whether kinase activity per se was required for Flt3-induced apoptosis, we generated Flt3K644R, reported to be a kinase-activity loss-of-function mutant (Sargin et al. 2007). Western blots of general tyrosine phosphorylated proteins (pTyr) were used to measure kinase activity. The levels of pTyr were highest 36–48 h after Flt3 transfection (Fig. 3c). The mean pTyr levels in HEK293wt 24 h after transfection ($n=3$, cells still viable) relative to control based on Western blot densitometry analyses, when normalized to actin, were as follows: Flt3K644R (1.5)< Flt3Y599F (2.4)<Flt3wt (5.3)<Flt3D835Y (24.5). A representative Western blot is shown in Fig. 3d and the average of three experiments illustrated in Fig. 3f. The Flt3 expression level was higher in cells transfected with Flt3-D835Y compared to the other Flt3 constructs, and this could, to some extent, account for the observed regulation of protein levels.

Flt3-dependent decrease in proliferation and viability was less pronounced in p53-null HL-60 and SAOS-2 cells (Fig. 2); therefore, we investigated whether stabilization of p53 could be involved in Flt3-induced cell death in HEK293wt cells. The p53 protein levels relative to control were as follows: Flt3Y599F (0.8)<Flt3K644R (1.1)<Flt3wt (1.2)< Flt3D835Y (1.3) (Fig. 3e, f). A slight increase in p53-Ser15 phosphorylation that could contribute to p53 stabilization (Shieh et al. 1997) was observed with Flt3D835Y expression. The pro-apoptotic Bax protein level was slightly attenuated by Flt3wt, Flt3D835Y, and Flt3Y599F expression (Fig. 3e, f).

Flt3 functional mutants decrease proliferation in HEK293wt cells

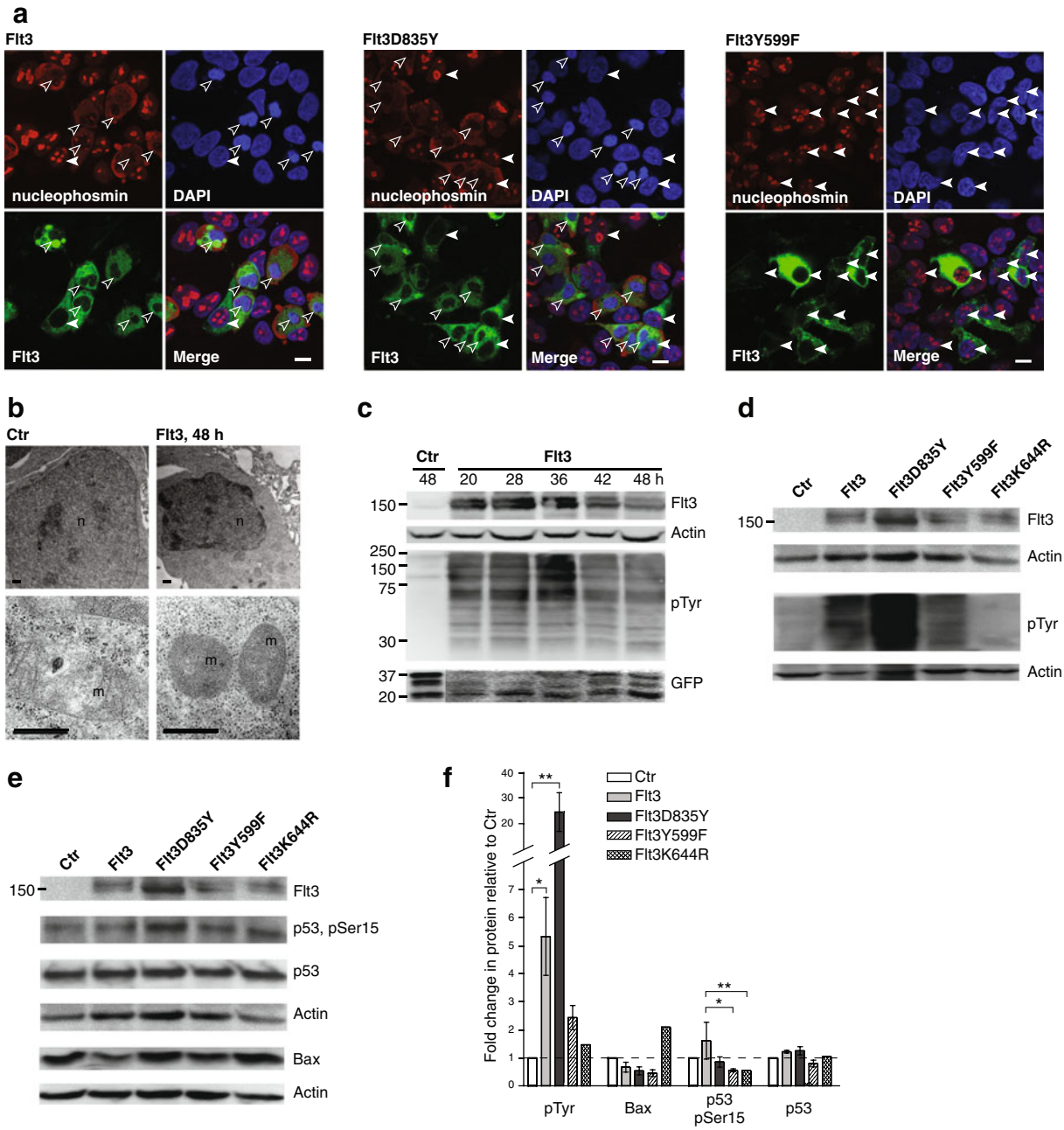
HEK293wt cells were monitored by a ^{3}H-thymidine incorporation cell growth assay 48 h after transfection with various constructs. Flt3wt expression promoted a significantly lower ($p<0.05$) cell proliferation rate (0.7-fold) compared to the control (Fig. 4a); this is consistent with the fact that enforced Flt3 expression subsequently leads to apoptosis. Interestingly, Bcl-2 co-expression rescued the Flt3-induced reduction in proliferation ($p<0.005$). Constitutively active Flt3D835Y-expressing cells showed a lower proliferation rate (0.85-fold) than the control, but did not reach statistical significance. Flt3ITD is clinically more relevant in AML (Nakao et al. 1996), and we therefore included Flt3ITD constructs in our proliferation study. Intriguingly the Flt3ITD constructs resulted in significantly lower proliferation of the HEK293 cells than the control ($p<0.005$). Flt3Y599F- or Flt3K644A expression had no significant effects on HEK293wt cell proliferation, indicating that Flt3 cell signaling through its tyrosine kinase activity is required to induce apoptosis.

Flt3-induced reduction in proliferation is suppressed by siRNA knockdown of Bax in HL-60 cells

Nucleophosmin has been shown to interact with the pro-apoptotic Bcl-2 family member Bax and is believed to be important for Bax activation and translocation in apoptosis (Kerr et al. 2007; Thompson et al. 2008). A decreased Bax/Bcl-2 ratio increased the tolerance for Flt3 in HEK293wt cells, and to investigate this ratio in HL-60 cells, co-transfection experiments of Flt3 and siRNAs targeting Bax were performed. Flt3-expressing cells with concurrent Bax knockdown showed a 1.8-fold increased proliferation rate ($p<0.001$) compared to cells transfected with Flt3 alone (Fig. 4b).

Discussion

Activating Flt3 mutations are strongly associated with aggressive myeloid leukemias like AML, and Flt3-targeting small molecule kinase inhibitors are in clinical trials (Pratz et al. 2009). However, efforts to establish stable cell lines expressing clinically relevant



gain-of-function Flt3 mutants have been surprisingly challenging. While ectopic Flt3 expression is easily performed in the IL-3-dependent hematopoietic mouse cell lines Ba/F3 and 32D (Fenski et al. 2000; Hayakawa et al. 2000), constitutive expression of Flt3 has, to our knowledge, not been previously reported in human hematopoietic cell lines. The leukemic cell lines exposed to Flt3 transduction in this study (HL-60, NB4, Kg1a, Molm-13, and MV4-11) have a wide range of endogenous Flt3 expression levels, different mutational status, and have been immortalized by different genetic aberrations (Oveland et al. 2009). AML cells harbor mutations in multiple genes that potentiate pro-survival signal transduction in contrast to chronic myeloid leukemia that is predominantly driven by the Bcr-Abl protein. We hypothesize that ectopic

Fig. 3 Flt3-induced cell death involves nucleophosmin translocation and is executed by apoptosis in HEK293wt as seen by electron microscopy and protein modifications. **a** HEK293wt cells were transfected with constructs containing Flt3wt (active), Flt3D835Y (constitutive active), or Flt3Y599F (inactivated auto-phosphorylation site) and co-immunostained 48 h after transfection for nucleophosmin (*red*) and Flt3 (*green*). Nuclei were stained with DAPI, and the cells were visualized by confocal microscopy. *Arrowheads* indicate transfected cells, apoptosis with chromatin condensation, and nucleophosmin translocation (*open*) and living cells with normal nuclear morphology and unaltered nucleophosmin localization (*closed*). Size *bars*, 10 μm. **b** HEK293wt cells were transfected with Flt3wt (Flt3-IRES-GFP) or control (Ctr, IRES-GFP), areas with >75% transfection efficiency (GFP) were marked using a grid glued on underneath the wells. Cells were fixed for transmission electron microscopy after 48 h, and cells in areas with high transfection efficiency were embedded. Nuclear and mitochondrial morphology were examined by electron microscopy, and a representative cell from Flt3-transfected populations is compared to a cell from the more homologous control (IRES-GFP) populations. *m* mitochondrium, *n* nucleus. Size *bars*, 0.5 μm. **c** HEK293wt cells were transfected with IRES-GFP and Flt3-IRES-GFP, harvested at time points as indicated, and total lysates were used for Western blotting as indicated. **d**, **e** HEK293wt cells were transfected with IRES-GFP, Flt3-IRES-GFP, Flt3D835Y-IRES-GFP, Flt3Y599F-IRES-GFP, Flt3K644A-IRES-GFP, harvested after 24 h and total lysates were applied to gels for Western blotting as indicated. **f** Bar plot of the protein levels determined by Western blotting in **d** and **e**. Values are the mean of independent experiments (three experiments except ≤2 for Flt3K644R) and normalized to (1) the respective actin loading control and (2) IRES-GFP (set to 1); *error bars* represent SEM. Molecular weights in kDa are indicated to the left on the Western blots

Flt3 expression is incompatible with AML cell lines due to a signal overload that leads to oncogene-induced cell death. This is in agreement with recent findings that excessively elevated signaling by the oncoproteins Ras, Raf, and Myc can induce senescence and/or apoptosis (reviewed in Hanahan and Weinberg 2011). This is believed to reflect intrinsic cellular defense mechanisms that protect cells against an overload of certain types of signaling.

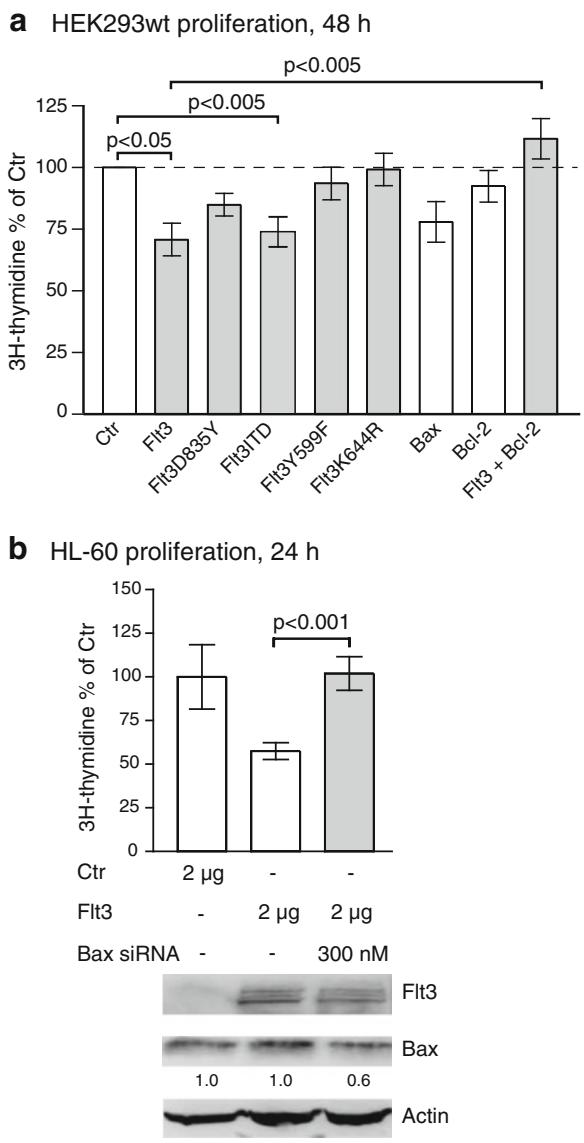
The anti-proliferative effect of Flt3 expression applied to various human cell lines was shown by Flt3 transfections in both the p53 null cell lines (HL-60 and SAOS-2), the p53 high-expression HEK293 derivative, and different AML cell lines (Figs. 2 and 4). But p53 was not significantly modulated in Flt3-transfected HEK293wt cells (Fig. 3ef), and the importance of p53 in Flt3-induced cell death remains unclear.

To further elucidate the Flt3-induced anti-proliferative effect, we used the easily transfectable HEK293 cells. Flt3 expression was shown to induce apoptosis as determined by chromatin condensation, cellular and mitochondrial shrinkage, and nucleophosmin translocation (Fig. 3a, b). The appearance of apoptosis coincided with elevated global tyrosine phosphorylation. Transfection with a constitutive active mutant, Flt3D835Y, increased the rate of apoptosis even more than Flt3wt (Fig. 3a) but did not significantly decrease cell proliferation in the ^{3}H-thymidine incorporation assay (Fig. 4a). This may reflect the ambiguity of oncogene-induced cell death (Huang et al. 2002; Koonin et al. 1996; Okura et al. 1998; Tikhomirov and Carpenter 2004). Captivatingly, Flt3-ITD significantly reduced the proliferation rate even more than Flt3-D835Y. This observation indicates that Flt3-ITDs, which transduce stronger survival signaling in AML in vivo, are even more lethal to the cell lines when ectopically expressed.

Transfection with the kinase-inactive construct Flt3K644A had no significant effect on proliferation or rate of apoptosis (Fig. 4a). Furthermore, transfection with Flt3Y599F, a SH2 domain binding-site knockout, did not induce apoptosis in HEK293wt cells (Figs. 3a and 4a). The knockout of the SH2 domain, normally involved in activation of protein tyrosine kinases like Src (Heiss et al. 2006), also resulted in lowered global tyrosine phosphorylation compared to wild-type and constitutively active Flt3 (Fig. 3d).

The constitutively active mutant, Flt3D835Y, provided stronger RTK signaling in comparison to Flt3wt overexpression as indicated by a fivefold increase in the level of tyrosine phosphorylation (Fig. 3d). Furthermore, an approximately fivefold increase in frequency of translocation of nucleophosmin was observed (Fig. 3a). However, it should be noted that the expression levels of Flt3D835Y were generally higher than Flt3 even though the cells were transfected with the same amount of plasmid DNA. Interestingly, in AML the prognostic influence of Flt3D835Y is unclear, but it is suggested that it is connected to other aberrancies, including nucleophosmin. Nucleophosmin mutation and translocation to the cytoplasm may cooperate with Flt3 expression/mutation and thereby allow for high Flt3 signaling in AML (Alcalay et al. 2005; Falini et al. 2005).

Nucleophosmin and Bax co-translocation has been assigned a role in the induction of apoptosis (Kerr et al. 2007; Thompson et al. 2008). The role of Bax was



◀ **Fig. 4** Both overexpression of Bcl-2 and downregulation of Bax prevent Flt3-induced decrease in proliferation. **a** HEK293wt cells were transfected with IRES-GFP, Flt3-IRES-GFP, Flt3D835Y-IRES-GFP, Flt3ITD (four different Flt3ITD constructs in four experiments, see "Materials and methods" section for details), Flt3Y599F-IRES-GFP, Flt3K644A-IRES-GFP, Bax, Bcl-2, Flt3-IRES-GFP+Bcl-2 and used in a ^{3}H-thymidine incorporation assay after 48 h (percentage of viability relative to the control, IRES-GFP, set to 100 %). Statistical significance (p) was calculated using one-way ANOVA and Tukey's multiple comparison test; *error bars* represent SEM of three individual experiments ($n \geq 6$). **b** HL-60 cells used in a ^{3}H-thymidine incorporation assay (18-h incubation) 24 h after Amaxa transfection with control plasmid (Ctr, IRES-GFP), Flt3 (Flt3-IRES-GFP), or Flt3 plus Bax siRNA. Statistical significance (p) was calculated using two-tailed unpaired t test ($n = 11$; extreme outliers were excluded, ≤3); *bars* represent SEM of three individual experiments. Representative Western blots of corresponding transfections, *numbers* indicate Bax levels relative to Ctr

apparent in Flt3-mediated cell death due to the ability to rescue Flt3-transfected HL-60 cells from inhibition of proliferation with Bax siRNA (Fig. 4b). Furthermore, Flt3-transfected HEK293wt cells were rescued by increased Bcl-2 expression (Fig. 4a). Results from ^{3}H-thymidine assays indicate a connection between the Flt3 and Bcl2 families of proteins; however, further investigation is needed in order to be able to understand the molecular mechanisms involved. In AML, Bcl-2 expression correlates with lowered rates of complete remission, and the ratio of Bax to Bcl-2 is predictive of long-term survival, as discussed by Irish et al. (2007).

RTK-induced apoptotic cell death has previously been reported for the class II EGF receptors: EGF receptors induce apoptosis in agreement with receptor tyrosine phosphorylation and by upregulation of p53, p21^{WAF}, and Myc, in addition to downregulation of Bcl-2 (Huang et al. 2002). A p53-independent p38 mitogen-activated protein kinase-driven apoptotic pathway has also been associated with high levels of EGF receptor activation (Tikhomirov and Carpenter 2004). Furthermore, activation of the PDGF receptor results in apoptosis in cultured vascular smooth muscle cells in a time- and dose-dependent manner, involving decreased levels of Bcl-2 and Bcl-xL mRNA (Okura et al. 1998). Overexpression of tyrosine kinases might be associated with a decrease in cell proliferation, as exemplified by upregulation of the Bcr-Abl protein due to amplification of the BCR-ABL gene in human imatinib-resistant cell lines (Desplat et al. 2005).

In conclusion, despite the fact that Flt3 activity is strongly associated with chemoresistance and relapse in AML, long-term ectopic expression of Flt3 appears to be detrimental to IL-3-independent human leukemic cell lines. We speculate that the difficulties in establishing human cell models of Flt3 expression are reflected by our limited understanding of Flt3 signaling networks. This would partly explain the slow progress experienced in the development of Flt3-directed therapy.

Acknowledgments We are grateful to Linn Hodneland, Lena Fjellstad Hansen, Atle Bredhaug, Ann Kristin Frøyset, Lise

Fismen, and Hege Avsnes Dale for valuable technical assistance and Ian F. Pryme for proofreading the manuscript. Confocal imaging and electron microscopy were performed at the Molecular Imaging Center (FUGE, Norwegian Research Council), University of Bergen. This work was supported by grants from the Helse Vest Research Fund and The Norwegian Cancer Society (B.T.G.).

References

Alcalay M, Tiacci E, Bergomas R, Bigerna B, Venturini E, Minardi SP, Meani N, Diverio D, Bernard L, Tizzoni L, Volorio S, Luzi L, Colombo E, Lo Coco F, Mecucci C, Falini B, Pelicci PG. Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. Blood. 2005;106:899–902.

Beham A, Marin MC, Fernandez A, Herrmann J, Brisbay S, Tari AM, Lopez-Berestein G, Lozano G, Sarkiss M, McDonnell TJ. Bcl-2 inhibits p53 nuclear import following DNA damage. Oncogene. 1997;15:2767–72.

Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature. 2001;411:355–65.

Bredholt T, Dimba EA, Hagland HR, Wergeland L, Skavland J, Fossan KO, Tronstad KJ, Johannessen AC, Vintermyr OK, Gjertsen BT. Camptothecin and khat (Catha edulis Forsk.) induced distinct cell death phenotypes involving modulation of c-FLIPL, Mcl-1, procaspase-8 and mitochondrial function in acute myeloid leukemia cell lines. Mol Cancer. 2009;8:101.

Bruserud O, Hovland R, Wergeland L, Huang TS, Gjertsen BT. Flt3-mediated signaling in human acute myelogenous leukemia (AML) blasts: a functional characterization of Flt3-ligand effects in AML cell populations with and without genetic Flt3 abnormalities. Haematologica. 2003;88:416–28.

Brustugun OT, Fladmark KE, Doskeland SO, Orrenius S, Zhivotovsky B. Apoptosis induced by microinjection of cytochrome c is caspase-dependent and is inhibited by Bcl-2. Cell Death Differ. 1998;5:660–8.

Desplat V, Belloc F, Lagarde V, Boyer C, Melo JV, Reiffers J, Praloran V, Mahon FX. Overproduction of BCR-ABL induces apoptosis in imatinib mesylate-resistant cell lines. Cancer. 2005;103:102–10.

Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, La Starza R, Diverio D, Colombo E, Santucci A, Bigerna B, Pacini R, Pucciarini A, Liso A, Vignetti M, Fazi P, Meani N, Pettirossi V, Saglio G, Mandelli F, Lo-Coco F, Pelicci PG, Martelli MF. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352:254–66.

Fearnhead HO, McCurrach ME, O'Neill J, Zhang K, Lowe SW, Lazebnik YA. Oncogene-dependent apoptosis in extracts from drug-resistant cells. Genes Dev. 1997;11:1266–76.

Fenski R, Flesch K, Serve S, Mizuki M, Oelmann E, Kratz-Albers K, Kienast J, Leo R, Schwartz S, Berdel WE, Serve H. Constitutive activation of FLT3 in acute myeloid leukaemia and its consequences for growth of 32D cells. Br J Haematol. 2000;108:322–30.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.

Hayakawa F, Towatari M, Kiyoi H, Tanimoto M, Kitamura T, Saito H, Naoe T. Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. Oncogene. 2000;19:624–31.

Heiss E, Masson K, Sundberg C, Pedersen M, Sun J, Bengtsson S, Ronnstrand L. Identification of Y589 and Y599 in the juxtamembrane domain of Flt3 as ligand-induced autophosphorylation sites involved in binding of Src family kinases and the protein tyrosine phosphatase SHP2. Blood. 2006;108:1542–50.

Hovland R, Gjertsen BT, Bruserud O. Acute myelogenous leukemia with internal tandem duplication of the Flt3 gene appearing or altering at the time of relapse: a report of two cases. Leuk Lymphoma. 2002;43:2027–9.

Huang GC, Hobbs S, Walton M, Epstein RJ. Dominant negative knockout of p53 abolishes ErbB2-dependent apoptosis and permits growth acceleration in human breast cancer cells. Br J Cancer. 2002;86:1104–9.

Irish JM, Anensen N, Hovland R, Skavland J, Borresen-Dale AL, Bruserud O, Nolan GP, Gjertsen BT. Flt3 Y591 duplication and Bcl-2 overexpression are detected in acute myeloid leukemia cells with high levels of phosphorylated wild-type p53. Blood. 2007;109:2589–96.

Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud O, Gjertsen BT, Nolan GP. Single cell profiling of potentiated phosphoprotein networks in cancer cells. Cell. 2004;118:217–28.

Kerr LE, Birse-Archbold JL, Short DM, McGregor AL, Heron I, Macdonald DC, Thompson J, Carlson GJ, Kelly JS, McCulloch J, Sharkey J. Nucleophosmin is a novel Bax chaperone that regulates apoptotic cell death. Oncogene. 2007;26:2554–62.

Koch S, Jacobi A, Ryser M, Ehninger G, Thiede C. Abnormal localization and accumulation of FLT3-ITD, a mutant receptor tyrosine kinase involved in leukemogenesis. Cells Tissues Organs. 2008;188:225–35.

Koonin EV, Altschul SF, Bork P. BRCA1 protein products … functional motifs. Nat Genet. 1996;13:266–8.

Lee BH, Williams IR, Anastasiadou E, Boulton CL, Joseph SW, Amaral SM, Curley DP, Duclos N, Huntly BJ, Fabbro D, Griffin JD, Gilliland DG. FLT3 internal tandem duplication mutations induce myeloproliferative or lymphoid disease in a transgenic mouse model. Oncogene. 2005;24:7882–92.

Lorens JB, Bennett MK, Pearsall DM, Throndset WR, Rossi AB, Armstrong RJ, Fox BP, Chan EH, Luo Y, Masuda E, Ferrick DA, Anderson DC, Payan DG, Nolan GP. Retroviral delivery of peptide modulators of cellular functions. Mol Ther. 2000;1:438–47.

Lowenberg B, van Putten W, Theobald M, Gmur J, Verdonck L, Sonneveld P, Fey M, Schouten H, de Greef G, Ferrant A, Kovacsovics T, Gratwohl A, Daenen S, Huijgens P, Boogaerts M. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. N Engl J Med. 2003;349:743–52.

Lyman SD, James L, Zappone J, Sleath PR, Beckmann MP, Bird T. Characterization of the protein encoded by the flt3 (flk2)

receptor-like tyrosine kinase gene. Oncogene. 1993;8:815–22.

Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, Sonoda Y, Fujimoto T, Misawa S. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996;10:1911–8.

Okura T, Igase M, Kitami Y, Fukuoka T, Maguchi M, Kohara K, Hiwada K. Platelet-derived growth factor induces apoptosis in vascular smooth muscle cells: roles of the Bcl-2 family. Biochim Biophys Acta. 1998;1403:245–53.

Oveland E, Fladmark KE, Wergeland L, Gjertsen BT, Hovland R. Proteomics approaches to elucidate oncogenic tyrosine kinase signaling in myeloid malignancies. Curr Pharm Biotechnol. 2006;7:185–98.

Oveland E, Gjertsen BT, Wergeland L, Selheim F, Fladmark KE, Hovland R. Ligand-induced Flt3-downregulation modulates cell death associated proteins and enhances chemosensitivity to idarubicin in THP-1 acute myeloid leukemia cells. Leuk Res. 2009;33:276–87.

Ozeki K, Kiyoi H, Hirose Y, Iwai M, Ninomiya M, Kodera Y, Miyawaki S, Kuriyama K, Shimazaki C, Akiyama H, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Ueda R, Ohno R, Emi N, Naoe T. Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. Blood. 2004;103:1901–8.

Pratz KW, Cortes J, Roboz GJ, Rao N, Arowojolu O, Stine A, Shiotsu Y, Shudo A, Akinaga S, Small D, Karp JE, Levis M. A pharmacodynamic study of the FLT3 inhibitor KW-2449 yields insight into the basis for clinical response. Blood. 2009;113:3938–46.

Sargin B, Choudhary C, Crosetto N, Schmidt MH, Grundler R, Rensinghoff M, Thiessen C, Tickenbrock L, Schwable J, Brandts C, August B, Koschmieder S, Bandi SR, Duyster J, Berdel WE, Muller-Tidow C, Dikic I, Serve H. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. Blood. 2007;110:1004–12.

Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell. 1997;91:325–34.

Small D, Levenstein M, Kim E, Carow C, Amin S, Rockwell P, Witte L, Burrow C, Ratajczak MZ, Gewirtz AM, et al. STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34+ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. Proc Natl Acad Sci U S A. 1994;91:459–63.

Stommel JM, Kimmelman AC, Ying H, Nabioullin R, Ponugoti AH, Wiedemeyer R, Stegh AH, Bradner JE, Ligon KL, Brennan C, Chin L, DePinho RA. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. Science. 2007;318:287–90.

Swift S, Lorens J, Achacoso P, Nolan GP. Current protocols in immunology. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology, vol Unit 10.17C, Suppl. 31. New York: Wiley; 1999. p. 14–29.

Thompson J, Finlayson K, Salvo-Chirnside E, Macdonald D, McCulloch J, Kerr L, Sharkey J. Characterisation of the Bax-nucleophosmin interaction: the importance of the Bax C-terminus. Apoptosis. 2008;13:394–403.

Tikhomirov O, Carpenter G. Ligand-induced, p38-dependent apoptosis in cells expressing high levels of epidermal growth factor receptor and ErbB-2. J Biol Chem. 2004;279:12988–96.

Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Saito H, Ueda R, Ohno R, Naoe T. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001;97:2434–9.