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Constitutive Activation of Akt by Flt3 Internal Tandem Duplications Is Necessary for Increased Survival, Proliferation, and Myeloid Transformation

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Abstract

Up to 30% of patients with acute myeloid leukemia (AML) harbor internal tandem duplications (ITD) within the *FLT3* gene, encoding a receptor tyrosine kinase. These mutations induce constitutive tyrosine kinase activity in the absence of the natural Flt3 ligand and confer growth factor independence, increased proliferation, and survival to myeloid precursor cells. The signaling pathways and downstream nuclear targets mediating leukemic transformation are only partly identified. Here, we show that the presence of Flt3-ITD constitutively activates Akt (PKB), a key serine-threonine kinase within the phosphatidylinositol 3-kinase pathway. Constitutive activation of Akt phosphorylated and inhibited the transcription factor Foxo3a. Restored Foxo3a activity reversed Flt3-ITD-mediated growth properties and dominant-negative Akt prevented Flt3-ITD-mediated cytokine independence. Conditional Akt activation targeted to the cell membrane induced cytokine-independent survival, cell cycle progression, and proliferation. Importantly, Akt activation was sufficient to cause *in vitro* transformation of 32D myeloid progenitor cells and *in vivo* promoted the development of a leukemia-like myeloid disease. Akt phosphorylation was found in myeloid blasts of 86% of AML patients, suggesting an important role in leukemogenesis. In summary, Akt is necessary for increased survival, proliferation, and leukemic transformation by Flt3-ITD, possibly by inactivation of Foxo transcription factors. These findings indicate that Akt and Foxo transcription factors are attractive targets for therapeutic intervention in AML. (Cancer Res 2005; 65(21): 9643-50)

Introduction

Flt3 is a receptor tyrosine kinase expressed on early myeloid progenitors of the bone marrow. Up to 30% of patients suffering from acute myeloid leukemia (AML) harbor internal tandem duplications (ITD) within the juxtamembrane domain of Flt3 (hereafter Flt3-ITD), which is encoded by exons 14 and 15 of the *FLT3* gene (1, 2). The presence of Flt3-ITD in AML patients is associated with a poor prognosis (3–6). In addition, point mutations have been described within the activation loop of the tyrosine kinase domain, including point mutations at Asp⁸³⁵ (D835) and I⁸³⁶ (6–8).

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Activating Flt3 mutations have been reported in all AML subtypes and represent the most common molecular defect in *de novo* AML. Oncogenic N-Ras and K-Ras mutations are found in a quarter of all cases of AML but rarely occur together with Flt3-ITD (9), suggesting functional overlap.

Oncogenic Ras has been shown to activate the phosphatidylinositol 3-kinase (PI3K; ref. 10), which leads to accumulation of phosphatidylinositol (3–5) trisphosphate at the cell membrane and binding of the serine-threonine kinase Akt via its pleckstrin homology domain. At the cell membrane, Akt undergoes a conformational change and is phosphorylated on Thr³⁰⁸ by PDK1 and Ser⁴⁷⁸ by an unknown kinase, leading to Akt activation (11). Akt has been shown to mediate survival and proliferation, partly through inhibition of Foxo transcription factors (11).

Foxo3a is a member of the Foxo subfamily of forkhead transcription factors that plays a critical role in apoptosis and cell cycle regulation. The three members of the forkhead family, Foxo1 (FKHR), Foxo3a (FKHRL1), and Foxo4 (AFX) represent mammalian orthologues of a *Caenorhabditis elegans* transcription factor, DAF16. Constitutively active Akt and Akt activated in response to growth factor stimulation phosphorylates Foxo3a. Through binding to 14-3-3 scaffolding proteins, Foxo3a is retained in the cytoplasm and its transcriptional activity is prevented (12, 13).

We and others (14, 15) have found that expression of Flt3-ITD in myeloid 32Dcl3 cells leads to interleukin-3 (IL-3)-independent survival and proliferation. Furthermore, constitutive signaling via the signal transducers and activators of transcription 5 (STAT5) transcription factor has been observed (14, 16, 17). However, the mechanisms of Flt3-ITD-mediated transformation are still only partially understood.

Given the emerging importance of the PI3K/Akt/Foxo pathway, we were interested to determine its relevance in Flt3-ITD-mediated transformation of myeloid cells. Here, we provide evidence that Flt3-ITD induced constitutive Akt activation and, in turn, inactivated its substrate Foxo3a. Conditionally active Akt mediated survival, proliferation, and leukemic transformation of myeloid cells both *in vitro* and *in vivo*, likely through inactivation of Foxo3a transcriptional activity. Our data provides a potential mechanism of Flt3-ITD-mediated transformation.

Materials and Methods

Cell Lines and Plasmids

The IL-3-dependent murine myeloid 32Dcl3 cell line (subsequently referred to as 32D) was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 containing 10% FCS, 10% WEHI-conditioned medium (as a source of IL-3) and

supplemented with antibiotics. Recombinant human Flt3 ligand and recombinant murine IL-3 were purchased from PeproTech (Rocky Hill, NJ).

The generation of 32D cells stably transfected with wild-type Flt3 or Flt3-ITD has been previously described (14, 18). Briefly, the full-length coding sequence of Flt3 from a patient with ITD mutation as well as the full-length wild-type sequence from the Oci-AML5 cell line were amplified, cloned into the pAL expression vector, and stably transfected into 32D cells (14, 18). The expression of these receptors was regularly measured by immunoblotting and fluorescence-activated cell sorting (FACS).

We generated 32D cells stably expressing a conditionally active form of Akt as previously described (19, 20). In brief, the pleckstrin homology domain of Akt was replaced by the c-Src myristylation-targeting sequence (M^+ , hereafter referred to as M^+Akt), leading to PI3K-independent membrane localization. Conditionality was conferred by fusing M^+Akt to a modified form of the mouse estrogen receptor (ER) that binds 4-hydroxytamoxifen (4-OHT) but is refractory to estrogen (ER^*). Addition of 4-OHT competitively removes sequestered heat shock proteins and activates the fusion protein (21). As a control, we expressed a myristylation-defective form of the protein ($M^-Akt:ER^*$), which is not membrane associated and therefore inactive (20). Both 32D- $M^+Akt:ER^*$ and conditional 32D- $M^+Akt:ER^*$ cell lines used for experiments were pooled populations.

A mutant form of Akt that contains alanine substitutions at residues Ser⁴⁷³ and Thr³⁰⁸ was kindly provided by Dr. D. Stokoe (22). To delete all kinase activity, we substituted K179 with alanine by site-directed mutagenesis, thereby creating a dominant-negative Akt/K179A-T308A-S473A mutant (referred to as dnAkt).

A mutant form of Foxo3a that contains alanine substitutions at three phosphorylation sites can neither be phosphorylated nor sequestered in the cytosol when Akt is activated (12, 13, 23). We generated 32D cells stably transfected with pcDNA-Foxo3a/A3:ER^{*}, a plasmid which was kindly provided by Dr. P. Coffey (23). The phosphorylation sites Thr³², Ser²⁵³, and Ser³¹⁵ are mutated to alanine, and Foxo3a/A3 is fused to ER^{*}. Thereby, Foxo3a can be activated by addition of 4-OHT. 32D cells coexpressing Flt3-ITD and Foxo3a/A3:ER^{*} were pooled populations.

Immunoblot Analysis and Antibodies

Cells were lysed in buffer containing 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 1% NP40, 1 mmol/L EDTA, 1 mmol/L EGTA, 25 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L DTT, and protease inhibitor cocktail (Complete mini; Boehringer, Mannheim, Germany) at 4°C. Lysate was centrifuged (10,000 × *g*) to remove insoluble components. Equal amounts of protein were loaded onto polyacrylamide gels and run by standard SDS-PAGE. Proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA) and blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBS-T). Primary antibodies were anti-phospho-Ser⁴⁷³-Akt, anti-phospho-Foxo, (total) Akt, (total) Foxo (purchased from Cell Signaling Technology, Inc., Beverly, MA), and β-actin (Sigma, Deisenhofen, Germany). This was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (obtained from Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Proteins were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech) and exposed to film.

Luciferase Reporter Assays

To determine Foxo transcriptional activity, we used the firefly luciferase reporter construct pGL2-6xDBE (containing six Foxo3a binding sequences) kindly provided by Dr. P. Coffey (23). 32D cells expressing either wtFlt3, Flt3-ITD, $M^-Akt:ER$, or $M^+Akt:ER$ were electroporated (300 V/975 μF) with 15 μg of pGL2-6xDBE or empty vector (pGL2) and cotransfected with 25 ng of a *Renilla* luciferase plasmid (pRL-TK). Luciferase activity was measured using the Dual Luciferase Reporter System (Promega, Madison, WI). The values were normalized for transfection efficiency to the respective values of *Renilla* luciferase. Each data point represents the mean ± SD of three independent experiments.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium Cell Viability Assays

The CellTiter 96 Aqueous Proliferation Assay (Promega, Madison, WI) is a colorimetric method for determining cell viability in a 96-well

format. Fifteen microliters of the substrate 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) are added to 100 μL of cell culture for 1 hour and MTS is metabolized into formazan by living cells. The quantity of formazan product as measured by the amount of 490-nm absorbance is proportional to the number of living cells in culture. Experiments were repeated at least thrice.

[³H]-thymidine Incorporation

A total of 4×10^4 cells per well were plated in 200-μL medium containing 0.5% FCS. Cells were stimulated with either 20 ng/mL Flt3 ligand or 2 ng/mL IL-3 or cultured without cytokines for 24 and 48 hours. Six hours before each time point, 1 μCi (0.037 MBq) of [³H]-thymidine was added. Genomic DNA of the cells was harvested on glass fiber filters, and β-emission of the bound DNA was detected with a scintillation counter. Experiments were repeated at least thrice.

Flow Cytometry

Cell cycle analysis. 32D cells were washed in PBS containing 0.1% bovine serum albumin (PBS-B) and fixed in 70% ethanol at 4°C for 1 hour. The cells were resuspended in PBS-B and treated with 5 mg/mL RNase A for 10 minutes. Propidium iodide (50 μg/mL, Boehringer) was added, incubated for 30 minutes at room temperature, and analyzed by FACS.

Annexin V/propidium iodide staining. Cells were washed once with PBS, resuspended in 250-μL calcium-binding buffer containing propidium iodide (1.5 μg/mL) and Annexin V/FITC (0.05 μg/mL; purchased from Immunotech, Marseilles, France), and incubated on ice for 30 minutes. FACS analysis was done by FACSCalibur (BD, Heidelberg, Germany), using CellQuest software.

Colony Assays

Growth in semisolid methylcellulose was taken as being representative of *in vitro* leukemic transformation. We analyzed clonal growth of 1×10^4 32D cells per dish in 1 mL of culture mix containing Iscove's modified Dulbecco's medium (Life Technologies, Grand Island, NY), 1% methylcellulose, and 20% FCS. 32D cells expressing $M^-Akt:ER^*$ or conditionally active $M^+Akt:ER^*$ were treated with 2 μmol/L 4-OHT. The colonies were counted on day 8.

Animal Experiments

Nine-week-old female C3H/HeJ mice, which are syngeneic to 32Dcl3 cells, were used to assess the *in vivo* development of leukemia-like disease. 32D-Flt3-ITD cells or 32D- $M^+Akt:ER^*$ cells (1×10^6) were injected into the tail vein. Mice injected with 32D- $M^+Akt:ER^*$ were treated by daily injection of either tamoxifen (which is metabolized to 4-OHT in the liver) or solvent (peanut oil), as previously reported (24). Moribund animals were sacrificed. Femurs and organs were removed, formalin fixed, and paraffin embedded. The experimental protocols were reviewed and approved by the local Committee on Animal Experimentation.

Tissue Array and Primary Acute Myeloid Leukemia Samples

The tissue array construction of 90 patients diagnosed with AML was done as recently described (25). The tissue array contained samples from 66 patients diagnosed with primary, untreated AML, 19 patients with relapsed AML, and five patients with refractory AML. A diagnostic Giemsa-stained section served to enable the definition of areas with the highest amount of blast cells. Two punches were arrayed per patient to control for intratumoral heterogeneity (26). Tissue sections were mounted on SuperFrost/Plus slides and dewaxed in xylene. The phospho-Ser⁴⁷³-Akt (p-Akt) antibody was tested on PTEN-mutant U87 cells that had been similarly fixed and embedded. The tissue array section was microwaved in 10 mmol/L citrate buffer (pH 6.0, 10 minutes, 120°C). After washing in PBS, the section was incubated with the primary p-Akt antibody (purchased from Cell Signaling Technology) at 1:100 dilution. The D-APAAP method was used for detection and a semiquantitative scoring system ranging from 0% to 100% (in 10% increments) was used to detect blast staining, as well as the nuclear and membrane/cytosolic localization. Approval for the use of primary AML samples was granted by the local Ethics Committee.

Results

Expression of Flt3 internal tandem duplications constitutively activates Akt and inactivates Foxo3a. 32D cells expressing wtFlt3 or Flt3-ITD were starved for 14 hours and stimulated with either Flt3-ligand or IL-3. Both stable cell lines expressed Flt3 at equal levels (data not shown). To analyze activation of Akt and inactivation of Foxo transcription factors, we did immunoblot analysis with phospho-specific antibodies. Figure 1A (left) showed Ser⁴⁷³ phosphorylation of Akt (i.e., activation of Akt) in response to 15 minutes of stimulation with Flt3 ligand or IL-3 in wtFlt3-expressing 32D cells. In contrast, Flt3-ITD-expressing cells showed Ser⁴⁷³ phosphorylation of Akt, irrespective of any stimulation. In Fig. 1A (right), cells were maximally stimulated with a high concentration of Flt3 ligand for 5 minutes and washed in Flt3 ligand-free media. Maximal Flt3 ligand stimulation induced a strong Akt activation in wtFlt3. Importantly, the time course revealed a rapid loss of Akt activity in wtFlt3-expressing cells, whereas in Akt activity in Flt3-ITD-expressing cells is maintained. Together these data suggest constitutive activation of Akt. In addition, maximal Flt3 ligand stimulation led to minor additional Akt activation in Flt3-ITD-expressing cells (Fig. 1A, right).

As Akt phosphorylates Foxo3a on Thr³², we asked whether this was true for Flt3-ITD-expressing cells. Phospho-Thr³²-Foxo3a was detected under all conditions where Akt was active (Fig. 1A, left). Importantly, expression of Flt3-ITD induced constitutive phosphorylation of Foxo3a, irrespective of additional stimulation with Flt3 ligand or IL-3. On the other hand, wtFlt3 stimulated with Flt3 ligand induced only a minor increase in Foxo phosphorylation. To measure Foxo transcriptional activity, we did luciferase assays using a luciferase reporter construct containing six Foxo DNA binding elements (23). Luciferase activity was markedly reduced when Flt3-ITD was expressed or when wtFlt3-expressing cells were stimulated with IL-3 (and to a lesser extent Flt3 ligand; Fig. 1B). This suggests that, in contrast to wtFlt3, expression of Flt3-ITD functionally inactivates Foxo-dependent transcriptional activity under all examined conditions.

Dominant-negative Akt reverses Flt3 internal tandem duplication-mediated interleukin-3 independence. The 32D cell line is an IL-3-dependent murine myeloid precursor cell line. Expression of Flt3-ITD in 32D cells confers IL-3 independence (14, 18). On the contrary, wtFlt3-expressing cells are dependent on either Flt3 ligand or IL-3 (14, 18). As shown in Fig. 2C, we expressed

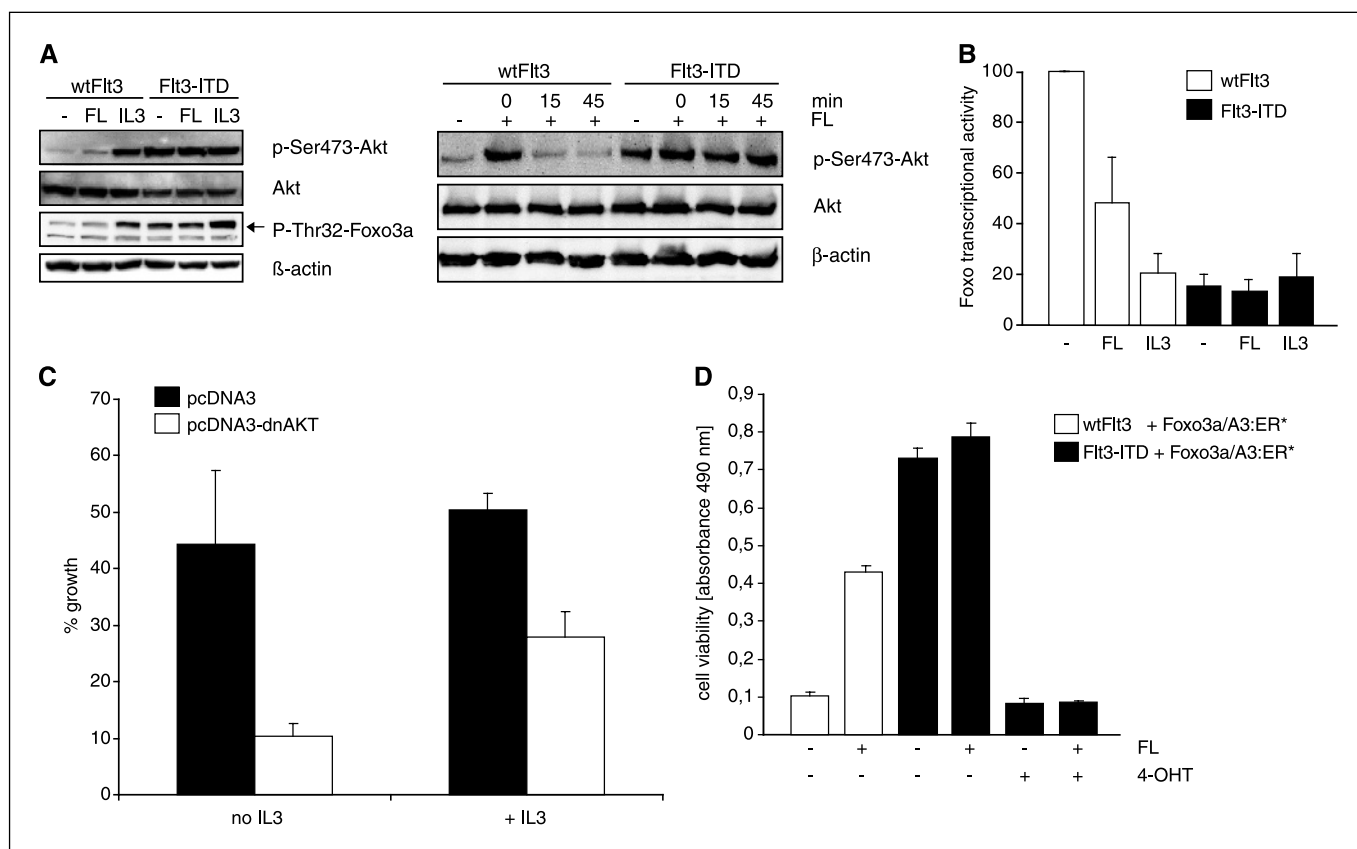


Figure 1. Expression of Flt3-ITD constitutively activates Akt and inactivates Foxo3a. *A*, 32D cells stably expressing wtFlt3 or Flt3-ITD were starved for 14 hours. *Left*, cells were stimulated for 15 minutes with either 50 ng/mL Flt3 ligand (FL) or 2 ng/mL IL-3 as indicated. *Right*, cells were maximally stimulated with 200 ng/mL Flt3 ligand for 5 minutes, washed, resuspended in medium without Flt3 ligand, and lysates were taken at the indicated time points. Lysates were separated by SDS-PAGE. Immunoblotting was done for p-Ser⁴⁷³-Akt, total Akt, and p-Thr³²-Foxo3a. β -Actin staining was done to show equal loading. *B*, analysis of Foxo transcriptional activity on a luciferase reporter construct containing six Foxo DNA binding domains (6 \times DBE). 32D-wtFlt3 and 32D-Flt3-ITD cells were electroporated with 6 \times DBE and pRL followed by either growth factor withdrawal or stimulation with Flt3 ligand or IL-3 for 17 hours, as indicated. Luciferase activity was measured using the dual luciferase reporter system. The values were normalized for transfection efficiency to the respective values of *Renilla* luciferase. Starved, nonstimulated cells were set at 100% Foxo transcriptional activity. *Columns*, means of three independent experiments; *bars*, \pm SD. *C*, 32D-Flt3-ITD cells were electroporated with empty vector (pcDNA3) or pcDNA3-dnAkt (K179A-T308A-S473A) and underwent selection with G418. MTS cell viability assays were done at 72 hours. *Columns*, means of two independent experiments in triplicate; *bars*, \pm SD. Growth of mock-electroporated 32D cells without G418 were defined as 100% growth. *D*, MTS cell viability assays were done with 32D cells expressing either wtFlt3-Foxo3a/A3:ER* or Flt3-ITD-Foxo3a/A3:ER* and stimulated with 50 ng/mL of Flt3 ligand for 24 hours where indicated. Foxo3a activity was conditionally activated by addition of 4-OHT for 24 hours. *Columns*, means of three independent experiments; *bars*, \pm SD.

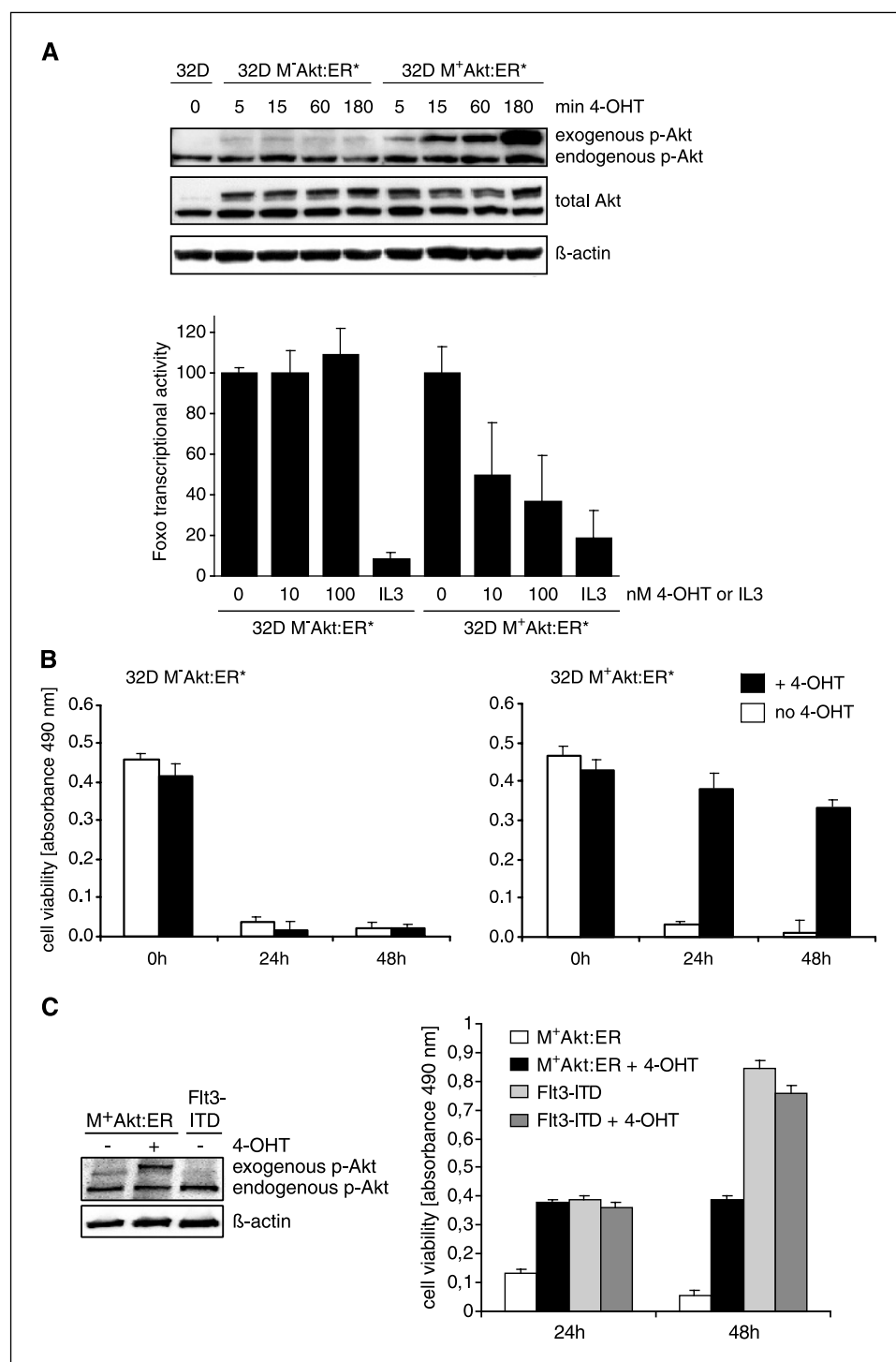


Figure 2. Conditional activation of Akt mediates Foxo inactivation and confers survival, proliferation, and transformation of myeloid cells. **A**, 32D cells stably expressing (as pools) either myristylation-defective M⁺Akt:ER* or myristylation-competent M⁺Akt:ER* were treated for 5 minutes to 3 hours with 1 μ mol/L 4-OHT. Lysates were separated by SDS-PAGE and immunoblotted for p-Ser⁴⁷³-Akt and total Akt, and β -actin served as loading control (*top*). The luciferase reporter construct 6 \times DBE was transfected into starved 32D-M⁺Akt:ER* or 32D-M⁺Akt:ER cells. Cells were either treated with 2 ng/mL of IL-3 or indicated amounts of 4-OHT for 18 hours (*bottom*). Starved, nonstimulated cells were set at 100% Foxo transcriptional activity. *Columns*, means of three independent experiments; *bars*, \pm SD. **B**, MTS cell viability assays were done with 32D cells expressing either M⁺Akt:ER* or M⁺Akt:ER* and treated with or without 1 μ mol/L 4-OHT for 24 and 48 hours. *Columns*, means of three independent experiments; *bars*, \pm SD. **C**, 32D cell clones expressing either inducible M⁺Akt:ER* or M⁺Akt:ER* were generated. Clone H10 expressing M⁺Akt:ER* and 32D cells expressing Flt3-ITD (*top and bottom*). After treatment with 10 nmol/L 4-OHT lysates were separated by SDS-PAGE followed by immunoblotting (*left*). In addition, MTS cell viability assays were done for 24 and 48 hours (*right*).

dominant-negative Akt (22) in 32D cells stably expressing Flt3-ITD and observed a 75% decrease in IL-3-independent viability. Interestingly, the effects of dnAkt were partly rescued by the addition of IL-3, which argues for a specific requirement of Akt in Flt3-ITD-mediated survival.

Furthermore, five separate attempts to generate 32D cells stably coexpressing dnAkt and Flt3-ITD were unsuccessful, suggesting a biological disadvantage.

Restoration of Foxo activity prevents Flt3 internal tandem duplication-mediated growth properties. To examine whether

forced Foxo activity could overcome Flt3-ITD-mediated growth properties, we used a conditional Foxo3a construct (23). The expression plasmid contains phosphorylation-insensitive Foxo3a fused to the estrogen receptor (Foxo3a/A3:ER*) that responds to 4-OHT. We generated 32D cells expressing conditional Foxo3a/A3:ER* and either wtFlt3 or Flt3-ITD. Induction of phosphorylation-resistant and therefore noninhibitable Foxo3a/A3 in Flt3-ITD-expressing cells prevented cell viability after 24 hours (Fig. 1D). Upon Foxo3a activation, 32D-Flt3-ITD cells underwent apoptosis, irrespective of concomitant Flt3 ligand stimulation. Taken together, the

data presented in Fig. 1 suggest a linear pathway, whereby expression of oncogenic Flt3-ITD constitutively activates Akt, which in turn phosphorylates Foxo3a and inactivates its transcriptional activity. Restoration of Flt3-ITD-resistant Foxo3a activity overcomes the Flt3-ITD-mediated growth advantages.

Conditional activation of Akt mediates Foxo inactivation and protects from cytokine withdrawal. We were interested to know whether the altered growth and survival properties conferred by Flt3-ITD were, at least in part, mediated by activation of Akt. To test this, we used a conditionally active form of Akt targeted to the cell membrane (M^+ Akt:ER*; ref. 20). The myristylation-defective and inactive form of the same construct (M^- Akt:ER*) served as a control and we stably expressed both forms in separate pools of 32D cells.

As shown in Fig. 2A, the M^- Akt:ER* and M^+ Akt:ER* forms were expressed at equal levels as measured by total Akt detection. Whereas exogenous Akt in M^+ Akt:ER*-expressing cells was rapidly activated within 15 minutes of 4-OHT treatment, the myristylation-defective M^- Akt:ER* was not. Concomitantly, Foxo-dependent transcriptional activity (as measured by luciferase reporter activity) was dose dependently inhibited in 32D M^+ Akt:ER* cells, whereas it was unaffected in 32D M^- Akt:ER* control cells after 4-OHT treatment (Fig. 2A).

IL-3 withdrawal of IL-3-dependent 32D cells resulted in near-complete cell death within 24 and 48 hours (Fig. 2B, *open columns*). Similarly, 4-OHT treatment of M^- Akt:ER*-expressing cells (leading to mislocalized and inactive Akt) did not result in increased cell viability (Fig. 2B, *left, black columns*). In contrast, cells induced to express membrane-targeted and active Akt (M^+ Akt:ER*) survived the cytokine withdrawal (Fig. 2B, *right, black columns*).

In an additional experiment, we generated stable clones of M^+ Akt:ER*-expressing 32D cells. In response to 4-OHT, several clones induced Akt activity that was quantitatively very similar to the Akt activation of Flt3-ITD (Fig. 2C). At these induction levels, Akt activation mediated a strong factor independence (Fig. 2C, *right*). In conclusion, activation of Akt at levels that are comparable with Flt3-ITD induced activation levels protect 32D cells from cytokine withdrawal.

Conditional activation of Akt promotes survival, cell cycle progression, and proliferation. To distinguish between protection from apoptosis or increased proliferation of a subset of cells (Fig. 2B and C), we did cell cycle analyses of 32D M^+ Akt:ER* cells. When cultured in the presence of IL-3, the cells were alive (sub- G_1 fraction of 1%) and rapidly dividing, with an S-phase population of 39% (Fig. 3A, *left*). After 24 hours of IL-3 withdrawal (Fig. 3A, *middle*), a large proportion of dead cells accumulated in the sub- G_1 fraction (40%), whereas only 10% and 12% cells progressed to S and G_2 -M phase, respectively. Upon Akt activation (Fig. 3A, *right*), the sub- G_1 population was markedly reduced (20%). The G_0 - G_1 population was evenly distributed (40%, 37%, and 41%; Fig. 3A, from *left to right*). Interestingly, more Akt-expressing cells were found in the S phase (20%) and the G_2 -M phase (20%) of the cell cycle, suggesting that Akt increases cell cycle progression (Fig. 3A, *middle*).

The survival advantage was confirmed by Annexin V/propidium iodide staining. As expected, only a small number of Annexin V-positive cells were detected in IL-3-treated cells (Fig. 3B, *left*). After 20 hours of IL-3 withdrawal, however, 16% of cells were Annexin V positive/propidium iodide negative (i.e., early apoptotic) and 21% were Annexin V/propidium iodide double positive (i.e., late apoptotic; Fig. 3B, *middle*). Under IL-3 withdrawal and addition of 4-OHT (i.e., Akt activation), both populations decreased to 6% and 12%, respectively (Fig. 3B, *right*). Therefore, Akt partially

rescued cell death after IL-3 withdrawal, although not to the extent seen in IL-3-treated cells. This suggests that Akt fulfills some but not all requirements of IL-3-dependent survival. In agreement with Fig. 2B, 32D M^- Akt:ER* cells rapidly underwent apoptosis after IL-3 withdrawal, irrespective of 4-OHT treatment (data not shown).

In addition, proliferation was measured by [3 H]-thymidine incorporation. Upon Akt activation, DNA synthesis was increased in surviving cells (Fig. 3C, *right*). Taken together, the data document a combination of increased survival and increased proliferation of surviving cells upon Akt activation.

Conditional activation of Akt promotes transformation. Finally, transformation can be measured by growth in semisolid methylcellulose. As a positive control, the IL-3-dependent 32D cells were treated with IL-3 and formed colonies as previously observed (Fig. 3D; ref. 14). Importantly, colony formation was observed in 32D- M^+ Akt:ER* cells treated with 4-OHT to activate Akt, whereas 4-OHT-treated 32D- M^- Akt:ER* cells formed no colonies (Fig. 3D). In addition, colonies from Akt-expressing cells seemed smaller in size than IL-3-treated colonies (data not shown), suggesting a weaker Akt-mediated proliferation when compared with IL-3. Nevertheless, Akt activation was sufficient to promote *in vitro* anchorage-independent growth and transformation of myeloid 32D cells.

Conditional activation of Akt *in vivo* promotes a myeloid leukemia-like disease. We have previously used a syngeneic model with C3H/HeJ mice to induce Flt3-ITD-dependent leukemia-like disease (14, 27). Because we were particularly interested to dissect Akt as an important pathway downstream of Flt3-ITD, we injected C3H/HeJ mice with either 32D-Flt3-ITD or 32D- M^+ Akt:ER* cells. I.p. injection with tamoxifen (which is metabolized to 4-OHT in the liver) of 32D- M^+ Akt:ER* carrying mice was done daily. The first mice started to die from a leukemia-like disease 33 days after injection. As can be seen from Fig. 4A, mice treated with tamoxifen were particularly prone to develop a leukemia-like disease (median survival, 42 days). Similarly, mice carrying Flt3-ITD rapidly developed a leukemia-like disease (median survival, 51 days). Only 2 of 10 mice injected with 32D- M^+ Akt:ER* but treated with solvent as control died within the 8 weeks of the experiment (Fig. 4A). Survival of the control mice differed significantly from Flt3-ITD or Akt-ER* + tamoxifen mice ($P < 0.05$, log-rank test), whereas the two latter did not differ significantly ($P = 0.61$, log-rank test). Gross anatomic examination showed hepatomegaly, splenomegaly, and lymphadenopathy. Histologic evaluation revealed that all the mice with activated Akt showed massive infiltration of p-Akt-positive blasts in the bone marrow (Fig. 4B), as well as in the liver and spleen (data not shown).

Activation of Akt in human acute myeloid leukemia. We made use of a tissue array containing bone marrow samples from 90 patients diagnosed with AML and stained for p-Ser⁴⁷³-Akt (Fig. 5A). The tissue array contained samples from 66 patients diagnosed with primary, untreated AML, 19 patients with relapsed AML, and five patients with refractory AML. These data revealed that 86% were positive for p-Akt staining, with a mean of 42% positive blasts per sample. Of these, 85% (mean, 49%) showed staining of the nuclei and 82% (mean, 54%) of the membrane and cytoplasm (Fig. 5B). No correlation with p-Akt status was found with respect to FAB subtype, % blast infiltration of the bone marrow, cytogenetic abnormalities, Flt3 status, or when comparing untreated versus relapsed/refractory AML. Normal bone marrow did not show positive staining for p-Akt (data not shown). These results suggest a more general role of Akt activation in AML.

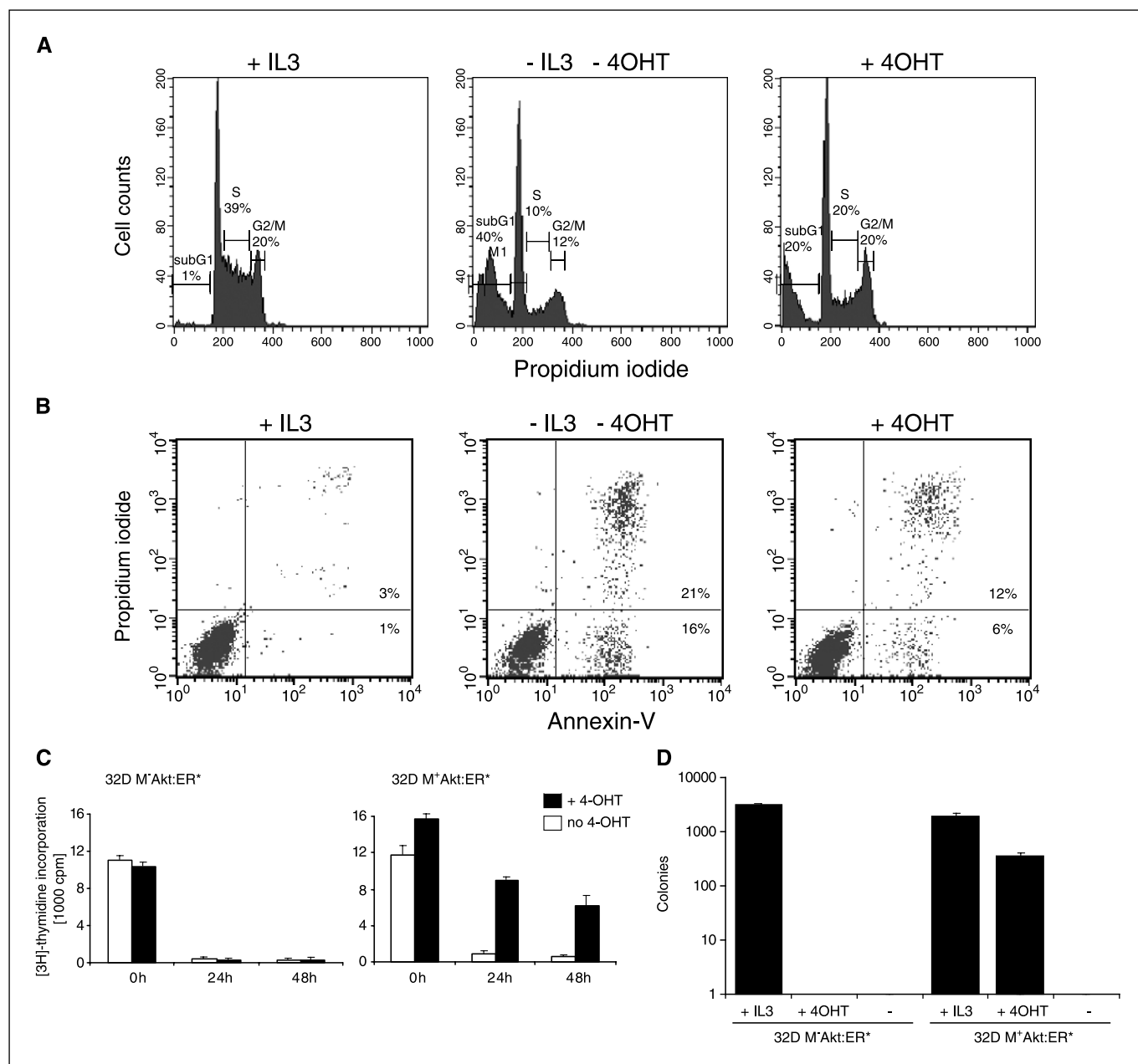


Figure 3. Biological consequences of Akt activation. *A*, cell cycle analysis by propidium iodide staining was done on 32D-M⁺Akt:ER⁺ cells 24 hours after withdrawal of IL-3 or treatment with either 2 ng/mL of IL-3 or 1 μ M 4-OHT (to induce Akt), as indicated. The percentage of cells in the sub-G₁ fraction and the different cell cycle phases were measured by FACS. *B*, apoptotic 32D-M⁺Akt:ER⁺ cells were stained with Annexin V and propidium iodide and subjected to flow cytometry. *C*, DNA synthesis was measured by [³H]-thymidine incorporation into 32D cells expressing either M⁻Akt:ER⁺ or M⁺Akt:ER⁺. Cells were starved for 24 or 48 hours with or without 1 μ M 4-OHT. Six hours before each time point, 1 μ Ci of [³H]-thymidine was added, DNA harvested on glass fiber filters, and absolute β -emission quantified in a scintillation counter. Columns, means of three independent experiments; bars, \pm SD. *D*, clonal growth of 1×10^4 32D-M⁻Akt:ER⁺ and 32D-M⁺Akt:ER⁺ cells were plated in 1 mL of semisolid methylcellulose with or without 2 ng/mL of IL-3 or 2 μ M 4-OHT, as indicated. The colonies were counted on day 8. Triplicates of one of three independent experiments.

Discussion

Activating Flt3 mutations promote proliferation and survival of myeloid cells through the activation of multiple signaling pathways (14). We and others have reported activation of Akt in Flt3-ITD-expressing cells (14, 28). We were interested in elucidating the consequences and importance of Akt activation in myeloid precursor cells. We showed here that Flt3-ITD constitutively activated Akt, irrespective of additional stimulation with growth factors. The transcription factor Foxo3a is phosphorylated by Akt on residues

Thr³², Ser²⁵³, and Ser³¹⁵ (12, 13). In agreement with this, we observed constitutive phosphorylation of Foxo3a in Flt3-ITD-expressing cells (Fig. 1A). In addition, our reporter assays revealed that expression of Flt3-ITD or Akt inhibited Foxo-mediated transcriptional activity (Figs. 1B and 2A). Importantly, expression of dominant-negative Akt largely prevented cytokine independence conferred by Flt3-ITD, suggesting that Akt is necessary for this biological function of Flt3-ITD. However, growth was not prevented in 25% of remaining cells, suggesting additional important signal transduction pathways

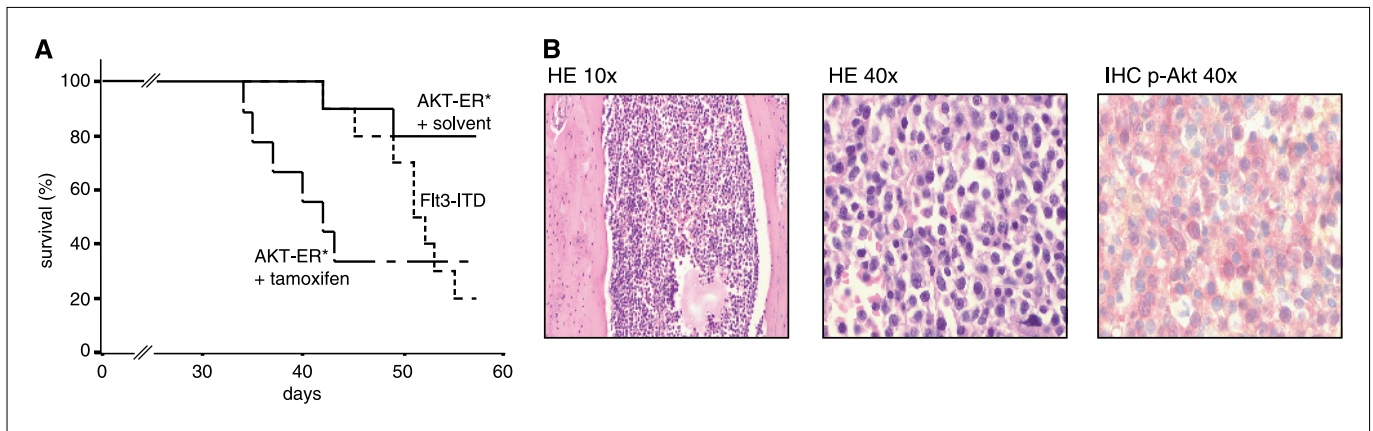


Figure 4. Conditional activation of Akt *in vivo* promotes a myeloid leukemia-like disease. Female C3H/HeJ mice, ages 9 weeks, were used for *in vivo* tumorigenesis experiments. 32D-Flt3-ITD or 32D-M*Akt:ER* (1×10^6) cells were inoculated by tail vein injection. **A**, survival of injected mice is shown as Kaplan-Meier survival plots over the 8 weeks of the experiment. Mice injected with 32D-M*Akt:ER* cells were treated daily with either i.p. tamoxifen (in peanut oil) or solvent (peanut oil), whereas 32D-Flt3-ITD-injected mice were left untreated. **B**, histologic examination of bone marrow from 32D-M*Akt:ER* injected mice treated with tamoxifen. The paraffin embedded femurs were stained with H&E (*left* and *middle*), and immunohistochemistry was done using a p-Ser⁴⁷³-Akt-antibody (*right*).

activated by Flt3-ITD. These pathways are likely to include the STAT and mitogen-activated protein kinase pathways. Interestingly, the effects of dnAkt were partly rescued by the addition of IL-3, which argues for a specific requirement of Akt in Flt3-ITD-mediated survival. Furthermore, restoration of Foxo3a activity prevented Flt3-ITD-mediated growth properties (Fig. 1D). Together, our data showed a linear pathway of Akt activation by Flt3-ITD, which in turn led to phosphorylation and inactivation of Foxo3a.

Because Akt activation has been shown to mediate survival and proliferation in several nonmyeloid cell systems (22, 23, 29, 30), we wondered about the biological consequences in myeloid precursor cells. We generated myeloid cells to conditionally activate Akt and target it to the cell membrane. As a control, we used a myristylation-defective form, which leads to mislocalization and cannot be activated by addition of 4-OHT (Fig. 2A). We showed that activated Akt targeted to the cell membrane protected myeloid 32D cells from

cytokine withdrawal (Fig. 2B) and apoptosis (Fig. 3A and B). The observed effects were not due to unphysiologically high levels of Akt activity, as similar observations were made with individual clones titrated to Akt activity observed in Flt3-ITD (Fig. 2C).

Interestingly, Akt affected proliferation by allowing progression from the G₁ into S and G₂-M phase of the cell cycle (Fig. 3A), with increased DNA synthesis in the S phase (Fig. 3C). This proliferative advantage of surviving cells allowed for clonal growth in semisolid methycellulose (Fig. 3D). However, Akt-mediated colonies were smaller in size when compared with IL-3-treated or Flt3-expressing colonies, suggesting that Akt does not fully complement the proliferative function of IL-3 or of Flt3-ITD. Correct localization of Akt to the cell membrane was an absolute requirement, as the myristylation-defective (mislocalized) form of Akt was inactive (Fig. 2A and B and Fig. 3A-D). We previously showed that wtFlt3-expressing 32D cells did not form

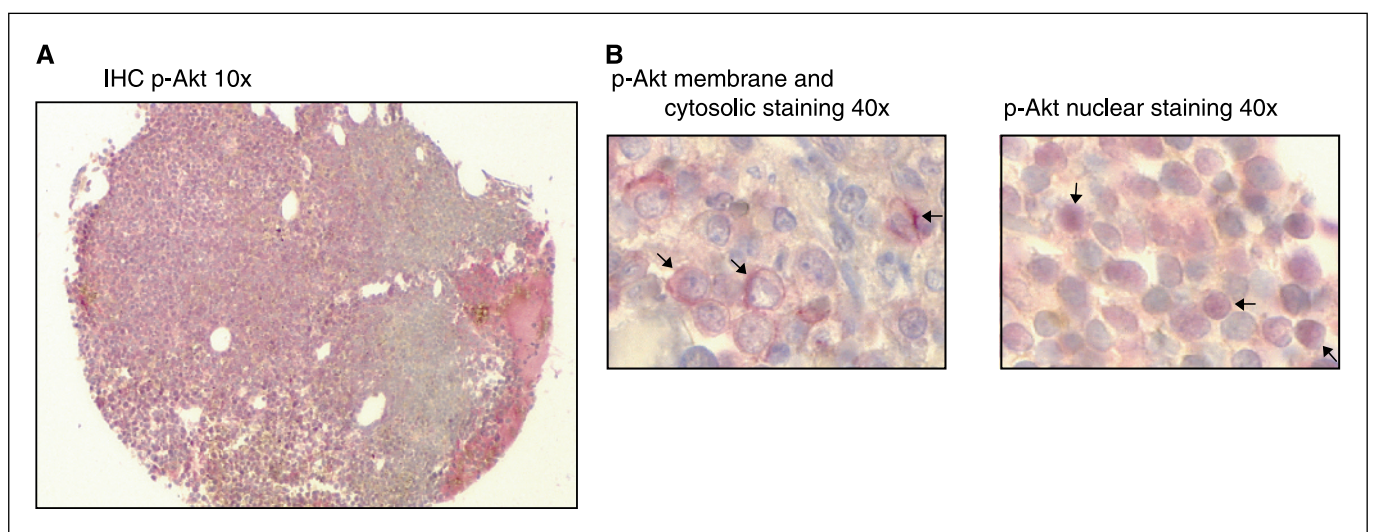


Figure 5. Activation of Akt in primary human AML. Tissue array analysis of p-Ser⁴⁷³-Akt expression by immunohistochemistry. **A**, overall, bone marrow biopsies of 90 AML patients were arranged as a single paraffin block. Sections were cut and each bone marrow biopsy was represented twice on each slide. Expression levels of p-Ser⁴⁷³-Akt were analyzed by immunohistochemistry, as shown for a bone marrow biopsy of an individual patient. **B**, examples of membrane/cytosol staining (*left*) and nuclear staining (*right*).

colonies after treatment with Flt3 ligand (14). As Akt activation was observed after Flt3 ligand stimulation (Fig. 1A), we speculate that sustained activation of Akt above a certain threshold is necessary for myeloid transformation, as provided by Flt3-ITD or by myristylated Akt.

The important and novel finding that activation of Akt was sufficient to mediate myeloid transformation *in vitro* was further underlined by our *in vivo* results (Fig. 4A; refs. 14, 27). In previous work, we showed that Flt3-ITD expression promoted a leukemia-like disease, whereas expression of wtFlt3 did not (14). As shown in Fig. 4A, activation of Akt was sufficient to cause a rapid onset of a leukemia-like disease and death, similarly to Flt3-ITD. Histologic examination revealed massive infiltration of the bone marrow with p-Akt-positive blasts (Fig. 4B), as well as of the liver and spleen. Onset of disease and death in activated Akt-ER* mice was observed earlier than in Flt3-ITD-bearing mice (Fig. 4A), which may be due to high Akt activation *in vivo*. Because Flt3-ITD mediate the activation of multiple aberrant signaling pathways, it would be interesting to compare the effects of Akt with Flt3-ITD in primary bone marrow.

Frequent activation of the PI3K/Akt pathway has been observed in lysates from AML patients (31) and found to predict poor overall survival (32). However, it remained unclear whether the blast population itself, or surrounding stroma contained the majority of p-Akt. Immunohistochemical stainings of our tissue array revealed that the blast population contained the majority of p-Akt, whereas normal bone marrow did not. Interestingly, p-Akt staining was nuclear in a large portion of the samples (Fig. 5B). The significance

of this finding is unknown. Importantly, p-Akt was found in AML samples with wtFlt3, suggesting a more general role of Akt in leukemogenesis. Other signaling pathways such as the Ras pathway are likely to activate Akt in human AML. Oncogenic N-Ras and K-Ras mutations are found in a quarter of all cases of AML but rarely occur together with Flt3-ITD (9), suggesting functional overlap. As oncogenic Ras is known to activate the PI3K pathway (10), it is tempting to speculate that Akt may be a necessary point of convergence of oncogenic signaling required for leukemic transformation.

In conclusion, our data suggest that Akt activation and Foxo inactivation are important features of myeloid leukemogenesis. As Akt is necessary for transformation, the Akt kinase and Foxo transcription factors may be an attractive targets for therapeutic intervention.

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