**Title:** Alternative modes of GM-CSF receptor activation revealed using activated mutants of the common β-subunit.

**Running Title:** Alternative modes of GM-CSF receptor activation.

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**Text Word Count:** 4234 words

**Abstract work count:** 200 words

**Number of Figures:** 5 figures

**Number of References:** 48 references

**Scientific Category:** Hematopoiesis and Stem Cells

**Keywords:** cytokine signaling, GM-CSF receptor, Acute Myeloid Leukemia, Src Family Kinases
Abstract

GM-CSF promotes growth, survival, differentiation and activation of normal myeloid cells and plays an important role in myeloid leukemias. The GM-CSF receptor (GMR) shares a common signaling subunit, βc, with IL-3 and IL-5 receptors and has recently been shown to induce activation of JAK2 and downstream signaling pathways via formation of a unique dodecameric receptor complex. In this study we use two activated βc mutants that display distinct signaling capacity, and have a differential requirement for the GM-CSF receptor α-subunit (GMRα), to dissect the signaling pathways associated with GM-CSF response. The V449E trans-membrane mutant selectively activates JAK/STAT5 and MAPK pathways resulting in a high level of sensitivity to JAK and MEK inhibitors while the extracellular mutant (FIΔ) selectively activates the PI3K/Akt and IκKβ/NFκB pathways. We also demonstrate a novel and direct interaction between the SH3 domains of Lyn and Src with a conserved proline-rich motif in GMRα, and show a selective requirement for Src Family Kinases (SFKs) by the FIΔ mutant. We relate the non-overlapping nature of signaling by these two activated mutants to the structure of the unique GM-CSF receptor complex and propose alternative modes of receptor activation, differentially dependent on JAK2 and SFK, and acting synergistically in the mature liganded receptor complex.
Introduction

The GM-CSF, IL-3 and IL-5 receptors are key contributors to the regulation of normal hematopoiesis mediating growth and survival of haemopoietic progenitor cells and the production and activation of mature haemopoietic cells. GM-CSF in particular can provide both permissive and instructive signals for myeloid differentiation and has been shown to play a critical role in dendritic cells (DC). While dispensible for steady state hematopoiesis, it has an important accessory role in radioprotection by donor hematopoietic cells. It also has a non-redundant role in surfactant clearance by alveolar macrophages resulting in lung disease in GM-CSF null animals. Null animals display compromised antigen-specific and LPS induced T-cell responses and IFNγ production, have defects in macrophage function, and are susceptible to various infectious agents. GM-CSF and IL-3 have important roles in leukemia with autocrine production and over-expression of their ligand-binding subunits (IL-3Rα and GMRα) documented in AML. Constitutive activation of GM-CSF survival pathways has been reported in AML and GM-CSF has also recently been shown to play an important role in primary resistance in CML.

GM-CSF induces activation of JAK2 and downstream signaling pathways via formation of a unique dodecameric receptor complex. Activation of JAK2/STAT5, Ras-Raf-MAPK, and PI3K-Akt pathways by the mature GM-CSF receptor have been well characterised both in normal hematopoiesis and in disease where aberrant signaling has been shown to contribute to dysregulated myelopoiesis. However, extensive signaling redundancy and cross-talk among cytokine receptors (CRs) has made it difficult to link individual pathways to specific functional outcomes such as cell survival, proliferation and differentiation. Activation of one pathway often feeds into another resulting in a networked signaling response, the final outcome of which is influenced by the cell context, stage of differentiation, transformation and microenvironment. It is also clear that many signaling pathways converge on the same signaling molecules and some biological effects can be mediated by multiple effectors.
In part, signaling redundancy of CRs can be attributed to the sharing of receptor subunits. GM-CSF, IL-3 and IL-5 receptors, share the common beta subunit (βc) which is the primary signaling subunit that is pre-associated with JAK2 and contains several tyrosines which upon phosphorylation provide protein docking sites for activation of signal transduction. Specificity of this class of CRs is achieved through ligand-specific alpha subunits that enable affinity conversion of the receptor complex in the presence of a specific ligand.15 Although essential for receptor function, determining the precise signaling contribution of the α–subunits has been hampered by the lack of understanding of the role of interacting molecules. Deletion of the short cytoplasmic domain of the GM-CSF receptor α subunit (GMRα) abolishes ligand-induced signaling with no effect on ligand binding.16 The membrane-proximal region, and in particular, the SH3 binding site/PROX-like (SBP) motif17 is essential for GM-CSF-induced activation of JAK2.18

Some signaling molecules have been shown to associate with the GMRα cytoplasmic domain including the p85 regulatory subunit of PI3K,19 and IκKβ,20 however, the nature and the role of these interactions remains poorly characterised. Nevertheless, it is interesting to note that although GMRα is essential for GM-CSFR activation it does not signal by itself, an important feature that emphasises the importance of the GMRα interaction with βc.

To reduce the level of signaling complexity and dissect the contribution of signaling events activated by GMR we have used two constitutively activated mutants of βc (V449E and F1Δ) that deliver a subset of the proliferation, survival and differentiation signals induced by the ligand-activated GMR and display a differential requirement for the GMRα.21,22 Specifically, the strict requirement of the F1Δ mutant for GMRα suggests a minimal heterodimeric structure26 in contrast to the V449E transmembrane mutant which is thought to signal as a βc homodimer, independently of GMRα.26 The differential activity of these two mutants is also clear in vivo, where expression of the extracellular mutant, F1Δ, induces a chronic myeloproliferative disorder (MPD), whereas expression of the V449E transmembrane mutant by mouse BM reconstitution results in an acute
myeloid leukemia (AML).\textsuperscript{23} To understand the differences in signaling between these two classes of activating mutants we have used the bi-potential murine myeloid cell line, FDB1, which switches between growth or granulocyte-macrophage (GM) differentiation in response to IL-3 or GM-CSF respectively.\textsuperscript{22,24} Expression of h\(\beta_c\) mutants, V449E and FI\(\Delta\), in these cells induces factor independent proliferation or GM differentiation respectively, mimicking the functional response to cytokines. Here we describe non-overlapping signaling signatures of two \(\beta_c\) activated mutants and propose a model whereby each initiates a subset of the signaling activated by the mature ligand-bound dodecameric GMR. We report an important contribution of JAK2-independent signaling from the FI\(\Delta\) mutant and demonstrate novel and potentially critical interactions of both Lyn and Src with GMR\(\alpha\). We propose that these interactions are critical in modulating signals generated by GMR and suggest an important role for accessory subunits in modulating signaling of other CRs.
Material and Methods

Cell Culture and Transfections. The culture conditions of FDB1 cells, the construction of FIA and V449E retroviral expression plasmids, and the generation of stable cell lines were conducted as previously described. FIA and V449E receptor expression was maintained through the addition of puromycin to culture media, and assessed by flow cytometry using the anti-flag M2 antibody (Sigma-Aldrich, NSW, Australia). Prior to treatment of cells with inhibitors, cells were washed three times and starved of growth factors for 12-16h in medium containing serum. Stimulation was carried out for 5 min at 37°C using 500U mIL-3 or mGM-CSF. Unless otherwise indicated the final concentration of inhibitors was 50µM PD98069; 25µM AG490, Jak2InhII, U0126, LY294002, PP1 and Lyn Peptide Inhibitor; 0.5µM Wortmannin; and 5µM SU6656. All inhibitors were purchased from Merck (Darmstadt, Germany) with the exception of the Lyn Peptide Inhibitor which was obtained from Tocris Bioscience (Bristol, UK). Transient transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Co-Immunoprecipitation and Western Immunoblot Analysis. Cells were lysed in Modified RIPA Lysis Buffer (MRLB), separated by SDS-PAGE, and transferred to nitrocellulose. Proteins of interest were detected using the antibodies described below and SuperSignal West Pico or West Dura substrates. Rabbit polyclonal anti-JAK2 [pYpY1007/1008] was purchased from Biosource (Camarillo, CA). The mouse monoclonal anti-phospho-STAT5A/B (Tyr694/699), STAT3 and STAT5 antibodies were obtained from Upstate Cell Signaling Solutions (Lake Placid, NY). Rabbit polyclonal Phospho-IκBα (Ser32/36), Phospho-Akt (Ser473), Akt, Phospho-p44/42 Map Kinase (Thr202/Tyr204) and p44/42 Map Kinase, Phospho-Src family (Y416), Phospho-Lyn, Src, Lyn, and p85 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Secondary IgG antibodies and SuperSignal West Pico and West Dura detection substrates were obtained from Pierce Biotechnology (Rockford, IL). The secondary anti-
mouse FITC conjugated antibody was purchased from Sigma Aldrich (St Louis, MO). For co-immunoprecipitation, lysates were incubated overnight with 5µg of 4H1 antibody (kind gift from Tim Hercus) or IgG1 isotype control and 50µl of protein A/G sepharose. The sepharose was washed with MRLB, boiled in SDS load buffer and immunoblotted.

**GST Pull-Downs.** Glutathione-S-transferase proteins containing SH3 domains from Src (aa 83-150), Lyn (aa 62-122) and p85 (aa 3-79) were affinity purified on 4MB-glutathione sepharose (Amersham). 500µg cell lysate from HEK293T cells expressing hGMRA or hGMRA-APVA was incubated with GST-SH3-sepharose conjugates overnight at 4°C. Precipitates were washed with MRLB three times and proteins were eluted in SDS-PAGE load buffer.

**Intracellular Flow Cytometry.** Cells were fixed using a final concentration of 1.6% formaldehyde at 37°C for 15 min, permeabilised in 1ml ice cold methanol on ice for 30 min and incubated with the 5µg/µl primary antibody (unless otherwise stated) for 30 min on ice. Following incubation with corresponding FITC-conjugated secondary antibodies on ice for 30 min, samples were analysed by flow cytometry (Beckman-Coulter Epics Elite ESP Flow Cytometer/Cell Sorter).

**Fluorescence Polarization.** GST fusion proteins were prepared for mLyn, mSrc and mp85 SH3 domains. A black 96-well plate was blocked with casein for 1 hour at 37°C. Samples were incubated in triplicate with 100nM fluorescein labelled mGMRA peptide (mGMRA-pep; fluorescien-RLFPPIPGI, mGMRA-pep) and 25µg GST-SH3. A mGMRA-pep only and mGMRA-pep + GST were used. The assay plate was incubated for 5 minutes at room temperature prior to reading on the FLUOstar OPTIMA™ (BMG Labtechnologies) fluorescence plate reader at an excitation wavelength of 520nm. Data was analysed using FLUOstar Galaxy software and polarisation values of the controls, were subtracted from the other sample values to obtain the relative change in polarisation (ie. ΔmP value) for the test samples.
Results

Signaling from the GM-CSF receptor FID mutant is predominantly JAK2 and ERK independent.

GM-CSF signaling is primarily mediated by activation of JAK2 associated with the cytoplasmic domain of βc. JAK2 phosphorylation is dependent on an intact PXXP motif in the GMRα subunit which, when mutated, abolishes JAK2/STAT5 signaling.25 Given this requirement for GMRα in JAK2 signaling, we tested the activation and requirement for JAK2 in signaling from two activated βc mutants (FID and V449E) which display a differential requirement for GMRα.22 Parental FDB1 cells and cell populations with stable expression of FID and V449E mutants were withdrawn from growth factor for 16h and lysates were prepared. As a control for signaling pathway activation, cell populations were also treated for 5 min with mIL-3 or mGM-CSF. Western immunoblot analysis was used to determine the phosphorylation status of JAK2 and its downstream effector STAT5A/B (Figure 1A). As predicted, we observed JAK2 and STAT5A/B phosphorylation in all FDB1 cell populations responding to IL-3 stimulation. While we observed robust constitutive JAK2 phosphorylation in FDB1 cells expressing the activated V449E mutation, FDB1 cells expressing FID did not display constitutively activated JAK2 or STAT5A/B (Figure 1A). We observed negligible constitutive phosphorylation of ERK1/2 in FDB1 FID cells compared to the robust constitutive activation of ERK1/2 seen in FDB1 V449E cells (Figure 1A). Growth factor stimulation of FID and V449E expressing cells often results in lower levels of tyrosine phosphorylation than in the GF-stimulated parental FDB1 cells. This maybe associated with the constitutive nature of signalling in these cells and a potential reduction in wild-type receptor levels, or from a feedback pathway from the constitutive receptors that suppresses receptor responses. A more sensitive single cell phosho-profiling approach27 was also used to detect STAT5A/B and ERK1/2 phosphorylation (Figure 1B). Consistent with western immunoblot analyses, these results confirmed that FID signals independently of STAT5 and ERK1/2 in the absence of growth factor, and that V449E
activates both STAT5 and ERK1/2, despite its lack of requirement for GMRα (Figure 1B).

To further assess the requirement for JAK2 and ERK signaling in the cellular response to the βc mutants, we assessed viability of FDB1 cell populations treated for 24hrs with JAK inhibitors, AG490 and JAK2 Inhibitor II (J2InhII), and the MEK inhibitors, U0126 and PD98059 (Figure 1C). A 24 hour time point for viability was chosen as parental FDB1 cells die by 24 hours post growth factor withdrawal and all functional attributes of the cells at this time are presumed to be attributed the signaling of the activated receptors. FDB1 FIAΔ cells undergo differentiation so effects at later time points may not be inhibitor-specific and may represent changes that occur as the cells differentiate. Murine IL-3 stimulation following GF starvation was used as a control in these experiments as the transient activation of endogenous murine receptors provides an indication of starvation efficiency. Treatment of FDB1 cell populations with 25µM AG490 and 25µM J2InhII resulted in a significant reduction in the levels of phospho-JAK2 and phospho-STAT5A/B as detected by immunoblot analysis (Supplementary Figure 1A). An average of 30% and 48% reduction in the viability of FDB1 V449E cells was observed following AG490 and J2InhII treatment respectively (Figure 1C). In contrast, the JAK inhibitors had a lesser effect on the viability of FDB1 FIAΔ cells following growth factor withdrawal, consistent with the generation of JAK2-independent signals by the FIAΔ mutant receptor (Figure 1C).

MEK kinases lie upstream of MAPK and treatment with MEK inhibitors results in inhibition of ERK1/2 activity. We tested the effect of two MEK inhibitors, PD98059 and U0126 on the viability of all FDB1 cell populations. No effect was seen on the viability of parental or FDB1 FIAΔ cells following growth factor-withdrawal and treatment with 50µM PD98059 or 25µM U0126, consistent with the phosphorylation status of ERK1/2 in these cells (Figure 1D). Conversely, FDB1 V449E cells, showed a marked decrease in cell viability to approximately 40% by 24 hours following MEK inhibitor treatment (Figure 1D). Treatment of FDB1 cells with U0126 and PD98059 resulted in a significant
reduction in the levels of phospho-ERK1/2 as detected by immunoblot analysis (Supplementary Figure 1B). These inhibitor studies suggest a major contribution of both JAK2/STAT5 and ERK1/2 pathways in promoting survival of cells expressing the V449E mutant consistent with previous reports implicating these pathways in cell survival and proliferation.²⁸

**Differential activation of p85/Akt and IκKβ/NFκB Pathways by the activated βc mutants**

We next assessed activation of Akt, a downstream effector of p85, and IκB, a signaling molecule downstream of IκKβ that de-represses the NFκB survival pathway, in response to FIA and V449E expression in FDB1 cells. Lysates from FDB1 cell populations, starved and stimulated with mIL-3 or mGM-CSF, were immunoblotted with phospho-Akt and phospho-IκKβ antibodies to measure activation of these pathways. Interestingly, FIA cells displayed high levels of constitutive Akt and IκB phosphorylation in contrast to V449E expressing cells which showed minimal constitutive phosphorylation of either Akt or IκB (Figure 2A). Cells expressing the V449E mutant showed little phosphorylation of either Akt or IκB when stimulated with mIL-3 or mGM-CSF, compared to parental or FDB1 FIA cells (Figure 2A). FDB1 cells expressing FIA were sensitive to two PI3K inhibitors, showing a significant decrease in cell viability over a 24 hour period to 28% with 25µM LY294002 and 42% with 0.5µM Wortmannin (Figure 2B). Wortmannin and LY294002 had less significant effect on the viability of FDB1 V449E cells consistent with the lack of constitutive Akt phosphorylation by this mutant and with the presence of an alternative survival signal mediated by JAK2/STAT5 and ERK1/2 pathways. All cells cultured in IL-3 maintained good viability irrespective of inhibitor treatment, emphasising the redundancy of the multiple pathways activated by IL-3 (Figure 2B). All cell populations treated with PI3K inhibitors showed a significant reduction in the levels of phospho-Akt as detected by immunoblot analysis (Supplementary Figure 1C).

*Role of GMRα and SFKs in FIA signaling*
Given the strict requirement of the FIΔ mutant for GMRα and the lack of JAK2 activation by this mutant we next wished to identify molecules associated with GMRα that may contribute to constitutive activation of this mutant. The regulatory subunit of PI3K, p85, has been previously shown to associate with the GMRα subunit cytoplasmic domain, an interaction that is dependent upon the SH3 domain of p85 binding to the membrane-proximal proline-rich sequence in the short cytoplasmic domain of the GMRα. Additionally, IκKβ, has been shown to directly interact with the GMRα in a yeast-2-hybrid screen. With a view to identifying the signaling components that are present in the GMRα:FIΔ complex we used several approaches to identify proteins binding to the GMRα-subunit cytoplasmic domain. Immunoprecipitation was used to confirm previously reported associations of p85 and IκKβ with GMRα. Co-immunoprecipitation of GMRα with p85 and IκKβ was readily observed in HEK293T cells transiently expressing human GMRα consistent with published reports (Figure 3A and B). Using a form of GMRα in which the two outer prolines in the PPVP motif are mutated to alanine (APVA), we show that the interaction of p85 with GMRα is dependent on the intact SBP motif (Figure 3C). Association of IκKβ with GMRα is consistent with activation of this pathway by FIΔ and with several reports of NF-κB activation by GM-CSF and IL-3 playing an important role in modulating survival in myeloid cells. We postulated that the interaction of GMRα with IκKβ is unlikely to involve the SBP motif as the sequence/structures of IκKβ do not have known proline-interaction domains (SH3 or WW domains). Consistent with this, we show that IκKβ can still interact with the GMRα-APVA mutant (Figure 3D).

In addition to confirming previously reported interactions with GMRα, we aimed to identify novel interactions with GMRα (Figure 4A). As the SBP motif of GMRα includes an excellent match to the SH3 binding consensus we used a direct binding assay to identify interacting proteins with SH3 domains. Incubation of filters containing immobilised, recombinant SH3 domains (150 proteins, Panomics), with a fluorescently
labelled peptide corresponding to the proline-rich core of the human GMRα SBP motif, identified binding to the SH3 domains of Lyn, Src, CrkD2, and Nck2 (data not shown). Lyn and Src have known roles in cytokine receptor signalling and were thus analysed further. Co-immunoprecipitation studies confirmed the interaction of Lyn with GMRα in HEK293T cells (Figure 4A) and this interaction was lost with the GMRα-APVA mutant (Figure 4B). Similarly, we show co-immunoprecipitation of Src with GMRα, and reduced binding to GMRα-APVA (Figure 4C). GST pull-down experiments were also used to demonstrated binding of Lyn and Src with GMRα. GST fusion proteins of Lyn and Src SH3 domains were generated and used as “bait” to capture GMRα transiently over-expressed in HEK293T cells. Interactions were confirmed for both Lyn SH3 and Src SH3 domains with GMRα, and were abrogated with GMRα-APVA (Supplementary Figure 2). Additionally, the use of a fluorescence polarisation assay with a fluorescently-labelled human GMRα-SBP peptide demonstrated selective and direct binding of the peptide to GST-Lyn SH3 and GST-Src SH3 domain fusion proteins with a weaker interaction (not significant) observed for p85-SH3 (Figure 4D). Given that JAK2 and p85 have been shown to physically associate with Lyn, it is possible that p85 binds to GMRα through Lyn. Using several binding assays we were unable to detect an interaction between JAK2 with GMRα (data not shown), also reported elsewhere.

The direct association of Lyn and Src with GMRα suggests the possibility that the SFKs may play an important role in the response to GM-CSF and be particularly important for the FIΔ activated mutant. We next used SFK-selective inhibitors to test whether the SFKs are selectively contributing to FIΔ-mediated survival and differentiation of FDB1 cells. We utilized two independent SFK inhibitors, 25μM PP1 and 5μM SU6656, that reduce the activity of both Src and Lyn (Supplementary Figure 1D). Importantly, in the absence of growth factor FDB1 FIΔ cells showed sensitivity to SFK inhibitors with a clear reduction in viability (reduced to ~40% with both inhibitors; Figure 4E). In order to further assess the role of Lyn specifically we used a Lyn-binding, peptide inhibitor which comprises a hβc sequence known to interact with Lyn, thus resulting in Lyn sequestration.
and preventing its activation by GMR.\textsuperscript{33,34} Treatment of FDB1 FlΔ cells with the Lyn peptide inhibitor resulted in a significant reduction in viability at the two concentrations used (10µM and 25µM), and had no effect on FDB1-V449E cells (Figure 4F). The observation that V449E-mediated survival is not affected by treatment with SFK or Lyn-specific inhibitors suggests this pathway is not critical for signaling from this mutant. The inhibitors are presumably not acting via a direct or indirect affect on JAK2 activity as cells expressing hGMRα and hβc maintain robust JAK2 activation in the presence of IL-3 and GM-CSF when treated with SFK inhibitor (data not shown). Additionally, treatment of the FDB1 FlΔ cells with the SFK inhibitors did not affect IκB or Akt phosphorylation (Supplementary Figure 1D). The increased sensitivity of FDB1 FlΔ cells to SFK inhibition and the selective requirement for GMRα, together with the reduced sensitivity to JAK inhibition and the lack of JAK/STAT activation are consistent with an important signaling role for the SFKs in the FlΔ-mediated response. This mutant thus provides a tool for dissecting the roles of SFKs in GM-CSF receptor signaling.
Discussion

GM-CSF has recently been demonstrated to induce a unique receptor subunit configuration involving formation of a dodecameric complex, which results in JAK2 dimerisation and activation of JAK/STAT, ERK1/ERK2, PI3K/Akt, IκB/NFκB downstream pathways. In normal cells these pathways cooperate to induce growth, survival and/or differentiation responses that are cell type and context dependent. In leukemic cells these GM-CSF-induced pathways make an important contribution to proliferation and survival. In particular, constitutive serine phosphorylation of GM-CSF receptor has been reported in primary AML cells, associated with an unknown mechanism of receptor activation.

To dissect the relative contribution of GMRα and βc to pathway activation, and the role of each of these pathways in particular cell responses, we have used βc activated mutants which differentially require GMRα, and that activate non-redundant subsets of pathways and responses allowing association of signaling with alternative receptor configurations and cellular outcomes. This has provided evidence for JAK2-independent and SFK-dependent signals associated with GMRα. Taken together with evidence of association of GMRα with the SFKs Lyn and Src this suggests a novel contribution of GMRα to responses downstream of GMR.

A comprehensive biochemical analysis revealed non-overlapping signaling profiles of the βc mutants FIΔ and V449E. In the FIΔ mutant the survival response is mediated predominantly by the PI3K/Akt and IκKβ/NFκB pathways which are activated in the absence of detectable JAK2 activation. Whilst highly sensitive to PI3K inhibitors, the FIΔ mutant displayed relative resistance to two JAK inhibitors in keeping with the limited requirement for activation of JAK2. We and others have shown recruitment of the p85 regulatory subunit of PI3K to the GMRα and this may facilitate activation of PI3K/Akt signaling in FIΔ in the absence of JAK2 activity. In contrast to the FIΔ mutant, signaling from V449E was associated with robust activation of JAK2/STAT5 and
ERK1/2, high relative sensitivity to MEK inhibitors, and a lack of detectable Akt or IκB phosphorylation. The lack of Akt activation by this mutant, which we have proposed previously to be associated with ligand-independent dimerisation (Figure 2A),26 is consistent with the observation that PI3K/Akt activity is not seen in BA/F3 cells expressing a βc/JAK2 chimera36 and suggests that JAK2 activation is not sufficient for activation of the PI3K/Akt pathway.

A major question from these finding relates to the nature of the primary signaling event associated with the FIΔ mutant. JAK-independent signaling is known to contribute to important outcomes from CRs, including βc.37 JAK2-independent signaling is also consistent with our previous analysis of serial truncations of the FIΔ mutant, which indicate that the primary signal is associated with a region distal to the Box 1 motif responsible for JAK2 association.32 Given the strict requirement of the FIΔ mutant for GMRα we explored the protein interactions with the GMRα cytoplasmic domain. We have confirmed here association of GMRα with the p85 regulatory subunit of PI3 kinase, and with IκKB, as reported previously.19,20 The finding that Src and Lyn associate with GMRα via the critical proline-rich SBP motif strongly implicates the SFKs as playing a key role in signaling from the FIΔ complex. This association of GMRα with Src and Lyn was demonstrated using both direct (GST pulldown and fluorescence polarization) and indirect (co-immunoprecipitation) methods and the role of the SBP motif was confirmed by site directed mutagenesis of the prolines in the SBP motif. The association of Lyn and Src with the α-subunit via the SBP motif is consistent with studies demonstrating an important role for SFKs in receptor-mediated responses in the myeloid lineage. Members of the SFK family have been linked to activation of cellular signaling in response to GM-CSF (Reviewed in 38); Lyn in particular is activated by IL-3, IL-5 or GM-CSF, and can phosphorylate tyrosine residues in hβc in vitro.34

Lyn in particular, may be critical in the JAK2-independent signaling from GMR. The FIΔ mutant displayed a selective sensitivity to a Lyn inhibitory peptide, consistent with Lyn
being a key initiator of signaling from the FIΔ mutant. The Lyn inhibitory peptide in particular, may be acting by inhibiting SFK associated with βc, however the selective sensitivity of the FIΔ mutant to this peptide together with the high relative sensitivity to the SFK inhibitors clearly demonstrates the differential requirement for SFKs in FIΔ signaling. In contrast, the resistance that V449E displays to treatment with SFK inhibitors, including the Lyn inhibitory peptide, indicates that it may generate additional or alternative SFK-independent survival signals (probably JAK2 mediated). These are primarily mediated via the MAPK pathway as treatment with MEK inhibitors dramatically and selectively affects the survival of V449E-expressing FDB1 cells. In the FDB1 system IL-3 survival responses are also relatively resistant to SFK inhibitor treatment and we propose that both JAK- and SFK-dependent survival pathways are being activated by the wild-type ligand-induced receptor complexes, with extensive redundancy. In vivo studies suggest that JAK2-independent signaling from hβc will support only limited responses; while myeloid progenitors from the fetal liver of JAK2-deficient mice show a lack of growth responsiveness to IL-3 and GM-CSF, survival responses of JAK2 knockout progenitors in response to these growth factors have not been studied in detail.

These signaling properties of the two activated mutants are consistent with the two classes of mutant representing alternative receptor complexes as proposed previously. These complexes deliver at least some of the proliferative, survival, self-renewal and differentiative signals activated by a mature GM-CSF receptor complex. In Figure 5 we summarise the properties of these mutants in the context of the unique GM-CSF dodecameric receptor structure. We propose that the FIΔ and V449E activated receptor complexes initiate non-overlapping signaling events resulting in activation of complementary pathways and non-redundant signaling. These alternative events may be initiated within the WT dodecamer complex via the αββα tetramer structure or the αβ heterodimers (Figure 5). This would predict that the αββα complex signals predominantly through activated JAK2. It also raises the possibility that pathways downstream of JAK2 (eg Shc initiated activation of SHIP) suppress Akt activation from
the αβ heterodimer\textsuperscript{12} suggesting a mechanism for cross-regulation of signaling within the dodecamer. The tetrameric structure predicted for the FIΔ mutant would preclude JAK2 activation and thus signaling would occur predominantly via JAK-independent mechanisms, including those signals generated from the GMRα. Whether these alternative complexes shown in Figure 5 represent intermediates in formation of the mature GM-complex is not clear. The recent demonstration that low concentrations of GM-CSF induce a limited signalling response\textsuperscript{10,41} is consistent with a role for these alternative receptor configurations in certain contexts, including leukemic cell survival. Lyn is frequently activated in leukemic blasts from AML patients\textsuperscript{42} and in CML blast crisis;\textsuperscript{43} and these studies raise the possibility that this is related to aberrant GM-CSF or IL-3 receptor signaling. We suggest that further studies with these mutants will facilitate the identification of the role of SFKs in GM-CSF responses and may shed light on the mechanisms associated with aberrant GM-CSF and Lyn activation in leukaemia.

Acknowledgements
We acknowledge financial assistance from the NIH (R01 HL60657). We also thank Mrs. Sylvia Nobbs and Mr. Sandy McIntyre for their assistance with flow cytometry.

Authorship Contributions
M.P. and R.J.D. wrote the manuscript. M.P. performed the experiments and analyzed the data. D.G.S. provided experimental assistance. G.B, C.S. and T.R.H provided advice and technical assistance, A.L.B, M.H. and T.J.G. were involved in data interpretation and writing of the manuscript. A.F.L critically reviewed the manuscript.

Disclosure of Conflicts of Interest
There are no conflicts of interest to disclose.
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Figure Legends

Figure 1. The hβc FIΔ mutant generates JAK2- and ERK1/2-independent signals.
(A) FDB1 cell populations were cultured without growth factor (NF) for 16hr and then stimulated with mIL-3 or mGM-CSF for 5 min. Whole cell lysates (WCL) were subjected to western blot analysis with the indicated antibodies. (B) FDB1 cells were cultured in the absence of growth factor (NF), mIL-3 or mGM-CSF, then fixed, permeabilised and stained with the indicated primary antibodies (open histograms) or isotype matched control (grey histograms). (C) FDB1 cells were cultured with DMSO, JAK2 inhibitor II (J2InhII), or AG490. After 24hr, viability of cells was measured by 7-AAD staining and flow cytometry. (D) FDB1 cells expressing receptor mutants were cultured with DMSO, U0126, or PD98059 (PD). Viability was measured at 24hr by 7AAD staining and flow cytometry. Error bars represent standard error of the mean where n=3, statistical significance was calculated using a student t-test, *p<0.05  **p<0.001.

Figure 2. The hβc FIΔ mutant signals through Akt and NFκB pathways.
(A) FDB1 cells were cultured without growth factor (NF) for 16hr and then stimulated with mIL-3 or mGM-CSF for 5 min. Whole cell lysates were subjected to western blot analysis with the indicated antibodies. (B) FDB1 cells were cultured with DMSO, LY294002 (LY), or Wortmannin (Wort). After 24hr cell viability was measured by 7-AAD staining and flow cytometry. Error bars represent standard error of the mean where n=2, statistical significance was calculated using a student t-test, *p<0.05  **p<0.001.
**Figure 3. IκB and p85 interact with hGMRα.**

(A) Flow cytometric analysis to detect hGMRα and hGMRα-APVA following transfection of HEK293T cells. hGMRα was detected with 4H1 antibody (open histograms). An isotype-matched control is shown for comparison (grey histograms). (B) hGMRα, p85 and IκB detected in whole cell lysates (WCL) from HEK293T cells expressing hGMRα, and in 4H1 immunoprecipitates (4H1 IP) and minus antibody (-Ab) or IgG1 (IgG1) control immunoprecipitates. (C) hGMRα and p85 detected in WCL from HEK293T cells expressing hGMRα or hGMRα-APVA, and in 4H1 immunoprecipitates (4H1 IP) of the same cells. (D) hGMRα and IκB detected in WCL from HEK293T cells expressing hGMRα or hGMRα-APVA, and in 4H1 (4H1 IP) and minus antibody (-Ab) or IgG1 (IgG1) control immunoprecipitates of the same cells.

**Figure 4. Association of Lyn and Src SH3 domains with hGMRα.**

(A) hGMRα and Lyn detected in whole cell lysates (WCL) from HEK293T cells expressing hGMRα, and 4H1 immunoprecipitates (4H1 IP). (B) hGMRα and Lyn detected in WCL from HEK293T cells expressing hGMRα or hGMRα-APVA, and in 4H1 (4H1 IP) and IgG1 (IgG1) control immunoprecipitates of the same cells. (C) hGMRα and Src detected in WCL from HEK293T cells expressing hGMRα or hGMRα-APVA, and in 4H1 (4H1 IP) and IgG1 (IgG1) control immunoprecipitates of the same cells. (D) Fluorescence polarisation analysis of a fluorescein conjugated mGMRα.
peptide alone, with GST, or with p85, Lyn and Src SH3 domain GST fusions proteins. The change in millipolarization (Δmp) is given, where emissions of the mGMRα peptide alone have been subtracted from the other samples. (E) Trypan blue viability of FDB1 cells expressing receptor mutants after culture with DMSO, 0.5μM SU6656, or 25μM PP1 for 24hrs. (F) Trypan blue viability of FDB1 cells expressing receptor mutants after culture with DMSO, 10μM and 25μM Lyn peptide inhibitor (LI) for 24hrs. Error bars represent standard error of the mean where n=2, statistical significance was calculated using a student t-test, *p<0.05 **p<0.001.

**Figure 5. Model for assembly and activation of hβε mutants FIΔ and V449E.**

The high affinity complex of the GM-CSF receptor (GMR) is a dodecamer structure (centre) comprising two ligand bound hexamers. The central structure in the dodecamer complex enables JAK2 transphosphorylation and activation of STAT5- and Shc-mediated pathways. Proposed signaling through GMRα occurs in αβ heterodimers (outer structures in the dodecamer complex) which initiate activation of Akt and NFκB pathways. Also indicated is a possible negative feedback mechanism whereby activation of SHIP downstream of JAK2 may inhibit GMRα–induced Akt activation. The proposed V449E structure is represented as a βε tetramer which does not require GMRα and initiates a subset of the signals generated by the dodecameric GMR complex. V449E activated pathways are confined to those downstream of JAK2 and receptor tyrosine phosphorylation, and support survival and proliferation in the FDB1 cells. The proposed FIΔ structure comprises an αβ tetramer which precludes JAK2 transphosphorylation.
This complex generates signals predominantly through GMRα and results in activation of Akt and NFκB pathways, supporting FDB1 survival and differentiation.
Perugini et al – Figure 1

A.

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Phosphorylation of Indicated Target Protein

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C.

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D.

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Perugini et al – Figure 2

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![Western Blot Images]

- p-IκBα
- p-AKT
- AKT

B.

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<td>LY</td>
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* p < 0.05
** p < 0.01
Perugini et al – Figure 3

A. HEK 293T hGMRα and hGMRα-APVA Cell Number

anti-hGMRα 4H1

B. HEK hGMRα

WB: p85

WB: IκBα

WB: hGMRα (4H1)

WCL 4H1 IP

C. HEK hGMRα

WB: p85

WB: hGMRα (4H1)

WCL 4H1 IP Ab IgG

D. HEK hGMRα

WB: IκBα

WB: hGMRα (4H1)

WCL 4H1 IP Ab IgG
C.

<table>
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- p-Src
- p-Lyn
- β-Actin
- p-1xBα
- p-AKT
- β-Actin
Perugini et al – Supplementary Figure 2