Features of Ras activation by a mislocalized oncogenic tyrosine kinase: FLT3 ITD signals through K-Ras at the plasma membrane of acute myeloid leukemia cells

Susanne Köthe1, Jörg P. Müller1, Sylvia-Annette Böhmer1, Todor Tschongov1, Melanie Fricke1, Sina Koch2,3, Christian Thiede2, Robert P. Requardt4, Ignacio Rubio1,4 and Frank D. Böhmer1,*

1Institute of Molecular Cell Biology, Center for Molecular Biomedicine, Jena University Hospital, 07743 Jena, Germany
2Molecular Hematology Group, Department of Internal Medicine I, University Hospital Carl Gustav Carus, 01307 Dresden, Germany
3Department of Systemic Cell Biology, Max Planck Institute for Molecular Physiology, 44227 Dortmund, Germany
4Center for Sepsis Control and Care (CSCC), Jena University Hospital, 07743 Jena, Germany

*Author for correspondence (boehmer@med.uni-jena.de)

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Summary
FMS-like tyrosine kinase 3 with internal tandem duplication (FLT3 ITD) is an important oncoprotein in acute myeloid leukemia (AML). Owing to its constitutive kinase activity FLT3 ITD partially accumulates at endomembranes, a feature shared with other disease-associated, mutated receptor tyrosine kinases. Because Ras proteins also transit through endomembranes we have investigated the possible existence of an intracellular FLT3-ITD/Ras signaling pathway by comparing Ras signaling of FLT3 ITD with that of wild-type FLT3. Ligand stimulation activated both K- and N-Ras in cells expressing wild-type FLT3. Live-cell Ras–GTP imaging revealed ligand-induced Ras activation at the plasma membrane (PM). FLT3-ITD-dependent constitutive activation of K-Ras and N-Ras was also observed primarily at the PM, supporting the view that the PM-resident pool of FLT3 ITD engaged the Ras/Erk pathway in AML cells. Accordingly, specific interference with FLT3-ITD/Ras signaling at the PM using PM-restricted dominant negative K-RasS17N potently inhibited cell proliferation and promoted apoptosis. In conclusion, Ras signaling is crucial for FLT3-ITD-dependent cell transformation and FLT3 ITD addresses PM-bound Ras despite its pronounced mislocalization to endomembranes.

Key words: Oncogenic RTK, FLT3, FLT3 ITD, Leukemia, AML, Ras, Localization, Plasma membrane, Endomembrane

Introduction
Constitutively active receptor tyrosine kinases (RTKs) play a pathogenic role in a number of human diseases, including several malignancies. They are active in a ligand-independent manner, leading to aberrant and constitutive activation of downstream signaling pathways. RTKs are N-glycosylated proteins that undergo quality control in the endoplasmic reticulum (ER) and maturation of their carbohydrate moiety while trafficking in vesicles from ER through the Golgi to the plasma membrane (PM) (Helenius and Aebi, 2004). For a number of constitutively active RTKs such as constitutively active versions of FMS-like tyrosine kinase 3 (FLT3), c-Kit/Stem-cell factor receptor, anaplastic lymphoma kinase (ALK), fibroblast growth factor receptor 3 (FGFR3), and the angiopoietin receptor tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (TIE-2) the normal processing along this pathway is impaired (Choudhary et al., 2009; Lievens et al., 2006; Limaye et al., 2009; Mazot et al., 2011; Schmidt-Arras et al., 2005; Tabone-Eglinger et al., 2008; Xiang et al., 2007). Inefficient processing is caused by yet unknown mechanisms, but appears to be causally associated with constitutive tyrosine kinase activity. As a consequence, these RTKs are partially mislocalized in that they accumulate in intracellular ER/Golgi compartments. Importantly, both the intracellular and the PM pools of these RTKs can activate signal transduction. Differences in signaling quality of both pools have been identified, notably a preferred activation of STAT5 by intracellular RTK species (Choudhary et al., 2009; Schmidt-Arras et al., 2009; Schmidt-Arras et al., 2005).

FLT3 with internal tandem duplication of amino acid stretches in the juxtamembrane or kinase domain (FLT3 ITD), is a constitutively active version of FLT3 occurring in as many as 25–30% of patients with acute myeloid leukemia (AML) (Breitenbuecher et al., 2009; Fröhling et al., 2005; Kiyoi et al., 1998; Meshinchi and Appelbaum, 2009; Thiede et al., 2002). AML is the most frequent form of acute leukemias in adults and arises by malignant transformation of myeloid progenitor cells (Döhner et al., 2010). Mutations in FLT3 confer a proliferative advantage and are referred to as ‘class I mutations’. In addition to class I mutations, leukemogenesis requires further genetic lesions such as mutations that lead to blocked hematopoietic differentiation (‘class II mutations’) (Deguchi and Gilliland, 2002; Ishikawa et al., 2009; Cancer Genome Atlas Research Network, 2013). Another group of class I mutations are activating mutations in RAS genes (Fröhling et al., 2005; Tyner et al., 2009). RAS and FLT3-activating mutations are mutually exclusive, indicating functional equivalence in the pathogenesis of leukemia (Cancer Genome Atlas Research Network, 2013; Schlenk et al., 2008). Consistent with this notion, previous studies have suggested that Ras activation downstream of FLT3 ITD is
essential for cell transformation, in addition to activation of STAT5 and of the AKT/phosphoinositide 3-kinase (PI3K) pathway (Mizuki et al., 2000; Müller-Tidow et al., 2002; Takahashi, 2006; Toffalini and Demoulin, 2010). However, activation of Ras downstream of FLT3 ITD has never been directly analyzed, either biochemically or in terms of its spatiotemporal features. In particular it is not known whether FLT3 ITD addresses Ras at endomembranes or at the PM.

The three prototypical Ras proteins H-Ras, K-Ras and N-Ras (collectively referred to as Ras, from here on) are differentially expressed in different types of cells and tissues. Leukemia cells express predominantly N- and K-Ras with little or no expression of H-Ras (Omerovic et al., 2008). Furthermore, the various Ras isoforms exhibit distinct subcellular localization and trafficking properties owing to differences in the pattern of post-translational modifications (Omerovic et al., 2008). Notably, K-Ras is localized almost entirely to the PM, whereas N-Ras and H-Ras shuttle back and forth between the PM and endomembranes in the context of a palmitoylation/depalmitoylation cycle (Rocks et al., 2010). Activation of all three isoforms of Ras proteins can be assessed biochemically by employing the Ras-binding domain (RBD) of the Ras effector Raf as an affinity probe for Ras–GTP isolation. This strategy can also be exploited for visualizing the subcellular sites of Ras–GTP accumulation in intact cells by using fluorescently labeled RBD reporter probes (Augsten et al., 2006; Chiu et al., 2002; Rubio et al., 2010). In accordance with the notion that de-palmitoylated versions of H-Ras and N-Ras transit through Golgi and ER (collectively known as endomembranes) on their way to the PM (Choy et al., 1999), several studies reported the presence of active GTP-loaded Ras at endomembranes of growth factor challenged cells (reviewed by Fehrenbacher et al., 2009). However, although there is agreement that activation of Ras downstream of multiple types of receptors occurs initially at the PM, there is an ongoing controversy regarding the possible existence of meaningful amounts of Ras–GTP at intracellular sites, a debate fueled by the fact that endomembrane Ras activation has been detectable in cells that overexpress Ras (Chiu et al., 2002) but could not be observed in the case of native, endogenous Ras (Augsten et al., 2006; Fehrenbacher et al., 2009; Rubio et al., 2010).

Given the importance of Ras for FLT3-mediated transformation, we have systematically analyzed Ras signaling downstream of wild-type FLT3 and FLT3 ITD. Notably, we considered the possibility that FLT3 ITD, which is partially retained in intracellular membranes, could potentially functionally interact with endomembrane-resident Ras. Using a combination of biochemical and fluorescence live-cell microscopy approaches, we assessed the role of the different Ras isoforms in wild-type FLT3 and FLT3 ITD signaling. The results revealed that Ras activation takes place at the PM both for wild-type FLT3 and FLT3 ITD, and that K-Ras is the predominant Ras isoform activated downstream of the transforming variant FLT3 ITD. Moreover, Ras activation at the plasma membrane is required for cell proliferation and viability.

**Results**

**Different localization of wild-type FLT3 and FLT3 ITD in human AML cell lines**

To assess Ras activation downstream of FLT3, we chose two human leukemia cell lines, which harbor the same chromosomal translocation (4;11) leading to expression of a leukemia-associated fusion protein (MLL-AF4), but differ in their FLT3 status. RS4-11 cells express wild-type FLT3, whereas MV4-11 cells express solely FLT3 ITD. As shown in Fig. 1, localization of FLT3 in these cell lines is clearly different. Immunostaining and microscopy using confocal laser scanning microscopy (LSM) revealed that FLT3 in RS4-11 is predominantly localized at the PM, while FLT3 ITD in MV4-11 cells is to a large extent localized in intracellular membranes (Fig. 1A). Quantitative analyses using antibodies detecting an epitope in the FLT3 extracellular domain (CD135 antibody) and flow cytometry were in excellent agreement with this observation. A large fraction of FLT3 was detectable at the surface of RS4-11 cells and much less FLT3 ITD at the surface of MV4-11 cells. However, in both cell lines similar amounts of FLT3 became detectable upon permeabilization of the cells (Fig. 1B). We also employed 32D cells, an IL-3-dependent murine myeloid cell line, stably transfected with either wild-type FLT3 or FLT3 ITD for some analyses. A different localization of wild-type FLT3 and FLT3 ITD in these cell lines has been reported previously (Schmidt-Arras et al., 2009), with FLT3 ITD expressed at endomembranes and to a lesser extent at the cell surface.

**K-Ras and N-Ras are activated downstream of wild-type FLT3**

To evaluate which Ras isoforms are activated downstream of the native, wild-type FLT3 receptor we performed biochemical experiments using both 32D cells stably transfected with

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**Fig. 1. Aberrant localization of FLT3 ITD in intracellular membranes.**

(A) The human leukemia cell lines RS4-11 and MV4-11 harboring endogenous wild-type FLT3 or FLT3 ITD, respectively, were subjected to immunostaining with anti-FLT3 antibodies (red). Plasma membranes were decorated with Alexa-Fluor-488-labeled wheat germ agglutinin (WGA, green). Nuclei were stained with HOECHST 33342 (blue). Scale bars: 5 μm. (B) Quantitative analysis of FLT3 distribution, by flow cytometry. FLT3 was detected using antibodies (anti-CD135) recognizing a surface epitope. Cells were stained either intact, or after permeabilization to reveal the total FLT3 amounts, as indicated. The relative amount of surface FLT3 in the two cell lines is also given.
wild-type FLT3, and the human cell line RS4-11. As a first step, we analyzed the expression profile of H-Ras, K-Ras and N-Ras in these cell types. Although K-Ras and N-Ras proteins were present at similar levels in both myeloid cell lines (although detected expression was somewhat variable in different experiments, data not shown), H-Ras expression was not detectable by immuno blotting (data not shown), a pattern found also in other leukemia lines (Omerovic et al., 2008). Therefore, we confined our analysis to K-Ras and N-Ras. Activation of K-Ras and N-Ras was analyzed by using a glutathione S-transferase (GST) – RBD affinity probe to pull out active Ras from cell extracts (see Materials and Methods). The presence of activated K- or N-Ras proteins was subsequently detected by immunoblotting with isoform-specific Ras antibodies. Cells were starved of serum (and of IL3 in the case of 32D cells), and stimulated with FLT3 ligand (FLT) for different periods of time. As shown in Fig. 2 (FLT3 IP panels), this led to a rapid activation of FLT3, which remained high for up to 10 minutes of stimulation and was still detectable 30 minutes after ligand addition in RS4-11 cells. Both Ras isoforms, K-Ras and N-Ras, were activated downstream of FLT3 with similar kinetics to that of FLT3 activation (Fig. 2, Ras–GTP pulldown panels). Stimulation was also associated with a rapid activation of Erk1/2 (Fig. 2, lystate panels), which declined after 30 minutes. Thus, these experiments showed that ligand-stimulated wild-type FLT3 activates K-Ras and N-Ras with similar efficiency in two different cell models.

Whereas K-Ras activation is largely restricted to the PM in all experimental systems tested so far, N-Ras–GTP accumulation has been documented both at the PM and at endomembranes of stimulated cells (Chiu et al., 2002; Perez de Castro et al., 2004). To assess the localization of FLT3-induced K-Ras and N-Ras activation, we monitored the subcellular distribution of E3-R3(A/D), a trivalent fluorescent affinity probe for Ras–GTP (see the Materials and Methods for more details; shown in the figures in green; RBD) that has previously been employed to image Ras activation in a number of settings (Augsten et al., 2006; Leadsham et al., 2009; Rubio et al., 2010; Wu et al., 2010). The specificity of this probe for detection of activated Ras proteins in the employed cell system was confirmed by co-transfection with different N-Ras versions (supplementary material Fig. S1). To analyze FLT3-dependent Ras activation, RS4-11 cells were co-transfected with green fluorescent E3-R3(A/D) and red fluorescent Ras proteins (mCherry Ras, labeled in the Figures red as K- or N-Ras), challenged with FL and imaged confocally. As shown in Fig. 3, E3-R3(A/D) probe redistribution can be seen upon FL stimulation over time. Intense labeling of surface membranes by the E3-R3(A/D) probe was apparent both in the case of mCherry–K-Ras–expressing (Fig. 3A) and mCherry–N-Ras–expressing (Fig. 3B) cells. For both isoforms, Ras activation at the PM was abundant and relatively rapid. In the case of N-Ras an accumulation of E3-R3(A/D) at intracellular compartments was also visible (Fig. 3B). However, a quantitative analysis of cells expressing mCherry–N-Ras revealed that ~50% of cells featured those intracellular E3-R3(A/D)-illuminated dense and compact structures already before FL stimulation, whereas in 30–40% of cells E3-R3(A/D) decorated intracellular structures after FL stimulation. In these cells, accumulation of E3-R3(A/D) at endomembranes was most prominent and continued to rise at 60 minutes post-stimulation, a time point at which little Ras–GTP is left in the cell as judged by the biochemical analysis (Fig. 2). Thus, we assume that the illumination of intracellular structures by E3-R3(A/D) may have been provoked by the overexpression of mCherry–N-Ras. To further characterize the intracellular structures that harbor the RBD probe, their potential colocalization with several cellular compartments was assessed. We detected some overlap in localization of the compact probe accumulations with endosomal vesicles (supplementary material Fig. S2). In sum, these experiments confirmed that ligand-stimulated FLT3 activates both Ras isoforms, K-Ras and N-Ras, and that ligand-dependent Ras activation proceeded predominantly at the PM.

**K-Ras is prominently activated downstream of FLT3 ITD**

We were particularly interested in Ras activation downstream of FLT3 ITD since this pathway is considered to be critical for cell transformation. For the biochemical assays, we employed the murine cell line 32D, stably transfected with FLT3 ITD, and the human cell line MV4-11, which endogenously expresses FLT3 ITD. Constitutive FLT3 phosphorylation was abundant in both cell lines (Fig. 4A,B, FLT3 IP panels). A moderate constitutive
m-dependent redistribution of E3-R3(A/D) to the plasma membrane, indicating antibody pTyr591 (Fig. 4A,B) or an antibody against total by probing FLT3 phosphorylation with the site-selective kinase inhibitor cpd.102 (Mahboobi et al., 2006). Then, the FLT3 ITD, we treated the cells with the FLT3-selective tyrosine compound was washed out and Ras and Erk activation were monitored over time. Indeed, kinase inhibition led to complete inhibition of FLT3 autophosphorylation in both cell lines (Fig. 4A,B, lower panels). This is consistent with earlier reports on constitutive Erk1/2 activation in FLT3-ITD-expressing cells (Mizuki et al., 2000), which we also observed in both analyzed cell lines (Fig. 4A,B, lysate panels), consistent with the pattern of Ras activation.

Both cell lines were also subjected to the microscopic analysis of spatial aspects of Ras activation. Prominent K-Ras activation at the PM was readily observed in both cell lines and was completely suppressed by treating the cells with the FLT3 kinase inhibitor cpd.102 (Fig. 5A–E). To substantiate that K-Ras activation occurs by the PM-bound pool of FLT3 ITD, we employed a 32D cell line harboring a FLT3 ITD version that is completely trapped in endomembranes owing to the inclusion of an ER-retention signal (32D FLT3 ITD R3) (Schmidt-Arras et al., 2009). Indeed, no K-Ras activation was detectable in these cells (Fig. 5C).

Assessment of N-Ras activation by FLT3 ITD at the PM revealed that N-Ras activation was clearly weaker than activation of K-Ras, despite similar amounts of N-Ras or K-Ras (detectable as mCherry staining) in this location. In contrast, intracellular dense and compact structures ('spots') of the E3-R3(A/D) probe detecting activated N-Ras were frequently observed (Fig. 5F–I), raising the possibility that intracellular located N-Ras was being activated by FLT3 ITD. A quantitative comparison of the observed activation of K-Ras and N-Ras in the different cellular localizations is shown in Fig. 5J,K. To test the hypothesis that the apparent intracellular N-Ras activation in compact structures was driven by FLT3 ITD signaling, we again employed the FLT3 inhibitor. Treatment with cpd.102, however, did not significantly affect the colocalization of E3-R3(A/D) and mCherry–N-Ras at intracellular spots (Fig. 5F,H, and quantification in Fig. 5G,I). Moreover, in parental 32D cells co-expressing mCherry–N-Ras and E3-R3(A/D), formation of similar spot-like structures with apparently activated N-Ras could not, however, be causally linked to FLT3 ITD activity. Obviously, despite an intracellular mislocalization of a large fraction of FLT3 ITD, the oncoprotein appears incapable of activating N-Ras in endomembranes.

Ras activation was also detectable for both isoforms and both cell lines (Fig. 4A,B, Ras-GTP pulldown panels). This is consistent with earlier reports on constitutive Erk1/2 activation in FLT3-ITD-expressing cells (Mizuki et al., 2000), which we also observed in both analyzed cell lines (Fig. 4A,B, lower panels). To test the dependence of Ras activation on upstream activity of FLT3 ITD, we treated the cells with the FLT3-selective tyrosine kinase inhibitor cpd.102 (Mahboobi et al., 2006). Then, the compound was washed out and Ras and Erk activation were monitored over time. Indeed, kinase inhibition led to complete inhibition of FLT3 autophosphorylation in both cell lines as shown by immunoblotting. Identical results were obtained by probing FLT3 phosphorylation with the site-selective antibody pTyr591 (Fig. 4A,B) or an antibody against total phosphotyrosine (pY100, data not shown). In the presence of inhibitor, basal Ras activation was reduced in the case of K-Ras, and affected to a lesser extent in the case of N-Ras (Fig. 4C). Interestingly, Ras activation was strongly elevated after removal of the inhibitor. In contrast to the basal state, under these conditions, the magnitude of Ras activation was similar for both isoforms. At the level of Erk1/2 phosphorylation, a pronounced activation could also be observed after inhibitor washout (Fig. 4A,B, lysate panels), consistent with the pattern of Ras activation.

Activation of the plasma membrane pool of Ras is crucial for FLT3-ITD-mediated cell growth and cell survival

K- and N-Ras may play different roles downstream of FLT3 ITD. Also, PM and endomembrane pools of N-Ras could each transmit distinct signals in the context of FLT3-ITD-dependent transformation. In order to analyze the functional importance of the specific Ras isoforms for FLT3-ITD-mediated leukemic cell transformation, we initially employed a siRNA strategy. To selectively deplete K- or N-Ras or both Ras isoforms together in FLT3-ITD-expressing MV4-11 cells, Ras-isoform selective siRNAs were used. For comparison, cells were also depleted of FLT3 ITD by siRNA, or treated with the FLT3 kinase inhibitor.
As biological end points cell proliferation/viability and cell cycle progression were analyzed. As shown in supplementary material Fig. S3, although selective and efficient knockdown of the two Ras isoforms K-Ras and N-Ras was achieved, the effects of the Ras knockdowns on proliferation/viability and cell cycle progression were only very mild. In contrast, siRNA-mediated knockdown of FLT3 ITD or inhibition of FLT3 ITD kinase or MEK kinase by pharmacological inhibitors very strongly impaired cell proliferation/viability (supplementary material Fig. S3). We hypothesized that the low levels of Ras isoforms remaining in the siRNA-treated cells were presumably sufficient to still sustain FLT3-ITD-dependent cell proliferation and survival. Therefore, to rigorously prove that Ras played an important role in FLT3 signaling we resorted to a previously successfully applied dominant negative (dn) Ras strategy. However, using the commonly employed dn H-RasS17N (Mizuki et al., 2000; Müller-Tidow et al., 2002) to interfere with Ras signaling, it is not possible to discriminate the roles of differently localized Ras pools, because dn H-RasS17N blocks signal transduction through all three Ras isoforms and in all their cellular locations (Matallanas et al., 2003). Therefore, we used dn K-RasS17N. This molecule (like its wild-type counterpart) exclusively localizes to plasma membranes (Omerovic et al., 2008) and thus can interfere with Ras–GTP loading only in this location.

Fig. 4. Constitutive K-Ras activation in FLT3-ITD-expressing myeloid cells depends on FLT3 kinase activity. Murine FLT3-ITD-expressing 32D cells (A) or human MV4-11 cells endogenously expressing FLT3 ITD (B) were serum starved for 2 hours, then treated with the selective FLT3 inhibitor cpd.102 (1 μM) for 1 hour or left untreated (cpd.102 −, washout −). Then cells were washed twice with medium again containing cpd.102 (cpd. 102 +, washout +) or vehicle. At the indicated time points after washing and further incubation, cells were lysed and subjected to a Ras–GTP pulldown. The activation status of Ras, and ERK was sequentially determined in the same lysates, as described in the Materials and Methods. FLT3 activation was assessed in separate experiments under identical conditions. The same membrane sections were reprobed to detect the different Ras isoforms and to detect total proteins with pan-specific antibodies. Antibodies used for immunoprecipitation (IP) or immunoblotting (left side of panels) are indicated. pERK1/2 denotes anti-phospho-p44/42 MAPK; pTyr591, anti-phospho-FLT3 tyrosine 591. Six (A) or three (B) independent experiments, yielded consistent results. (C) Quantification of Ras-GTP levels in 32D mFLT3 ITD cells. Five blots for K-Ras and four blots for N-Ras were quantified by densitometry, and intensities of Ras-GTP after inhibitor cpd.102 treatment were normalized to levels without treatment.

Discussion
This study undertook the systematic comparison of biochemical and spatiotemporal features of Ras activation downstream of the hematopoietic RTK FLT3 and its leukemia-associated counterpart FLT3 ITD. Main findings include the reduced capacity of FLT3 ITD to activate N-Ras compared with wild-type FLT3, and the observation that FLT3 ITD-mediated Ras activation at the plasma membrane is predominant and required for cell transformation. Importantly, the mislocalized intracellular pool of activated FLT3 ITD appears incapable of activating Ras in endomembranes.
Fig. 5. FLT3 ITD causes constitutive K-Ras activation at the plasma membrane. 32D cells expressing human FLT3 ITD, or human MV4-11 cells endogenously expressing FLT3 ITD (as indicated) were co-transfected with plasmids encoding either mCherry–K-Ras or –N-Ras (red) together with the E3-R3(A/D) reporter for Ras–GTP (green, RBD). Cells were deprived of serum for 2 hours, treated with the selective FLT3 inhibitor cpd.102 or left untreated, and subjected to image analysis by confocal laser scanning microscopy. (A) Representative images of FLT3-ITD-expressing 32D cells co-transfected with the mCherry–K-Ras-encoding plasmid and E3-R3(A/D), and treated with the selective FLT3 inhibitor cpd.102 or left untreated, as indicated. (B) Corresponding quantitative comparisons of cpd.102-treated and non-treated cells. (C) A 32D cell line harboring an ITD version that is anchored to the endoplasmic reticulum by a C-terminal tag (FLT3 ITD R3) was also analyzed. (D,E) Representative images of (D) MV4-11 cells co-transfected with mCherry–K-Ras-encoding plasmid and E3-R3 and (E) corresponding quantitative comparisons of cpd.102-treated and non-treated cells. (F,H) Representative images of FLT3-ITD-expressing 32D cells (F) or MV4-11 cells (H) co-transfected with the mCherry–N-Ras-encoding plasmid and E3-R3(A/D), and treated with the selective FLT3 inhibitor cpd.102 or left untreated, as indicated. (G,I) Corresponding quantitative analysis of the effect of inhibitor treatment on N-Ras activation in intracellular compact structures (‘spots’). (J,K) Quantitative analysis of Ras isoform activation in different compartments in 32D cells expressing FLT3 ITD (J) or MV4-11 cells (K) transfected with either mCherry–K-Ras- or mCherry–N-Ras-encoding plasmids and E3-R3(A/D). PM, plasma membrane. For all quantifications and statistic analyses, 60 cells from three independent experiments were analyzed for each condition. Scale bars: 10 μm. Original magnification 63×. Note that some of the probe accumulates in the nucleus. The reason for this is not known, but it is unrelated to Ras activation (Augsten et al., 2006). *P<0.05; **P<0.01; n.s., not significant.
The presented findings illustrate that K-Ras and N-Ras are equally well activated by ligand stimulation of wild-type FLT3. Prominent K-Ras and N-Ras activation that could be causally linked to FLT3 receptor signaling, occurred at the PM. Some intracellular N-Ras activation may occur at late time-points of stimulation, possibly reflecting internalization of PM-associated N-Ras–GTP and its accumulation at an endosomal compartment. Consistent with this notion, partially overlapping localization of sites of intracellular N-Ras activation with an endosomal marker was observed in our study. A similar endocytotic process has been proposed to mediate the accumulation of K-Ras at early endosomes (Lu et al., 2009) and the presence of (active) H-Ras in the endocytic compartment in the context of growth factor signaling has also been documented in a number of cases (Fehrenbacher et al., 2009).

FLT3 ITD was able to activate Ras in the absence of any ligand stimulation. However, the biochemically detected steady-state levels of activated Ras in unstimulated FLT3 ITD-harboring cells were typically relatively low and only basal K-Ras but not basal N-Ras seemed to be under control of FLT3 ITD. Our data on the effects of transient receptor blockade indicate that a negative feedback mechanism downstream of FLT3 ITD keeps Ras–GTP levels at bay in these cells.

Several aspects of the topology of Ras activation by FLT3 ITD disclosed in the present study warrant special mention. Notably, despite the dominant intracellular localization of FLT3 ITD [data in Fig. 1 and published findings (Koch et al., 2008; Schmidt-Arras et al., 2009; Schmidt-Arras et al., 2005)], K-Ras activation proceeded at the PM. Accordingly, complete intracellular retention of FLT3 ITD using an ER-retention tag (‘R3 anchor’), abolished K-Ras activation (Fig. 5A,C). We conclude that the PM-bound pool of FLT3 ITD is necessary and sufficient to activate K-Ras at this location. Activation of Ras by FLT3 ITD at the PM is consistent with previous signaling analyses of differently localized FLT3 ITD pools. For example, FLT3 ITD, which was forced into an ER compartment by treatment of cells with Brefeldin A or by employing the aforementioned ER-specific targeting sequence, largely lost the ability to activate Erk1/2 (Choudhary et al., 2009; Schmidt-Arras et al., 2009).

N-Ras activation at the PM by FLT3 ITD was much less efficient than activation of K-Ras, at least in the employed settings using overexpressed Ras proteins. A possibly lower potency of FLT3 ITD in terms of N-Ras activation at the PM could be linked to the segregation of both proteins into different PM microdomains. Differential localization of Ras isoforms in micro- and nanodomains is extensively documented (Ashery et al., 2006; Omerovic et al., 2008). Of note, some apparent intracellular N-Ras–GTP formation was observed in FLT3 ITD-expressing cells. Since the appearance of those compact structures labeled by E3-R3(A/D) was, however, not reversed by FLT3 kinase inhibition, we hypothesize that it was, at least primarily, an incidental consequence of N-Ras and/or E3-R3(A/D) probe overexpression.

Cell biological experiments were performed to assess the role of Ras in FLT3-ITD-dependent signaling. The siRNA-based knockdown of N-/K-Ras revealed an unexpectedly low impact on FLT3-ITD-mediated cell survival and proliferation, probably due to residual Ras levels. The crucial role of Ras in FLT3-ITD-driven cell proliferation was, however, obvious in experiments using the dominant negative K-RasS17N as an alternative means of blocking Ras signaling. A profound requirement of Ras for...
survival and FLT3-ITD-induced DNA synthesis in these cells was detected. It is likely that this effect is at least partially mediated by preventing Erk1/2 activity, which is prominently dependent on FLT3 ITD signaling (Fig. 4) and downstream Ras activation (supplementary material Fig. S3). Furthermore, Ras downstream pathways, such as the phosphoinositide 3-kinase pathway, may also be involved, but have not been explored in this study. Since K-RasS17N is localized exclusively at the PM (Choy et al., 1999) and can only interfere with Ras signaling at this location, these data further underscored the critical role of plasma–membrane-bound Ras activation for FLT3 ITD signaling.

In conclusion, this study demonstrates that both wild-type FLT3 and FLT3 ITD can potently activate Ras in leukemic cells and that Ras activation is essential for proliferation and survival of FLT3-ITD-expressing AML cells. Moreover, in the light of recent data pointing to the existence of distinct signaling platforms for FLT3 ITD at the PM and endomembranes of myeloid leukemia cells (Choudhary et al., 2009; Schmidt-Arras et al., 2009), our present findings reinforce the notion that activation of Ras and Ras-effector pathways such as the Raf–MEK–Erb axis in the context of FLT3-ITD-dependent transformation originates at least predominantly at the PM.

The features of FLT3-ITD-mediated Ras activation described in the current study may be of broader relevance. Spatial features of Ras activation and its potential biological importance have not yet been assessed for any of the intracellular retained RTKs. We propose that Ras activation downstream of other activated and intracellular retained RTKs, e.g. activated c-Kit in gastrointestinal stroma tumors (Tabone-Eglinger et al., 2008) or activated ALK in neuroblastoma tumors (Mazot et al., 2011) may exhibit similar features, a hypothesis which can be tested using the methodology used in the present study.

Materials and Methods

Antibodies and reagents

Anti-pan-Ras (Ab-4) was from Oncogene Science (Cambridge, MA, USA). H-Ras (F235), K-Ras (F234) and N-Ras (F155) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For the simultaneous detection of K-Ras and N-Ras, a mixture of anti-pan-Ras (Ab-4) and anti-N-Ras (F155) antibodies was used, which was titrated for detection of all Ras isoforms with similar sensitivity using recombinant proteins. This antibody preparation is designated ‘anti-pan-Ras’ (Ab-4) was from Oncogene Science (Cambridge, MA, USA). H-Ras (F235), K-Ras (F234) and N-Ras (F155) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal goat anti-FLT3 antibody used for immunoprecipitation and immunoblotting of murine FLT3 (AF768) and monoclonal anti-human FLT3 (MAB812) for immunostainings were obtained from R&D Systems (Wiesbaden, Germany). Flow for flow cytometric analysis, R-phycocerythrin (R-PE)-conjugated mouse monoclonal anti-CD135 (hFL3; BD, Heidelberg, Germany); goats recognizes phospho-FLT3 Y591 (p#3461), total phosphotyrosine (pY100, #9411), and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204; #9106), were obtained from BD Transduction Laboratories (Heidelberg, Germany). Anti-pan-Ras (Ab-4) was from Oncogene Science (Cambridge, MA, USA). H-Ras (F235), K-Ras (F234) and N-Ras (F155) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal goat anti-FLT3 antibody used for immunoprecipitation and immunoblotting of murine FLT3 (AF768) and monoclonal anti-human FLT3 (MAB812) for immunostainings were obtained from R&D Systems (Wiesbaden, Germany). Flow for flow cytometric analysis, R-phycocerythrin (R-PE)-conjugated mouse monoclonal anti-CD135 (hFL3; BD, Heidelberg, Germany); goats recognizes phospho-FLT3 Y591 (p#3461), total phosphotyrosine (pY100, #9411), and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204; #9106), were obtained from BD Transduction Laboratories (Heidelberg, Germany). The cloning strategy led to removal of the IRES-EGFP cassette of LeGO-Ras fusion proteins were transferred into the vector LeGO-iG2 (Weber et al., 2008). The cloning strategy led to removal of the IRES-EGFP cassette of LeGO-iG2 (details available on request). As a reference vector LeGo+iV was used, driving expression of the yellow fluorescent protein Venus (http://www.lentivirus-vectors.de/vectors.htm).

Cell lines

32D cells stably expressing murine Flt3 wild-type (32D Flt3 wt) or murine Flt3 with human ITD (Mizuki et al., 2000) (32D Flt3 ITD) were kindly provided by Dr Rebeka Gründler and Prof. Justus Duyster (Technical University Munich, Germany). Parental 32D cells, and 32D cells stably expressing human FLT3 wild-type (32D hFL3 wt) or, the human FLT3 ITD variant (32D hFL3 ITD) were kindly provided by Prof. Huber (University of Münster, Germany). RS4-11 and MV4-11 cells were purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. All cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (BioWest, Essen, Germany). The medium for 32D cells was additionally supplemented with 20 mM HEPES, 1 mM sodium pyruvate and 1 ng/ml IL-3.

Immunodetection of FLT3 in leukemia cell lines

Detection of FLT3 localization by immunocytochemistry in RS4-11 and MV4-11 cells was done as previously described (Schmidt-Arras et al., 2009). In brief, the PM was stained on ice with WGA conjugated to Alexa Fluor 555 and MV4-11 cells were fixed with paraformaldehyde, quenched with ammonium chloride, permeabilized with 0.2% Triton X-100. Unspecific staining was blocked with 10% FCS in PBS. Cells were incubated for 1 hour at room temperature with anti-FLT3 antibodies (diluted 1:50), washed in PBS, and stained for 1 hour in the dark with Alexa-Fluor-555-conjugated secondary antibodies. Nuclei were stained with Hoechst 33342. The slides were then mounted with mounting solution and examined on a confocal Olympus FV 1000 microscope (Olympus, Hamburg, Germany) with a PlanApo 60×/1.4 NA oil objective or a 60×/1.35 NA oil objective, or on a Leica SP5 microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a HCX PLAPO lambda blue 63×/1.4 oil UV objective. Cell stainings to detect surface localized or total FLT3 by flow cytometry were performed exactly as described earlier (Schmidt-Arras et al., 2009). Cells were analyzed with a FACSCanto cytometer (Becton Dickinson, Heidelberg, Germany) and results were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Ras-GTP pulldown assay, and FLT3 immunoprecipitation

Cells were grown to a density of 10^6 cells/ml and were deprived of serum (for the time indicated in the figure legends) in RPMI 1640 supplemented with 0.2% fatty-acid-free/endoamine-low BSA, 1 mM sodium pyruvate and 50 mM HEPES pH 7.5, counted, and then resuspended at 2×10^7 cells/ml in the same solution. Cell suspensions were incubated in a water bath at 37°C and tubes were tipped every 2–3 minutes. After appropriate stimulation of wild-type FLT3-ITD-expressing cells with FL (20 ng/ml) or at the appropriate time after washing out cpd.102 from FLT3 ITD cells (see figure legends for details), 1 ml of the cell suspension was transferred to a 1.5 ml reaction vial, and quickly spun in a table-top centrifuge. The medium was aspirated, and the cell pellet was lysed with 1 ml ice-cold lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% NP-40) supplemented with protease and phosphatase inhibitors, 100 µM GDP and 30 µg GST-RBD protein. GDP and GST-RBD were included at this point to quench post-tx GTP loading or GAP-dependent Ras-bound GTP hydrolysis, respectively. Cell extracts were cleaved by centrifugation at 14,000 rpm at 4°C for 20 minutes, and GST-RBD-Ras-GTP complexes were collected on 40 µl glutathione-Sepharose beads (1:1 suspension) by incubation with end-over-end rotation at 4°C for 30 minutes. Beads were washed once with 500 µl lysis buffer, supplemented with protease and phosphatase inhibitors. Bound proteins were eluted with SDS-PAGE sample buffer and processed for immunoblotting. The supernatant of the Ras–GTP pulldown, once GST–RBD had been collected on glutathione-Sepharose was used to obtain total cell-lysate aliquots for immunoblotting. FLT3 immunoprecipitations were either performed using the same lysates or lysates prepared independently under identical conditions with 0.5 µg anti-FLT3 antibody or 0.8 µg anti-hFLT3 antibody, and protein-G– or -A-Sepharose beads, respectively.

Plasmids

PmCherry-K-Ras and pmCherry-N-Ras were generated as previously described (Rubio et al., 2010). A previously characterized multivalent fluorescent reporter of Ras–GTP, based on the trimerization of the Ras binding domain (RBD) of –Raf, was used to visualize Ras–GTP in live cells (Augusten et al., 2006; Rubio et al., 2010). Throughout this study we used EGFP3-xRBDx3 (abbreviated: E3-R3) and the attenuated version EGFP3-xRBD (R59A/N64D)x3 (abbreviated: E3-R3-A/D), as indicated in the figure legends.

Constructs for lentiviral expression of wild-type human K-Ras and the dn K-RasS17N were obtained by standard cloning procedures. EyFP-K-Ras was generated by replacing mCherry in mCherry-K-Ras (Rubio et al., 2010) with EyFP through AgeI–BorGI restriction cloning. The S17N mutation was introduced by site-directed mutagenesis using the QuikChange™ (Agilent, Waldbronn, Germany) method. Thereafter, the sequences encoding the corresponding EyFP–Ras fusion proteins were transferred into the vector LeGO-iG2 (Weber et al., 2008). The cloning strategy led to removal of the IRES-EGFP cassette of LeGO-iG2 (details available on request). As a reference vector LeGo+iV was used, driving expression of the yellow fluorescent protein Venus (http://www.lentivirus-vectors.de/vectors.htm).
Immunoblotting
Protein samples were separated on 12.5% (cell lysates, Ras-GTP pulldown) or 5%
(FLT3 immunoprecipitation) SDS-PAGE gels, and transferred to Hybond
Extra nitrocellulose membranes (GE Healthcare Life Sciences, Freiburg,
Germany). The membranes were incubated with primary antibody at 4°C
overnight, followed by development with horseradish peroxidase (HRP)-
conjugated secondary antibodies and enhanced chemiluminescence detection.
Images were acquired with a LAS 4000 CCD camera system (Fujiﬁlm, Düsseldorf,
Germany), quantiﬁed with Multi Gauge V3.0 software (Fujiﬁlm) and processed
using Adobe Photoshop CS3 extended Version 10.0 (Adobe Systems, San Jose,
CA).

Plasmid transfection and confocal live-cell imaging
For transfection, 6×10^5 cells were washed once in RPMI 1640 medium containing
10% FCS, then mixed with 10 µg of plasmid DNA in 300 µl culture medium in
0.4-cm gap cuvettes and electroporated using a GenePulserII instrument (BioRad,
Hercules, USA), with settings of 300 V and 960 µF and resistance set to inﬁnity,
24 hours after transfection, living cells were examined with a Zeiss LSM 510
Axiovert confocal microscope (Carl ZEISS GmbH, Jena, Germany) equipped with
a thermostated stage chamber (IBIDI, München, Germany) at 37°C. Confocal
images (1 µm optical slice) were acquired using a 63× water immersion objective
lens. mCherry and EGFP were excited with the HeNe 543 nm and the argon
488 nm line, respectively, in subsequent tracks. Emitted ﬂuorescence was
collected with a 560 nm longpass and a 505–550 nm bandpass ﬁlter, respectively.

Prior to experiments, cells were deprived of serum in RPMI 1640 supplemented
with 50 mM HEPEPS (pH 7.4), 1 mM sodium pyruvate, and 0.2% endotoxin-
free fatty acid-free BSA (Sigma-Aldrich, Taufkirchen, Germany) for 2 hours.

FLT3-ITD-expressing MV4-11 cells or 32D cells were treated with 1 µM
cpd.102 for the last 45 minutes of the starvation period, or left untreated. Cells
were plated on poly-L-lysine-coated (poly-L-lysine HBr; Mw 30,000–70,000; Sigma)
glass-bottomed (coverslip) 15-mm dishes 5 minutes before monitoring.
Confocal images of at least 20 cells per experiment were recorded. Only cells with
mCherry–Ras signals at the PM were considered for the analysis.

Wild-type FLT3-expressing RS4-11 cells were placed under the microscope,
then challenged with FL (100 ng/ml), and then a timed series of confocal images
of selected cells were acquired.

All images of a time series (for RS4-11 cells) or of experimental groups of cells
for comparison (for MV4-11 and 32D hFLT ITD cells) were exported as LSM
files, subjected to the same processing routine using Zeiss ZEN 2008 Light Edition
software (ZEISS, Jena, Germany), converted to TIFF files and processed again
identically using Adobe Photoshop CS3 extended Version 10.0 (Adobe Systems,
San Jose, CA). For presentation in the ﬁgures, parts of the images were enlarged to
reveal more details. Scale bars are depicted accordingly.

Role of Ras signaling for cell proliferation and viability
siRNAs were ON-TARGETPlus SMART pools (Thermo Scientiﬁc, Dharnaum,
Schwerte, Germany): control pool/non-targeting pool, cat. no. D-001810-10-20;
human FLT3 siRNA (KRAAS, cat. no. L-00596-00-00); human FLT3 (hFLT3),
cat. no. L-00319-00-0005; human FLT3 (hFLT3), cat. no. L-00137-00-0005.
Transfection was performed using the nucleofection device (Amaxa Inc.,
Cologne, Germany) with Nucleasefactor® kit V and the program A-30, according
to the manufacturer’s instructions. After 72 hours of culture, proliferation/viability
assays were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide (MTT) as described previously (Choudhary et al., 2009). The values were
calculated as means ± s.e.m. for comparison of groups in these assays, the two-tailed
-test was used. Calculations
were done with SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA).
P<0.05 (*) or P<0.01 (**), as indicated in the ﬁgure legends, was regarded as
statistically signiﬁcant.

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Author contributions
S. Köthe, I.R., and F.D.B. conceived and designed the experiments and wrote the manuscript. S. Köthe, J.P.M., S.A.B., T.T., M.F., S. Koch., C.T., R.P.R. and I.R. performed experiments and analyzed data. F.D.B. directed the project.

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Supplementary Figures

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Features of Ras activation by an aberrantly localized oncogenic tyrosine kinase: FLT3 ITD signals through K-Ras at the plasma membrane of acute myeloid leukemia cells
Figure S1. E3-R3(A/D) specifically reports the distribution of GTP-loaded N-Ras at the plasma membrane (PM) and endomembranes. The E3-R3(A/D) probe (labeled green RBD) was transiently expressed in RS4-11 cells by transfection of the corresponding expression plasmid in combination with plasmids coding for various mCherry-tagged N-Ras variants. These included wildtype N-Ras, the constitutively active N-Ras G12V mutant, and palmitoylation-less, endomembrane resident N-Ras C181S mutants. Further, N-Ras versions harboring these mutations in combination, and with or without the effector site mutation D38A, which is known to compromise the Ras-GTP-RBD interaction (Rubio et al., 2010) were also included, as indicated. 24 h after transfection cells were imaged confocally as described under Materials and Methods. Note that E3-R3(A/D) decorates GTP-loaded N-Ras both at the PM (N-Ras G12V) and endomembranes (N-Ras G12V, C181S). Ras-GTP visualization is specific because co-localization of probe (green) and Ras (red) signals was abrogated by the D38A mutation. Scale bars: 10 µm.
Figure S2. Intracellular sites of N-Ras activation in absence of FLT3 activity co-localize partially with an endosome marker. RS4-11 cells were transiently transfected with plasmids encoding untagged wildtype N-Ras, the reporter probe E3-R3(A/D) (indicated green as RBD), and organell markers for the endoplasmic reticulum (pECFP-ER, Clontech, Heidelberg, Germany) or endosomes (SARA-FYVE-ECFP (Hayakawa et al., 2004), kindly provided by Silvia Corvera, University of Massachusetts Medical School, Worcester, MA, USA). Endosomes were alternatively visualized by uptake of Alexa Fluor 555-labeled Transferrin (Life Technologies/Molecular Probes, Darmstadt, Germany; kindly provided by Christoph Kaether, Leibniz Institute for Age Research, Jena, Germany). To this end, cells were serum-starved for one hour, washed twice with serum-free medium and incubated with the probe in serum-free medium at 37°C for 45 min. Golgi membranes and mitochondria were stained using BODIPY TR ceramide or Mitotracker Red CMXRos (Life Technologies/Molecular Probes, Darmstadt, Germany), respectively, according to the instructions of the manufacturer. The cells with EGFP and red signals were subjected to confocal image analysis as described in Materials and Methods. To detect ECPF and EGFP labels, measurements were done with a Zeiss C-Apochromate 63x objective on a Zeiss LSM 710 laser scanning microscope. ECPF fluorescence of the respective organelle markers was excited with the 458 nm line of the Argon-laser, and organelle marker fluorescence was recorded in the range from 465-735 nm. EGFP fluorescence was excited with the 488 nm line of the Argon-laser and recorded in the
range of 495-735 nm. Cross talk between the CFP and GFP channel was minimal. To better visualize the co-localization ECFP (= organelle marker) fluorescence is shown in red, whereas EGFP fluorescence is displayed in green. Intracellular green compact structures represent sites of apparent N-Ras activation, which did not correlate with FLT3 activation. Some co-localization of these structures was detected with the endosome marker SARA-FYVE (white arrow). In some cells Ras activation at the PM is detectable, since cells were not extensively serum-starved before analysis to improve viability. Scale bars: 5 µm.
**Figure S3.** Role of Ras signaling for cell proliferation and viability - interference with FLT3 signaling by siRNA and pharmacological treatments. Human MV4-11 cells endogenously expressing FLT3 ITD were transfected with control siRNA, siRNA against KRAS, NRAS KRAS and NRAS or FLT3, or mock-transfected (no). Pharmacological treatments were with the FLT3 inhibitor cpd.102 (1 µM) or the MEK inhibitor UO126 (10 µM), or with solvent only (control) as indicated. (a) 48 hours later transfected cells were lysed and SDS-PAGE and immunoblotting were performed with the antibodies indicated. (b) Directly after siRNA transfection or initiation of inhibitor treatment cells were seeded into 96-well plates, and proliferation/viability assay was done using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after 72 hours of cultivation. The values were normalized to the control of mock-transfected cells as applicable. Results for depletion of K-Ras or both N- and K-Ras are labeled with arrows (n=5, means ± s.e.m.). (c, d) Cell cycle analysis by FITC-BrdU staining was done 48 hours after siRNA transfection or inhibitor treatment as indicated. The percentage of cells in different cell cycle phases was plotted as FITC-BrdU incorporation versus 7-AAD staining. (c) Examples of analysis results. (d) The percentage of cells in the S-phase is shown. Results for depletion of K-Ras or both N- and K-Ras are labeled with arrows (n=2, means ± s.e.m).

**References**
