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Abstract: Granulocyte-macrophage colony stimulating factor (GM-CSF) promotes the growth, survival, differentiation and activation of normal myeloid cells and is essential for fully functional macrophage differentiation in vivo. To better understand the mechanisms by which growth factors control the balance between proliferation and self-renewal versus growth-suppression and differentiation we have used the bi-potent FDB1 myeloid cell line, which proliferates in IL-3 and differentiates to granulocytes and macrophages in response to GM-CSF. This provides a manipulable model in which to dissect the switch between growth and differentiation. We show that, in the context of signaling from an activating mutant of the GM-CSF receptor β subunit, a single intracellular tyrosine residue (Y577) mediates the granulocyte fate decision. Loss of granulocyte differentiation in a Y577F second-site mutant is accompanied by enhanced macrophage differentiation, accumulation of β-catenin together with activation of Tcf4 and other Wnt target genes. These include the known macrophage lineage inducer, Egr1. Further, we show that forced expression of Tcf4 or a stabilised β-catenin mutant is sufficient to promote macrophage differentiation in response to GM-CSF, and that GM-CSF can regulate β-catenin stability. This study therefore identifies a novel pathway through which growth factor receptor signalling can interact with transcriptional regulators to influence lineage choice during myeloid differentiation.

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May 27, 2011

The Editor
Differentiation

Dear Dr Stewart

We are submitting for your consideration, a manuscript entitled 'The GM-CSF receptor utilizes β-catenin and Tcf4 to specify macrophage lineage differentiation'.

In this study we describe a novel function of the GM-CSF receptor to regulate members of the Wnt/β-catenin pathway and in-turn specify the level of macrophage differentiation in the context of a bi-potential granulocyte-macrophage progenitor. Specifically, we provide evidence that β-catenin and Tcf4 are central in promoting macrophage differentiation and activity of these is regulated through a specific tyrosine in the GM-CSF receptor.

We believe that as these findings suggest a new mechanism that may regulate differentiation within the myeloid compartment they are of significant interest to the readership of *Differentiation*.

All authors concur with the submission of this manuscript. This material contained in this manuscript is original research and has not been previously reported and is not under consideration for publication elsewhere.

Conflict of interest statement: The authors declare no competing financial interests.

Thank you for your consideration. We look forward to your response.

Sincerely,

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Original Article

Title: The GM-CSF receptor utilizes β-catenin and Tcf4 to specify macrophage lineage differentiation.

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Summary sentence: Signaling from the GM-CSF receptor modulates β-catenin and Tcf4, to influence macrophage lineage commitment.

Running title: GM-CSF and β-catenin in macrophage differentiation

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Abstract

Granulocyte-macrophage colony stimulating factor (GM-CSF) promotes the growth, survival, differentiation and activation of normal myeloid cells and is essential for fully functional macrophage differentiation *in vivo*. To better understand the mechanisms by which growth factors control the balance between proliferation and self-renewal versus growth-suppression and differentiation we have used the bi-potent FDB1 myeloid cell line, which proliferates in IL-3 and differentiates to granulocytes and macrophages in response to GM-CSF. This provides a manipulable model in which to dissect the switch between growth and differentiation. We show that, in the context of signaling from an activating mutant of the GM-CSF receptor β subunit, a single intracellular tyrosine residue (Y577) mediates the granulocyte fate decision. Loss of granulocyte differentiation in a Y577F second-site mutant is accompanied by enhanced macrophage differentiation, accumulation of β-catenin together with activation of *Tcf4* and other Wnt target genes. These include the known macrophage lineage inducer, *Egr1*. Further, we show that forced expression of Tcf4 or a stabilised β-catenin mutant is sufficient to promote macrophage differentiation in response to GM-CSF, and that GM-CSF can regulate β-catenin stability. This study therefore identifies a novel pathway through which growth factor receptor signalling can interact with transcriptional regulators to influence lineage choice during myeloid differentiation.

Abbreviations: GM, granulocyte macrophage; GMR, GM-CSF receptor; DC, dendritic cell; IPA, ingenuity pathway analysis; BIO, (2'Z,3'E)-6-Bromoindirubin-3'-oxime

Introduction

In this study we examine the process of granulocyte and macrophage lineage specification mediated via Granulocyte-macrophage colony stimulating factor (GM-CSF) signaling. GM-CSF is a hematopoietic growth factor that can provide both permissive and instructive signals for myeloid differentiation (Kondo et al., 2000) and has been shown to play a critical role in DC function (Conti and Gessani, 2008). *In vivo* administration of GM-CSF promotes increased production of granulocytes and macrophages demonstrating its key role *in vivo* in driving coordinated proliferation and differentiation of GM progenitors (Metcalf et al., 1987). In particular, under steady-state conditions, GM-CSF signaling has a non-redundant role in mature macrophage production, with loss of GM-CSF signaling in mouse and humans leading to pulmonary alveolar proteinosis due to defects in alveolar macrophages (Dranoff et al., 1994; Suzuki et al., 2008; Martinez-Moczygemba et al., 2008; Nishinakamura et al., 1995). Null animals also display compromised antigen-specific and LPS-induced T-cell responses and IFNγ production, which may be DC-mediated, have defects in macrophage function and are susceptible to various infectious agents (Enzler et al., 2003; Paine et al., 2000). The high affinity receptors for human GM-CSF (GMR), IL-3 (IL3R) and IL-5 (IL5R) are each comprised of unique ligand-specific α subunits (GMRα, IL3Rα or IL5Rα) and a shared β subunit ($h\beta_c$) which are all members of the cytokine receptor superfamily (for review see (Miyajima et al., 1993; Lopez et al., 2010). Each ligand binds to its specific α-subunit to form a low affinity intermediate which we, and others have shown to form a signaling complex that is likely to include a dimer of $h\beta_c$ and, at least in the case of the GMR, has been recently shown to form a higher order dodecameric complex for the full range of ligand induced signaling (McClure et al., 2001; McClure et al., 2003; Hansen et al.,

2008). $h\beta_c$ is the primary signaling subunit and mutation can result in constitutive activation with a range of mutants now described that display alternative phenotypes and signaling profiles (D'Andrea et al., 1998; McCormack and Gonda, 1999; Jenkins et al., 1995; Brown et al., 2004; Perugini et al.; 2010).

Mutational studies of the GMR have identified intracellular regions and key residues of the GMRα and hβc that are responsible for the signaling required for myeloid differentiation versus growth. In particular, the region of hβc containing Tyr577 is important for mediating GM-CSF induced myeloid differentiation of M1 and WEHI-3B D+ cells, where macrophage differentiation is induced in response to ligand, however particular residues in this region were not linked to the response (Smith et al., 1997). Studies with activated mutants of hβc, showing reduced signaling complexity compared to the wild type receptor, have facilitated dissection of signaling networks downstream of the GM-CSF receptor, and allowed particular signaling events to be assigned to cellular outcomes (Brown et al., 2004; Perugini et al., 2010; Jenkins et al., 1998). In this study we use the well-characterised activated hβc mutant, FIΔ, and a second-site mutant with a tyrosine to phenylalanine substitution at position 577 (Y577F) that selectively abolishes granulocyte differentiation and enhances macrophage differentiation (Brown et al., 2004). This has provided a model system in which to dissect GM differentiation induced through the GM-CSF receptor. The Tyrosine 577 residue of hβc has been previously shown to be a key signaling residue associated with binding of the Shc adapter molecule and is part of a small phosphorylation-dependent motif, which regulates alternative survival and proliferation pathways (Okuda et al., 1997; Powell et al., 2009; Guthridge et al., 2006; Ramshaw et al., 2007). Here we focus on defining downstream events, associated with the Tyr577 residue, and on linking these to the lineage-fate choice between granulocyte and macrophage differentiation. We show that the Y577F mutation is associated with enhanced β-catenin protein accumulation and *Tcf4* gene expression and we demonstrate

a central role for these factors in promoting macrophage differentiation at the expense of granulocyte differentiation.

Materials and Methods

Cell culture

The culture conditions of FDB1 cells, the construction of FIΔ and FIΔY577F retroviral expression plasmids, and the generation of stable cell lines have been previously described (Brown et al., 2004). Before treatment of cells with inhibitors, cells were washed 3 times and starved of growth factor for 16 hours in medium containing serum. Stimulation was carried out for 5 minutes at 37°C by the use of 500 bone marrow units (BMU)/mL mouse (m)IL-3 or mouse (m)GM-CSF. The GSK-3 Inhibitor IX , BIO, and control, MeBIO, (Merk Chemicals, Nottingham, UK) were dissolved in DMSO and used at a final concentration of 2 μM.

Microarray hybridisation and analysis

FDB1 cells expressing either FIΔ and FIΔY577F were washed 3 times to remove growth factor and a proportion of cells were harvested at time zero. Cells were then cultured without factor for 72 hours for the second time point. Two biological replicates were prepared for each cell line (supplemental Fig. 1). Comparisons were performed for each cell line over time and direct comparisons between cell lines done at 0 and 72 hours. Additional dye swaps were done with the FIΔ/ FIΔY577F 0 hour direct comparison and the FIΔ 0-72 hour comparison (supplemental Fig. 2). RNA preparation and microarray analysis and hybridisation were performed as described previously (Brown et al., 2006). Lineage specific gene lists were defined as follows. Neutrophil associated genes were defined as genes with a significant change over time in FIΔ expressing cells ($p<0.001$) but not FIΔY577F cells. Macrophage associated genes were defined as those with a significantly larger fold change over time in

FIΔY577F cells compared to FIΔ cells ($p<0.001$, supplemental Fig.2). We performed gene-set enrichment analysis as previously described (Brown et al., 2006). Briefly, to test for association of published TF target gene sets based on either differential expression (C/EBP) (Gery et al., 2005) or ChIP (TCF and β-catenin) (Hatzis et al., 2008; Yochum et al., 2007) with our data sets, we used the non-parametric Wilcoxon rank sum test as implemented in the R statistical program (http://www.r-project.org/). We constructed our reference set of genes based on ranking the FDR p-value for comparisons of reference-set and test-set. For the C/EBP comparison we generated a reference set by ranking all of the genes on our array using the FDR p-values for the change in expression over time in FIΔ expressing cells and examined the distribution of C/EBP target genes in our reference list. For the β-catenin and TCF4 comparisons we generated a reference set by ranking all of the genes on our array using FDR p-values for the change in expression over time in FIΔY577F expressing cells and examined the distribution of β-catenin and TCF4 target genes in our reference list. A significant result from the Wilcoxon rank sum test indicated that the gene-set of interest displays an association with the indicated reference-set. Unsupervised clustering and heatmaps of data were generated with MeV (http://www.tm4.org/mev/). The raw microarray data is available in the Gene Expression Omnibus (GSE25857).

QRT-PCR

FDB1 cells expressing either FIΔ and FIΔY577F were washed 3 times to remove growth factor and a proportion of cells were harvested at time zero. Cells were then cultured without factor and cells harvested at 24 hours and 72 hours. Parental FDB1 cells were also cultured in GM-CSF and harvested at 24, 72 and 120 hours. Two biological replicates of this experiment were performed. RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturers instructions. cDNA was prepared using the QuantiTect Reverse

Transcription Kit (Qiagen, Valencia, CA, USA). The sequences of the oligonucleotides used for PCR are listed in supplemental Table 1. Gene-specific PCR reactions were performed for 40 cycles using FastStart Taq (Roche diagnostics, Indianapolis, IN, USA) and recommended conditions. SYBR green (Invitrogen, Carlsbad, CA, USA) was added to a final concentration of 0.6x per reaction and PCR was performed on the Rotor-Gene 6000 and related software used for data collection and to determine mean expression values relative to beta-actin (Corbett Research, Version 5.0, Qiagen, Valencia, CA, USA). Amplification products were analyzed by melt curve and sequenced to confirm specificity.

Construction of retroviral expression plasmids and transduction of FDB1 cells

Constructs for FLAG-tagged full length TCF4 and ΔN-TCF4 in pcDNA3.1/Zeo were obtained from Hans Clever (van de et al., 2002). The cDNAs were amplified from the constructs using the oligos indicated in supplemental Table 1. The products were digested with SfiI and ligated into SfiI digested murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-green fluorescence protein (GFP) retroviral vector (modified to contain SfiI sites in the MCS). The construct for stabilised β-catenin (pCS2MMBCS33A) was obtained from Rolf Kemler (Rottbauer et al., 2002). The β-catenin cDNA contains 4 amino acid substitution mutations (Ser33Ala, Ser37Ala, Thr41Ala and Ser45Ala). β-catenin was amplified using the oligos indicated in supplemental materials and methods and cloned into MSCV-IRES-GFP as described above.

Virus production and FDB1 transduction was performed as previously described (Perugini et al., 2009). For the TCF constructs, cells expressing GFP were sorted using flow cytometry and allowed to recover overnight before using in assays. For the βcatS33A construct, cells expressing GFP were sorted to generate a line with stable βcatS33A expression. Cell sorting

was performed on Beckman-Coulter ALTRA (Beckman-Coulter, Brea, CA, USA) or Becton-Dickinson ARIA cell sorters (Becton-Dickinson, Franklin Lakes, NJ, USA).

Proliferation and differentiation assays

Growth was assessed using either CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), or trypan blue exclusion. The proportion of viable cells was determined by trypan blue exclusion. To assess differentiation, cytospin preparations were made, and slides stained with May-Grünwald Giemsa. 200 cells were scored microscopically for morphology. In addition, cell surface staining was performed using a rat anti-mouse cfms-PE conjugate and isotype control, IgG2a-PE, from eBiosciences (San Diego, CA, USA) and analyzed by flow cytometry using a FC500 or XL-MS analyser (Beckman-Coulter, Brea, CA, USA).

Western immunoblot analysis and antibodies

Cells were lysed in modified RIPA lysis buffer, separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Proteins of interest were detected by use of the appropriate antibodies and SuperSignal West Pico, West Dura or West Femto substrates (Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA). Mouse monoclonal anti-β-catenin was purchased from BD Biosciences (Becton-Dickinson, Franklin Lakes, NJ, USA). Rabbit polyclonal p38MAP Kinase and rabbit monoclonal GSK3β antibody, were purchased from Cell Signaling Technology (Beverly, MA, USA). Secondary immunoglobulin G (IgG) antibodies were obtained from Pierce Biotechnology (Thermo Scientific, Rockford, IL, USA).

Online Supplemental Material

Supplemental Table 1 includes sequences for oligonucleotides used for PCR and cloning. Supplemental Table 2 includes full lists of genes differentially regulated in macrophage and granulocyte lineages by Y577. Supplemental Table 3 includes the full list of β-catenin/Tcf4 target genes differentially regulated by Y577. Supplemental Fig. 1 contains mutant hβc expression and differentiation information for the cells used in the microarray analysis. Supplemental Fig. 2 contains microarray experimental design and volcano plots of genes differentially regulated by Y577. Supplemental Fig. 3 shows the gene set enrichment plot for β-catenin/Tcf4 target genes in FIΔ-Y577F regulated genes.

Results

A single tyrosine residue in GMR is required for granulocyte differentiation: a model of GM-CSF-induced lineage specification.

When cultured in GM-CSF, FDB1 cells form granulocytes and macrophages in approximately equal proportions and this can be mimicked in the absence of growth factor by ectopic expression of the hβc FIΔ activated mutant (McCormack and Gonda, 2000). To characterise the signaling pathways and downstream genes supporting granulocyte and macrophage differentiation induced by GMR we performed comparative gene expression profiling of the differentiation response for FDB1 cells expressing the FIΔ mutant and its derivative FIΔY577F, which supports differentiation predominantly to macrophages (Brown et al., 2004). Cultures of FDB1 cells expressing the parental FIΔ mutant or the Y577F derivative were cultured in the absence of growth factor for 72 hours and assessed for the expected morphological differentiation prior to RNA extraction (supplemental Fig. 1). Microarray hybridisation and processing was carried out on the Compugen Mouse Oligo Library (v2, 21,997 65mers comprising 21,587 unique genes) as previously described (Brown et al., 2006). The differential gene expression in each population was determined over this time period and gene expression changes associated with mutation of Tyr577 were determined by linear modelling (supplemental Fig. 2A) (Brown et al., 2006). Gene expression changes were defined as Tyr577-associated and involved in promoting granulocyte differentiation if the change in expression was selectively lost in cells responding to the FIΔY577 mutant (supplemental Fig. 2B). Genes with a significantly greater change over time in FIΔY577F compared to FIΔ (supplemental Fig. 2B) were defined as suppressed by Tyr577 signaling, and associated with promoting macrophage differentiation. Lists of differentially-expressed genes were generated using an FDR p-value of 0.001 and a fold change cut-off of

1.4. Using this approach we identified 153 up-regulated and 83 down-regulated genes associated specifically with intact Tyr577 signaling and differentiation of the granulocyte lineage and 166 up-regulated and 55 down-regulated genes associated with enhanced differentiation of the macrophage lineage in the absence of Tyr577 signaling **(**supplemental Table 2). The top 25 up and down regulated genes associated specifically with the granulocyte and macrophage lineages are shown in Fig. 1A and C respectively**.**

Consistent with the loss of neutrophil differentiation associated with the Y577 second-site mutant we identified differential expression of many known neutrophil genes amongst the Tyr577-regulated genes. These included granule proteins such as *Ltf*, *Ngp*, *Mmp8*, *Itgb2l*, *Camp*, *Prtn3*, *Lcn2*, *Ceacam1*, *S100a8*. Many of these genes are also targets of the C/EBP family of transcription factors several members of which, including C/EBPα and C/EBPε, are important regulators of this lineage. Indeed, using gene-set enrichment analysis we identified a significant association of these genes with the C/EBP target gene-set generated by Gery *et al* (Gery et al., 2005) (p=0.00023). Ingenuity pathway analysis (IPA) of the full neutrophil associated gene list identified significantly associated canonical pathways. These revealed a network of down-regulated genes involved in IL-4 signaling (and T and B cell differentiation and function) consistent with suppression of some alternative lineages, and up-regulation of genes associated with leukocyte function (Fig. 1B).

As predicted, the genes that show enhanced up-regulation over time in the presence of the Y577F mutation were strongly associated with macrophage differentiation. These included genes involved in macrophage function, such as proteins normally found in the lysosomal compartment of macrophages (e.g. *Ctss*, *Psap*, *Mmp12*, *Asahl*) or cell surface proteins involved in the innate immune response (e.g. *Clecsf12*, *Clecsf10*, *Trem2*, *Ccr2*, *Itgax*). Of

particular interest are known key regulators of macrophage differentiation such as CSF1 receptor and ligand, PKCδ and the transcription factors *Egr1*, *Fos* and *C/EBPβ* all of which displayed large increases in expression selectively in the Y577F mutant over the 72 hour time course (Fig. 1C-D). IPA canonical pathway analysis identified pathways associated with rheumatoid arthritis, a disease with known GM-CSF induced macrophage involvement (Hamilton, 2008) (Fig. 1D). In addition, the macrophage associated gene list also contained genes significantly associated with pathways of PPAR, IL-6, p38MAPK, GM-CSF and IL-3 signaling, that were not identified with the neutrophil associated gene list (Fig. 1B). Thus gene expression analysis using this FDB1 differentiation model is consistent with signaling from Tyr577 promoting a granulocyte differentiation program while suppressing key inducers of macrophage differentiation.

Tcf4 is a repressed target of Tyr577 signaling and a promoter of macrophage differentiation

Given that key regulators of macrophage differentiation are significantly up-regulated over time in cells expressing the FIΔY577 mutant we next used this characteristic pattern to identify additional potential regulators of macrophage differentiation. By microarray the transcription factor *Tcf4* showed significantly enhanced up-regulation in cells expressing the Y577F second-site mutant of FIΔ (FDR $p=1.8x10^{-11}$, Fig. 1C). A comparison of the change in expression for *Tcf4* and other macrophage lineage transcription factors (TFs) *(Egr1*, *Fos* and *C/EBPβ)* in FIΔ and FIΔY577F expressing cells was determined by quantitative RT-PCR (Q-PCR) and is shown in Fig. 2A. The expression changes of *Tcf4*, *Egr1*, *Fos* and *Cebpb* were consistent with the results obtained by microarray (Fig. 2A). Expression of these genes also increases during GM-CSF-directed bi-lineage terminal differentiation of FDB1 cells (which

proceeds more slowly than FIΔ-induced GM differentiation) over 120 hours (Fig. 2B). By QPCR *Tcf4* mRNA expression increased from 1.8 fold at 72 hours in FIΔ cells to 7 fold in FIΔY577F cells and by 3.8 fold in GM-CSF-treated parental FDB1 cells at 72 hours (Fig. 2 A,B). This is consistent with *Tcf4* being a repressed target of the Y577 signaling pathway with a potential role in promoting macrophage differentiation and/or suppressing granulocyte differentiation. *Tcf4* is itself a target gene activated by the Tcf4/β-catenin complex (Hatzis et al., 2008) and up-regulation of *Tcf4* expression is likely to be an indication of Wnt pathway activation. Further support for this was provided from IPA pathway analysis which identified an association between the genes associated with Tyr577 signaling and the Wnt/β-catenin pathway, in particular up-regulation of *Tcf4*, as well as down-regulation of the Wnt antagonist *Dkkl1* (Fig. 1D).

Tcf4 is a DNA-binding cofactor of β-catenin and a role for *Tcf4* has not previously been reported in promoting myeloid differentiation. To further investigate the possibility that β-catenin/TCF4 is involved in promoting macrophage differentiation in FDB1 cells we ectopically expressed full length *Tcf4* and *Tcf4* lacking the amino terminal β-catenin interaction domain (ΔN-*Tcf4*; Fig. 2C) using the MSCV-IRES-GFP (MIG) vector (Fig. 2D-F). FDB1 cells were transduced with MIG-*Tcf4*, MIG-ΔN-*Tcf4* or MIG vector control and infected cells (GFP+) enriched by flow cytometry. After sorting, cells were seeded into medium containing IL-3 (for growth) or GM-CSF (GM differentiation) and growth, viability and differentiation were measured over a 5 day period. Expression of *Tcf4* resulted in significantly reduced growth over 5 days in both IL-3 and GM-CSF (Fig. 2D) which was also associated with significantly reduced viability (as measured by trypan blue exclusion) at day 5 in IL-3, but not GM-CSF (data not shown). Interestingly, while *ΔN-Tcf4* was also able to affect the growth of cells in IL-3, there was no effect on cell expansion in response to GM-

CSF (Fig. 2D) indicating that the latter effect is dependent on the β-catenin interaction domain. The *Tcf4* and *ΔN-Tcf4* growth suppression in IL-3 was associated with a significantly decreased number of blasts and an increase in the number of granulocytes, although the majority of cells in IL-3 in all cases were still of undifferentiated morphology (Fig. 2E). Importantly, under conditions of GM-CSF-induced differentiation, we found that ectopic *Tcf4* expression significantly increased the number of cells differentiating to macrophages, a response that was not associated with expression of the *ΔN-Tcf4* mutant (Fig. 2E). This enhanced macrophage differentiation was also associated with increased cell surface expression of the macrophage marker, c-fms (Fig. 2F). These results show that *Tcf4* has the capacity, most likely via an interaction with β-catenin, to promote differentiation of the macrophage lineage.

GM-CSF receptor activation induces β-catenin stabilization that is negatively regulated by the Tyr577 pathway

We next determined the levels of β-catenin protein present in FDB1 cells expressing the FIΔ mutant or the Y577F derivative, or responding to GM-CSF over 72 hours. In cells responding to GM-CSF or expressing FIΔ we observed accumulation of β-catenin at 24 hours following IL-3 withdrawal, which was absent at 72 hours (Fig. 3A). In contrast, in cells expressing FIΔY577F, which differentiate predominantly to macrophages, β-catenin was constitutively present (Fig. 3A). This is consistent with mutation of Tyr577 disrupting a pathway that normally regulates the stability of β-catenin. To further examine the pathway regulating β-catenin stabilization we treated FDB1 cells with the Glycogen synthase kinase 3β (GSK3β) inhibitor BIO. GSK3β is a well-recognised negative regulator of β-catenin stability and one of the characterised effects of inhibition of GSK3β activity is constitutive β-catenin protein

stabilisation (Liu et al., 2002; Holmes et al., 2008). Parental FDB1 cells were treated with 2 μM BIO or the control N-methylated analogue (Me-BIO). As predicted, inhibition of GSK3β with BIO led to increased β-catenin protein levels measured at 16 hours (Fig. 3B). BIO treatment did not significantly affect viability of the cells, as measured by trypan blue exclusion (data not shown) but resulted in a significant suppression of growth in response to both IL-3 and GM-CSF (Fig. 3C). In response to GM-CSF, there was a significant increase in the number of macrophages with concomitant decreases in both blasts and granulocytes (Fig. 3D). This was also associated with a clear increase in the number of cells expressing c-fms (Fig. 3E). We also observed increased levels of macrophages in response to IL-3 in the presence of BIO although this increase was not significant.

β-catenin stabilization is sufficient to specify macrophage differentiation in FDB1 cells

We next tested the ability of β-catenin to directly mediate macrophage differentiation by ectopically expressing a stabilized form of β-catenin. For this we used the MIG vector to express an engineered version of β-catenin containing point mutations at the GSK3β and casein kinase phosphorylation sites (Ser33Ala, Ser37Ala, Thr41Ala, Ser45Ala) (Liu et al., 2002; Rottbauer et al., 2002). Expression of this β-catenin mutant (herein referred to as βcatS33A) has previously been shown to render the β-catenin protein resistant to degradation and consequently mimic a constitutive Wnt signal (Aoki et al., 2002; Rottbauer et al., 2002). FDB1 cells were transduced with MIG-βcatS33A or MIG vector control and infected cells (GFP+) enriched by flow cytometry. After sorting, cells were seeded into medium containing IL-3 or GM-CSF and growth, viability and differentiation were measured over a 5 day period. Western immunoblot analysis at day 5 confirmed the increase of β-catenin protein in cells transduced with MIG-βcatS33A, in both IL-3 and GM-CSF (Fig 4A). Over-expression of βcatS33A did not affect growth of FDB1 cells in IL-3, however in the presence of GM-CSF

we observed significantly enhanced growth of the MIG-βcatS33A FDB1 cell population (Fig. 4B). There was no difference in the viability of the cell populations (as measured by trypan blue exclusion) over the 5 day period (data not shown). Importantly, after 5 days in GM-CSF, differentiation of the MIG-βcatS33A cell population was associated with a significantly increased proportion of macrophages ($p=0.027$) compared to vector-control cells (Fig. 4C-D). While having a significant effect on differentiation in GM-CSF, βcatS33A was not sufficient to induce differentiation to macrophages in the presence of IL-3 (Fig. 4C-D). Taken together with the BIO-induction of differentiation this demonstrates that, in the context of GM-CSF signaling, stabilization of β-catenin promotes specification of macrophage differentiation in this FDB1 model system.

Tcf4/β-catenin target genes are enriched in Tyr577-regulated genes

To further investigate the link between Tyr577 signaling, Tcf4/β-catenin activity and macrophage differentiation we investigated whether other known β-catenin/Tcf4 target genes, are regulated in response to Tyr577 signaling. For this we used gene-set enrichment analysis to compare the full set of Y577-differentially regulated genes with a combined gene-set of direct TCF4 and β-catenin target genes (347 genes) derived from two chromatin immunoprecipitation (ChIP) studies in colorectal cancer cell lines (Hatzis et al., 2008; Yochum et al., 2007). This analysis identified a significant association between the 347 β-catenin/Tcf4 target genes and the genes significantly differentially regulated over time by FIΔY577F ($p=1.7x10^{-11}$, Wilcoxon rank-sum test, supplemental Fig. 3). The full list of significantly differentially expressed β-catenin/TCF4 targets (113 genes) is shown in supplemental Table 2. This analysis showed a clear association of the Tyr577-regulated genes with β-catenin and Tcf4 direct target genes. *Egr1* is the β-catenin/Tcf4 direct-target gene with

the largest-fold differential expression in FIΔY577F expressing cells over time suggesting a key macrophage promoting role for this lineage-specific TF downstream of the Tyr577 signaling pathway. Differential regulation of *Egr1* in the FIΔY577F mutant was also validated by QPCR (Fig. 2A). As shown in Fig. 5 *Egr1*, although not specified by IPA as a Wnt/β-catenin signaling-related gene, an identified ChIP target of the β-catenin/TCF4 complex (Yochum et al., 2007) and is independently connected to multiple β-catenin/Tcf4 targets, consistent with a model of macrophage differentiation that involves Wnt/β-catenin modulation of *Egr1* activity (Fig. 6).

Discussion

While genetic and functional studies provide clear evidence for the importance of GM-CSF signaling in macrophage differentiation, the pathways that contribute to this process are still incompletely defined. Here we define a novel signaling pathway from the GM-CSF receptor that we propose contributes to the lineage fate of bi-potential GM progenitors. The key intracellular Tyr577 residue in the common β subunit of the GM-CSF receptor has been shown previously to be a determinant of the extent of granulocyte and macrophage differentiation in the FDB1 cell line model (Brown et al., 2004). An *in vivo* mutant model of Tyr577 signaling also showed expansion of progenitor cells in response to GM-CSF consistent with a defect in GM progenitor differentiation (Ramshaw et al., 2007). Our analysis comparing gene expression in the activated (FIΔ) mutant of GMR, that mediates factor-independent macrophage and granulocyte differentiation, with that of the second-site mutant FIΔY577F, is consistent with the observed reduction in morphological granulocyte differentiation upon mutation of Tyr577 (Brown et al., 2004). Here we focused on *Tcf4* which displayed similar expression to other regulators of macrophage differentiation in the FDB1 system (Fig. 2). *Tcf4* is a member of the Tcf/Lef family that combines with β-catenin to form

transcriptional complexes that regulate target gene transcription. The Tyr577 mutation results in increased *Tcf4* expression and is associated with reduced granulocyte differentiation and enhanced differentiation to the macrophage lineage. Our data are most consistent with *Tcf4* functioning as a promoter of differentiation and tumour suppressor in the GM lineage. Forced expression of *Tcf4* in FDB1 cells responding to GM-CSF demonstrated a functional role in promoting macrophage differentiation. This effect was dependent on the β-catenin interaction domain, consistent with the known interaction and cooperation between Tcf4 and β-catenin.

Activation of *Tcf4* transcription downstream of GMR is most likely a result of β-catenin accumulation as *Tcf4* is itself a target gene of the Tcf4/β-catenin transcriptional complex (Hatzis et al., 2008). We found that β-catenin accumulated in the presence of the FIΔY577F mutant. We also observed transient accumulation of β-catenin at the 24 hour timepoint following GM-CSF stimulation of FDB1 cells (Fig. 3A) and expression of a stabilised β-catenin or inhibition of GSK3β with pharmacological inhibitors mimicked *Tcf4* over-expression and increased the number of cells differentiating to macrophages. Thus three independent approaches demonstrate that β-catenin/Tcf4 activity promotes macrophage differentiation in the FDB1 system. The ability of BIO to stabilize β-catenin downstream of GM-CSF signalling suggests that, as in other systems, GSK3β is key to regulating β-catenin protein levels and therefore activation of this pathway in response to GM-CSF. Modification of GSK3β signalling by the GMR has not been previously investigated and although not directly shown here, the accumulation β-catenin in cells expressing the FIΔY577F mutant suggests that this residue may normally have a role in regulating GSK3β activity in response to GM-CSF. Previous studies have linked Tyr577 to binding of Shc and activation and regulation of Gab2, Akt/PI3K and SHIP related pathways (Gu et al., 2000; Dijkers et al.,

1999; Ramshaw et al., 2007). Interestingly, mutation of this residue in different contexts has been associated with both reduced and enhanced activation of Akt (Dijkers et al., 1999; Ramshaw et al., 2007). Preliminary analysis of signalling from FIΔ and FIΔY577F in FDB1 cells suggests alterations in activation of both Akt and p38MAPK (data not shown) both of which are known regulators of the GSK3β/β-catenin axis (Liu et al., 2002; Cross et al., 1995; Thornton et al., 2008; Bikkavilli et al., 2008). Further mapping of this pathway downstream of the GM-CSF receptor in this cell line model will reveal the signalling events important for macrophage differentiation and modulation of β-catenin activity, and shed light on the interplay between growth factor receptor signalling and transcriptional regulation in myeloid differentiation.

While a role for β-catenin in stem cell function and progenitor commitment has been reported previously (Zhao et al., 2007), our observation of a differentiation-promoting and lineage modulation capacity represents a novel function for Tcf4/β-catenin in the hematopoietic system. Activation of β-catenin in response to M-CSF signaling supports the growth and survival of macrophages, consistent with our findings, although lineage specification was not examined in this context (Otero et al., 2009). Interestingly we also note that conditional activation of β-catenin in the hematopoietic system acts to reduce the number of GM progenitors, and colonies formed in response to GM-CSF overall, and blocks granulocyte colony formation completely, while maintaining permissive conditions for some macrophage colony formation (Scheller et al., 2006). These data support our hypothesis that β-catenin may have independent roles at different stages of haemopoiesis, including a role in committed progenitors for determining macrophage versus granulocyte differentiation decisions. To shed light on the possible mechanism of β-catenin/Tcf4 mediated macrophage differentiation we analysed the Tyr577-regulated genes and showed a significant enrichment

of β-catenin/TCF4 target genes. Of particular note was the known transcriptional mediator of macrophage differentiation, *Egr1* which displayed an 8-fold increase in expression over 72 hours during FIΔY577F-induced macrophage differentiation and was the most significant differentially regulated macrophage-associated gene. In primary myeloid progenitors *Egr1* is controlled by the macrophage master regulator *PU.1* and induction of *Egr1* promotes the transcription of macrophage genes, while repressing the alternative granulocyte lineage associated genes (Laslo et al., 2006). This provides a likely mechanism by which β-catenin/Tcf4 could influence macrophage differentiation. Recent reports have shown that Egr1 directly regulates *Tcf4* in endometrial carcinoma cells and link *Egr1* expression to progression of colorectal cancer, a process which also involves β-catenin/Tcf4 (Saegusa et al., 2008; Ernst et al.), suggesting the possibility of a positive feedback loop. Interestingly like *β-catenin*, *Egr1* is also important for hematopoietic stem cell homeostasis (Min et al., 2008; Zhao et al., 2007) suggesting that this regulatory loop may operate with different outputs at the level of the HSC and the committed myeloid progenitor. Further dissection of these roles will require stage or lineage specific deletion approaches to separate stem cell effects from those important for later cell fate decisions and will shed light on their role in GM lineage choice.

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Author Contributions

A.L.B. and R.J.D. wrote the manuscript. A.L.B designed the experiments, performed the experiments and analyzed the data; D.G.S., T.S. and M.P. performed experiments and analysed the data; C.W. and C.H.K. analysed microarray data; S.S. and T.S. provided technical assistance and advice; I.L. and T.J.G were involved in data interpretation and critical review of the manuscript.

Conflicts of Interest

The authors declare no competing financial interests.

References

Aoki, M., Sobek, V., Maslyar, D.J., Hecht, A., and Vogt, P.K., 2002. Oncogenic transformation by beta-catenin: deletion analysis and characterization of selected target genes. Oncogene 21, 6983-6991.

Bikkavilli, R.K., Feigin, M.E., and Malbon, C.C., 2008. p38 mitogen-activated protein kinase regulates canonical Wnt-beta-catenin signaling by inactivation of GSK3beta. J. Cell Sci. 121, 3598-3607.

Brown, A.L., Peters, M., D'Andrea, R.J., and Gonda, T.J., 2004. Constitutive mutants of the GM-CSF receptor reveal multiple pathways leading to myeloid cell survival, proliferation, and granulocyte-macrophage differentiation. Blood 103, 507-516.

Brown, A.L., Wilkinson, C.R., Waterman, S.R., Kok, C.H., Salerno, D.G., Diakiw, S.M., Reynolds, B., Scott, H.S., Tsykin, A., Glonek, G.F., Goodall, G.J., Solomon, P.J., Gonda, T.J., and D'Andrea, R.J., 2006. Genetic regulators of myelopoiesis and leukemic signaling identified by gene profiling and linear modeling. J.Leukoc.Biol. 80, 433-447.

Conti, L., and Gessani, S., 2008. GM-CSF in the generation of dendritic cells from human blood monocyte precursors: recent advances. Immunobiology 213, 859-870.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785-789.

D'Andrea, R.J., Harrison-Findik, D., Butcher, C.M., Finnie, J., Blumbergs, P., Bartley, P., McCormack, M., Jones, K., Rowland, R., Gonda, T.J., and Vadas, M.A., 1998. Dysregulated hematopoiesis and a progressive neurological disorder induced by expression of an activated form of the human common beta chain in transgenic mice. J.Clin.Invest 102, 1951-1960.

Dijkers, P.F., van Dijk, T.B., de Groot, R.P., Raaijmakers, J.A., Lammers, J.W., Koenderman, L., and Coffer, P.J., 1999. Regulation and function of protein kinase B and MAP kinase activation by the IL-5/GM-CSF/IL-3 receptor. Oncogene 18, 3334-3342.

Dranoff, G., Crawford, A.D., Sadelain, M., Ream, B., Rashid, A., Bronson, R.T., Dickersin, G.R., Bachurski, C.J., Mark, E.L., and Whitsett, J.A., 1994. Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. Science 264, 713-716.

Enzler, T., Gillessen, S., Manis, J.P., Ferguson, D., Fleming, J., Alt, F.W., Mihm, M., and Dranoff, G., 2003. Deficiencies of GM-CSF and interferon gamma link inflammation and cancer. J. Exp. Med. 197, 1213-1219.

Ernst, A., Aigner, M., Nakata, S., Engel, F., Schlotter, M., Kloor, M., Brand, K., Schmitt, S., Steinert, G., Rahbari, N., Koch, M., Radlwimmer, B., Weitz, J., and Lichter, P., 2011. A gene signature distinguishing CD133hi from CD133- colorectal cancer cells: essential role for EGR1 and downstream factors. Pathology (Phila). 43, 220-227.

Gery, S., Gombart, A.F., Yi, W.S., Koeffler, C., Hofmann, W.K., and Koeffler, H.P., 2005. Transcription profiling of C/EBP targets identifies Per2 as a gene implicated in myeloid leukemia. Blood 106, 2827-2836.

Gu, H., Maeda, H., Moon, J.J., Lord, J.D., Yoakim, M., Nelson, B.H., and Neel, B.G., 2000. New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. Mol. Cell. Biol. 20, 7109-7120.

Guthridge, M.A., Powell, J.A., Barry, E.F., Stomski, F.C., McClure, B.J., Ramshaw, H., Felquer, F.A., Dottore, M., Thomas, D.T., To, B., Begley, C.G., and Lopez, A.F., 2006. Growth factor pleiotropy is controlled by a receptor Tyr/Ser motif that acts as a binary switch. EMBO J. 25, 479-489.

Hamilton, J.A., 2008. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol 8, 533-544.

Hansen, G., Hercus, T.R., McClure, B.J., Stomski, F.C., Dottore, M., Powell, J., Ramshaw, H., Woodcock, J.M., Xu, Y., Guthridge, M., McKinstry, W.J., Lopez, A.F., and Parker, M.W., 2008. The structure of the GM-CSF receptor complex reveals a distinct mode of cytokine receptor activation. Cell 134, 496-507.

Hatzis, P., van der Flier, L.G., van Driel, M.A., Guryev, V., Nielsen, F., Denissov, S., Nijman, I.J., Koster, J., Santo, E.E., Welboren, W., Versteeg, R., Cuppen, E., van de Wetering, M., Clevers, H., and Stunnenberg, H.G., 2008. Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. Mol. Cell. Biol. 28, 2732-2744.

Holmes, T., O'Brien, T.A., Knight, R., Lindeman, R., Symonds, G., and Dolnikov, A., 2008. The role of glycogen synthase kinase-3beta in normal haematopoiesis, angiogenesis and leukaemia. Curr. Med. Chem. 15, 1493-1499.

Jenkins, B.J., Blake, T.J., and Gonda, T.J., 1998. Saturation mutagenesis of the beta subunit of the human granulocyte-macrophage colony-stimulating factor receptor shows clustering of constitutive mutations, activation of ERK MAP kinase and STAT pathways, and differential beta subunit tyrosine phosphorylation. Blood 92, 1989-2002.

Jenkins, B.J., D'Andrea, R., and Gonda, T.J., 1995. Activating point mutations in the common beta subunit of the human GM-CSF, IL-3 and IL-5 receptors suggest the involvement of beta subunit dimerization and cell type-specific molecules in signalling. EMBO J. 14, 4276-4287.

Kondo, M., Scherer, D.C., Miyamoto, T., King, A.G., Akashi, K., Sugamura, K., and Weissman, I.L., 2000. Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. Nature 407, 383-386.

Laslo, P., Spooner, C.J., Warmflash, A., Lancki, D.W., Lee, H.J., Sciammas, R., Gantner, B.N., Dinner, A.R., and Singh, H., 2006. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. Cell 126, 755-766.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X., and He, X., 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108, 837-847.

Lopez, A.F., Hercus, T.R., Ekert, P., Littler, D.R., Guthridge, M., Thomas, D., Ramshaw, H.S., Stomski, F., Perugini, M., D'Andrea, R., Grimbaldeston, M., and Parker, M.W., 2010. Molecular basis of cytokine receptor activation. IUBMB Life 62, 509-518.

Martinez-Moczygemba, M., Doan, M.L., Elidemir, O., Fan, L.L., Cheung, S.W., Lei, J.T., Moore, J.P., Tavana, G., Lewis, L.R., Zhu, Y., Muzny, D.M., Gibbs, R.A., and Huston, D.P., 2008. Pulmonary alveolar proteinosis caused by deletion of the GM-CSFRalpha gene in the X chromosome pseudoautosomal region 1. J. Exp. Med. 205, 2711-2716.

McClure, B.J., Hercus, T.R., Cambareri, B.A., Woodcock, J.M., Bagley, C.J., Howlett, G.J., and Lopez, A.F., 2003. Molecular assembly of the ternary granulocyte-macrophage colony- stimulating factor receptor complex. Blood 101, 1308-1315.

McClure, B.J., Woodcock, J.M., Harrison-Findik, D., Lopez, A.F., and D'Andrea, R.J., 2001. GM-CSF binding to its receptor induces oligomerisation of the common beta-subunit. Cytokine 13, 240-243.

McCormack, M.P., and Gonda, T.J., 1999. Myeloproliferative disorder and leukaemia induced in mice by different classes of constitutive mutants of the human IL-3/IL-5/GM-CSF receptor common beta subunit. Oncogene 18, 7190-7199.

McCormack, M.P., and Gonda, T.J., 2000. Novel murine myeloid cell lines which exhibit a differentiation switch in response to IL-3 or GM-CSF, or to different constitutively active mutants of the GM-CSF receptor beta subunit. Blood 95, 120-127.

Metcalf, D., Begley, C.G., Williamson, D.J., Nice, E.C., De Lamarter, J., Mermod, J.J., Thatcher, D., and Schmidt, A., 1987. Hemopoietic responses in mice injected with purified recombinant murine GM-CSF. Exp.Hematol. 15, 1-10.

Min, I.M., Pietramaggiori, G., Kim, F.S., Passegue, E., Stevenson, K.E., and Wagers, A.J., 2008. The transcription factor EGR1 controls both the proliferation and localization of hematopoietic stem cells. Cell Stem Cell 2, 380-391.

Miyajima, A., Mui, A.L., Ogorochi, T., and Sakamaki, K., 1993. Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5. Blood 82, 1960-1974.

Nishinakamura, R., Nakayama, N., Hirabayashi, Y., Inoue, T., Aud, D., McNeil, T., Azuma, S., Yoshida, S., Toyoda, Y., Arai, K., and et al., 1995. Mice deficient for the IL-3/GM-CSF/IL-5 beta c receptor exhibit lung pathology and impaired immune response, while beta IL3 receptor-deficient mice are normal. Immunity 2, 211-222.

Okuda, K., Smith, L., Griffin, J.D., and Foster, R., 1997. Signaling functions of the tyrosine residues in the beta chain of the granulocyte-macrophage colony-stimulating factor receptor. Blood 90, 4759-4766.

Otero, K., Turnbull, I.R., Poliani, P.L., Vermi, W., Cerutti, E., Aoshi, T., Tassi, I., Takai, T., Stanley, S.L., Miller, M., Shaw, A.S., and Colonna, M., 2009. Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and beta-catenin. Nat Immunol 10, 734-743.

Paine, R., III, Preston, A.M., Wilcoxen, S., Jin, H., Siu, B.B., Morris, S.B., Reed, J.A., Ross, G., Whitsett, J.A., and Beck, J.M., 2000. Granulocyte-macrophage colony-stimulating factor in the innate immune response to Pneumocystis carinii pneumonia in mice. J. Immunol. 164, 2602-2609.

Perugini, M., Brown, A.L., Salerno, D.G., Booker, G.W., Stojkoski, C., Hercus, T.R., Lopez, A.F., Hibbs, M.L., Gonda, T.J., and D'Andrea, R.J., Alternative modes of GM-CSF receptor activation revealed using activated mutants of the common beta-subunit. Blood 115, 3346-3353.

Perugini, M., Kok, C.H., Brown, A.L., Wilkinson, C.R., Salerno, D.G., Young, S.M., Diakiw, S.M., Lewis, I.D., Gonda, T.J., and D'Andrea, R.J., 2009. Repression of Gadd45alpha by activated FLT3 and GM-CSF receptor mutants contributes to growth, survival and blocked differentiation. Leukemia.

Powell, J.A., Thomas, D., Barry, E.F., Kok, C.H., McClure, B.J., Tsykin, A., To, L.B., Brown, A., Lewis, I.D., Herbert, K., Goodall, G.J., Speed, T.P., Asou, N., Jacob, B., Osato, M., Haylock, D.N., Nilsson, S.K., D'Andrea, R.J., Lopez, A.F., and Guthridge, M.A., 2009. Expression profiling of a hemopoietic cell survival transcriptome implicates osteopontin as a functional prognostic factor in AML. Blood 114, 4859-4870.

Ramshaw, H.S., Guthridge, M.A., Stomski, F.C., Barry, E.F., Ooms, L., Mitchell, C.A., Begley, C.G., and Lopez, A.F., 2007. The Shc-binding site of the betac subunit of the GM-CSF/IL-3/IL-5 receptors is a negative regulator of hematopoiesis. Blood 110, 3582-3590.

Rottbauer, W., Saurin, A.J., Lickert, H., Shen, X., Burns, C.G., Wo, Z.G., Kemler, R., Kingston, R., Wu, C., and Fishman, M., 2002. Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. Cell 111, 661-672.

Saegusa, M., Hashimura, M., Kuwata, T., Hamano, M., Watanabe, J., Kawaguchi, M., and Okayasu, I., 2008. Transcription factor Egr1 acts as an upstream regulator of beta-catenin signalling through up-regulation of TCF4 and p300 expression during trans-differentiation of endometrial carcinoma cells. J. Pathol. 216, 521-532.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M.M., Birchmeier, W., Tenen, D.G., and Leutz, A., 2006. Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. Nat Immunol 7, 1037-1047.

Smith, A., Metcalf, D., and Nicola, N.A., 1997. Cytoplasmic domains of the common á-chain of the GM-CSF/IL-3/IL-5 receptors that are required for inducing differentiation or clonal suppression in myeloid leukaemic cell lines. EMBO J. 16, 451-464.

Suzuki, T., Sakagami, T., Rubin, B.K., Nogee, L.M., Wood, R.E., Zimmerman, S.L., Smolarek, T., Dishop, M.K., Wert, S.E., Whitsett, J.A., Grabowski, G., Carey, B.C.,

Stevens, C., van der Loo, J.C., and Trapnell, B.C., 2008. Familial pulmonary alveolar proteinosis caused by mutations in CSF2RA. J. Exp. Med. 205, 2703-2710.

Thornton, T.M., Pedraza-Alva, G., Deng, B., Wood, C.D., Aronshtam, A., Clements, J.L., Sabio, G., Davis, R.J., Matthews, D.E., Doble, B., and Rincon, M., 2008. Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. Science 320, 667-670.

van de, W.M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der, H.K., Batlle, E., Coudreuse, D., Haramis, A.P., Tjon-Pon-Fong, M., Moerer, P., van den, B.M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H., 2002. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell. 111, 241-250.

Yochum, G.S., McWeeney, S., Rajaraman, V., Cleland, R., Peters, S., and Goodman, R.H., 2007. Serial analysis of chromatin occupancy identifies beta-catenin target genes in colorectal carcinoma cells. Proc. Natl. Acad. Sci. U. S. A. 104, 3324-3329.

Zhao, C., Blum, J., Chen, A., Kwon, H.Y., Jung, S.H., Cook, J.M., Lagoo, A., and Reya, T., 2007. Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 12, 528-541.

Figure Legends

Fig. 1. Y577 regulated genes

A. Heatmap showing the expression of the top 25 up and down regulated genes associated FIΔ induced factor independent granulocyte differentiation over 72 hours (see materials and methods for details of gene-set derivations). **B**. IPA defined canonical pathways significantly associated with the neutrophil gene set Lysine= lysine degradation, LES= leukocyte extravasation signaling, CES= caveolar-mediated endocytosis signaling. **C.** Heatmap showing the expression of the top 25 up and down regulated genes associated with FIΔ-Y577F induced factor independent macrophage differentiation over 72 hours. **D**. IPA defined canonical pathways associated with the macrophage gene set, AP= acute phase response signaling, RA= rheumatoid arthritis. The M value (log2 fold change) for both FIΔ or FIΔY577F expressing cells was converted to a colour scale with red indicating that gene expression increases between 0 and 72 hours and green indicating that the gene expression decreases with the colour intensity indicating the magnitude of regulation (range log2, -3 to +3). Black boxes indicate individual genes involved in the indicated pathway. Tcf7l2=Tcf4.

Fig. 2. *Tcf4* is regulated by GM-CSF and promotes macrophage differentiation

Quantitative real-time PCR was used to determine the expression of the indicated genes. Expression was normalised to β-actin and expression is shown relative to 0 hours. Results shown are the average of two experiments **A.** FDB1 cells expressing FIΔ and FIΔY577F were cultured without factor and RNA extracted at 0, 24 and 72 hours. **B.** Parental FDB1 cells were culture in GM-CSF and RNA extracted at 0, 24, 72 and 120 hours. **C**. Full length TCF4 and the engineered form lacking the N-terminal β-catenin interaction domain are depicted. The β-catenin interaction domain is indicated by a black box and the DNA binding domain is indicated by a grey box. **D**. FDB1 parental cells were transduced with a MSCV-IRES-GFP

retrovirus encoding TCF4, ΔN-TCF4 or a control retrovirus and selected for GFP expression by FACS. Cells were washed and placed in the indicated growth conditions. Expansion of GFP positive cells was determined by assessing viable cell number using trypan blue exclusion and flow cytometry to determine the proportion of GFP positive cells at the indicated time points. **E.** At day 5, cells were cytocentrifuged, Wright-Giemsa stained and 200 cells for each condition were scored microscopically for morphology. **F**. At day 5 cells were stained with PE-conjugated anti-c-fms and the percentage of c-fms positive cells in the GFP positive population was determined for each cell line. A representative flow histogram is shown. Error bars represent SEM (n=4) and *=p<0.05, **=<0.01, ***=p<0.001 (unpaired, two sided t-test).

Fig. 3. β-catenin stabilization is associated with increased macrophage differentiation

A. FDB1 cells expressing FIΔ and FIΔY577F were cultured without factor and parental cells in GM-CSF and cell lysates were made at 0, 24 and 72 hours. Lysates were western blotted with the indicated antibodies. Representative photo-micrographs were taken at the 72 hour timepoint for FDB1 FIΔ and FIΔY577F cells and at 120 hours for parental cells in GM-CSF. **B**. FDB1 cells growing in response to IL-3 or undergoing GM differentiation in response to GM-CSF were incubated with 2 μM of the GSK3β inhibitor BIO ((2'Z,3'E)-6-Bromoindirubin-3'-oxime) or N-methylated control analogue (Me-BIO). Activity of BIO at 16 hours was assessed by detection of β-catenin protein by western analysis. **C**. Cell growth was determined at days 2 and 5 of GSK3β inhibition by trypan blue exclusion. **D**. At day 5 cells were cytocentrifuged, Wright-Giemsa stained and 200 cells for each condition were scored microscopically for morphology. **E**. At day 5 cells were stained with PE-conjugated anti-c-fms and the percentage of c-fms positive cells was determined for each cell population.

Error bars represent SEM (n=3) and *=p<0.05, **=<0.01, ***=p<0.001 (unpaired, two sided t-test).

Fig. 4. **β-catenin directs macrophage differentiation in response to GM-CSF receptor signaling**

FDB1 cells were transduced with MIG encoding β-catenin with mutations S33A, S37A, T41A, S45A (βcatS33A) or vector alone. **A.** GFP positive cells were placed in IL-3 or GM-CSF and after 5 days were assessed for expression of stabilised β-catenin by western analysis. **B**. Growth was assessed over 4 days using AQueous one solution proliferation assay. **C**. At day 5 cells were cytocentrifuged, Wright-Giemsa stained and 200 cells for each condition were scored by morphology. Error bars represent SEM (n=2) and *=p<0.05 (unpaired, two sided t-test). **D**. Photomicrographs of cells at day 5.

Fig. 5. A TCF4/β-catenin target gene network in FIΔY577F regulated genes

A. The overlapping TCF4/β-catenin and macrophage genes with an FDR p<0.05 (113) were subjected to Ingenuity Pathway Analysis (IPA). The genes were subject to IPA mapping of network interactions and filtered for direct interactions and networks containing genes identified as involved in Wnt/β-catenin signaling. The colour indicates the direction of differential expression in our macrophage regulated gene list with red indicating that gene expression increases between 0 and 72 hours and green indicating that the gene expression decreases with the colour intensity indicating the magnitude of regulation. CTNNB1= β-catenin.

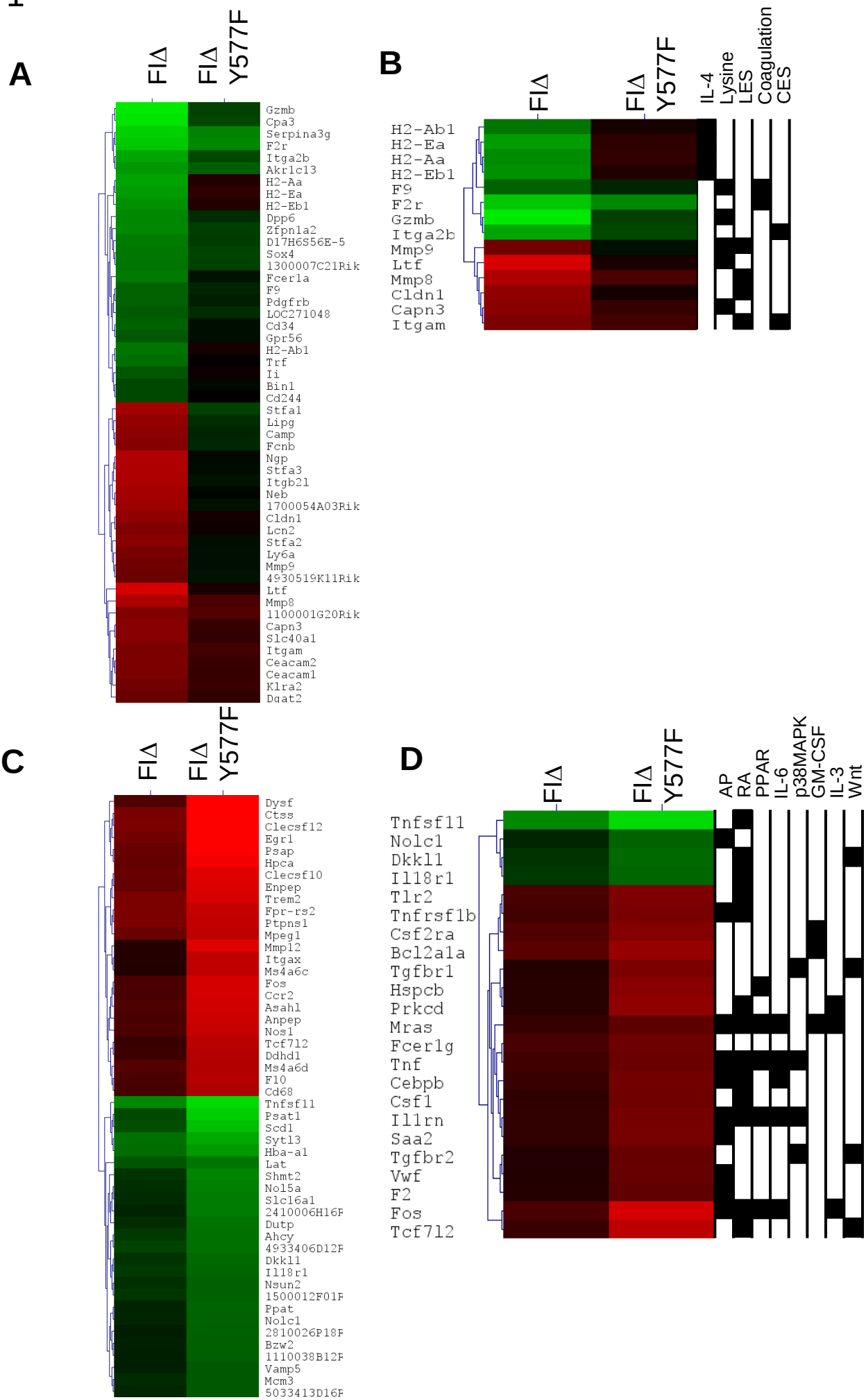
Fig. 6. Novel molecular interactions in monopoiesis regulated by GM-CSF signaling

Signaling emanating from Tyr577 of the GMR leads to stabilisation of the β-catenin protein

(possibly though GSK3β inactivation) which, in combination with Tcf4, leads to differentiation of the macrophage lineage at the expense of neutrophil lineage.

Fig1

Fig. 1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Dpp6
Zfpn1a2
D17H6S56E-5
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Fcer1a
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Cd34
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H2-Ab1
Trf
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Bin1
Cd244
Stfa1
Lipg
Camp
Fcnb
Ngp
Stfa3
Itgb21
Neb
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Cldn1
Lcn2
Stfa2
Ly6a
Mmp9
4930519K11Rik
Ltf
Mmp8
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Capn3
Slc40a1
Itgam
Ceacam2
Ceacam1
Klra2
Dgat2
B
FlΔ
FlΔ Y577F
IL-4
Lysine
LES
Coagulation
CES
H2-Ab1
H2-Ea
H2-Aa
H2-Eb1
F9
F2r
Gzmb
Itga2b
Mmp9
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Dysf
Ctss
Clecsf12
Egr1
Psap
Hpca
Clecsf10
Enpep
Trem2
Fpr-rs2
Ptpns1
Mpeg1
Mmp12
Itgax
Ms4a6c
Fos
Ccr2
Asah1
Anpep
Nos1
Tcf7l2
Ddhd1
Ms4a6d
F10
Cd68
Tnfsf11
Psat1
Scd1
Sytl3
Hba-a1
Lat
Shmt2
Nol5a
Slc16a1
2410006H16F
Dutp
Ahcy
4933406D12F
Dkkl1
Il18r1
Nsun2
1500012F01F
Ppat
Nolc1
2810026P18F
Bzw2
1110038B12F
Vamp5
Mcm3
5033413D16F
D
FlΔ
FlΔ Y577F
AP
RA
PPAR
IL-6
p38MAPK
GM-CSF
IL-3
Wnt
Tnfsf11
Nolc1
Dkkl1
Il18r1
Tlr2
Tnfrsf1b
Csf2ra
Bcl2a1a
Tgfbr1
Hspcb
Prkcd
Mras
Fcer1g
Tnf
Cebpb
Csf1
Il1rn
Saa2
Tgfbr2
Vwf
F2
Fos
Tcf7l2

Fig. 2

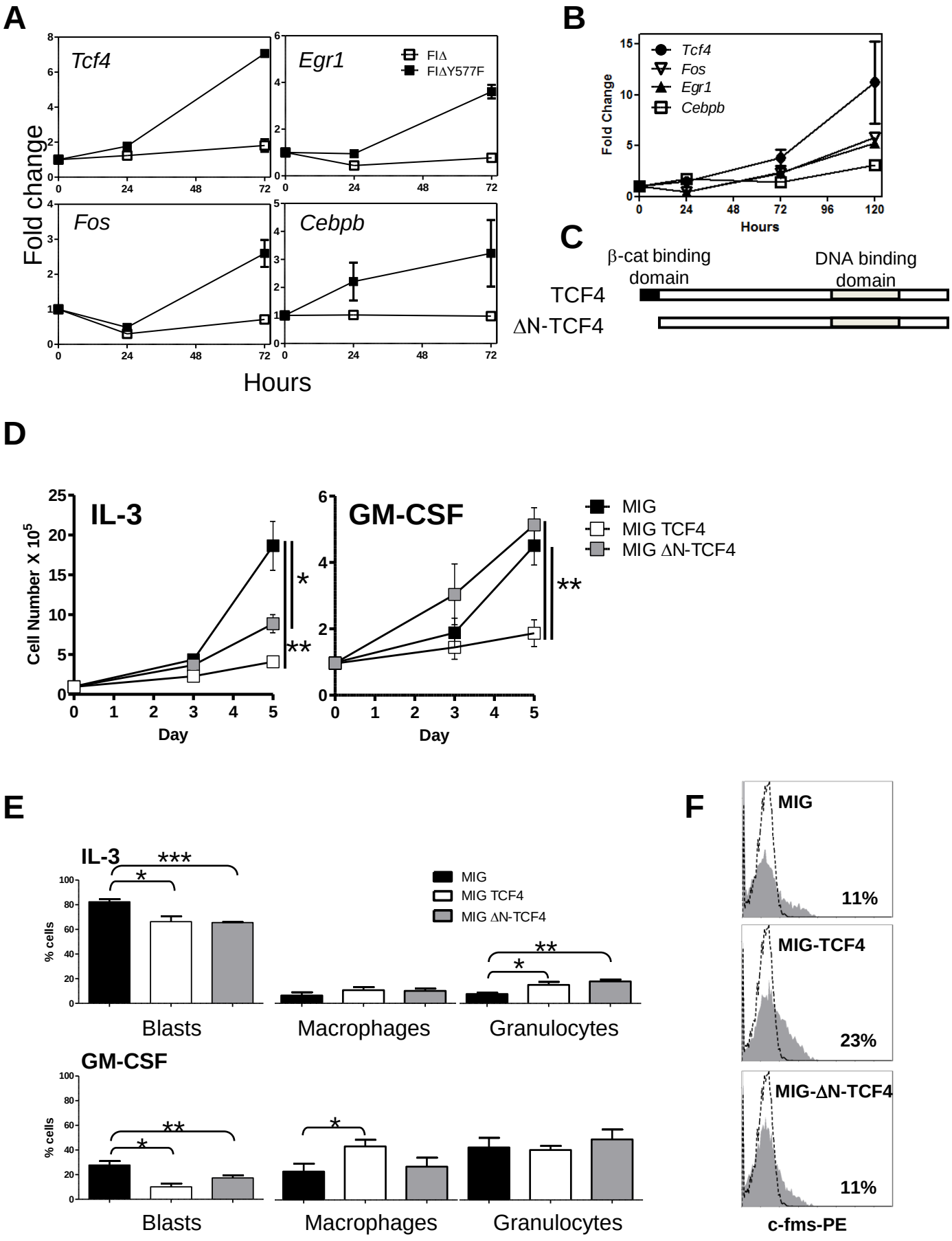

A
Tcf4
Egr1
Fos
Cebpb
FIΔ
FIΔY577F
Fold change
Hours
B
Tcf4
Fos
Egr1
Cebpb
Fold Change
Hours
C
β-cat binding domain
DNA binding domain
TCF4
ΔN-TCF4
D
IL-3
GM-CSF
MIG
MIG TCF4
MIG ΔN-TCF4
Cell Number X 10^5
Day
E
IL-3
GM-CSF
% cells
Blasts
Macrophages
Granulocytes
F
MIG
11%
MIG-TCF4
23%
MIG-ΔN-TCF4
11%
c-fms-PE

Fig. 3

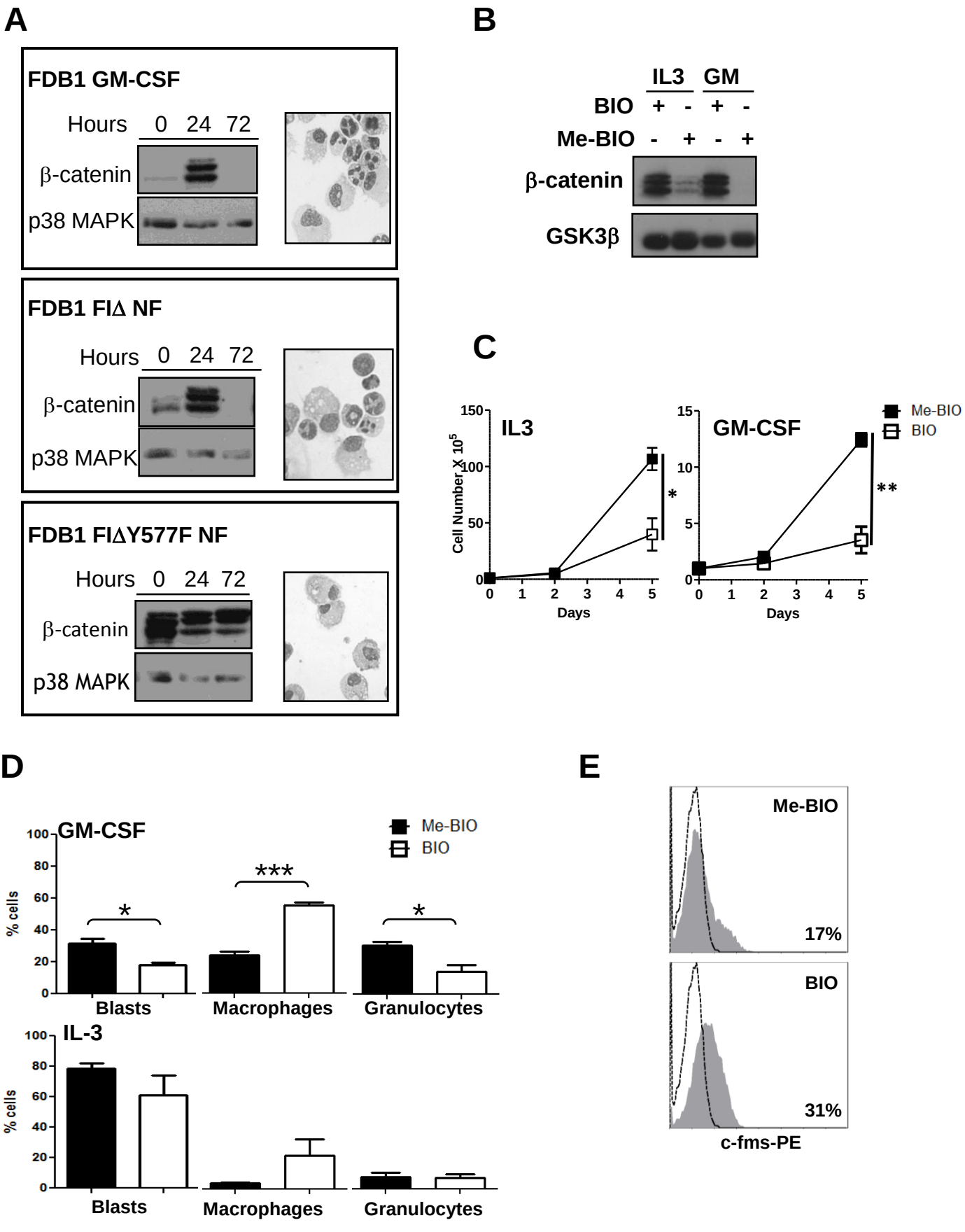

A
FDB1 GM-CSF
Hours 0 24 72
β-catenin
p38 MAPK
FDB1 FIΔ NF
Hours 0 24 72
β-catenin
p38 MAPK
FDB1 FIΔY577F NF
Hours 0 24 72
β-catenin
p38 MAPK
B
IL3 GM
BIO + - + -
Me-BIO - + - +
β-catenin
GSK3β
C
IL3
GM-CSF
Me-BIO
BIO
Cell Number X 10^5
Days
Days
*
**
D
GM-CSF
Me-BIO
BIO
% cells
*
***
*
Blasts
Macrophages
Granulocytes
IL-3
% cells
Blasts
Macrophages
Granulocytes
E
Me-BIO
17%
BIO
31%
c-fms-PE

Fig. 4

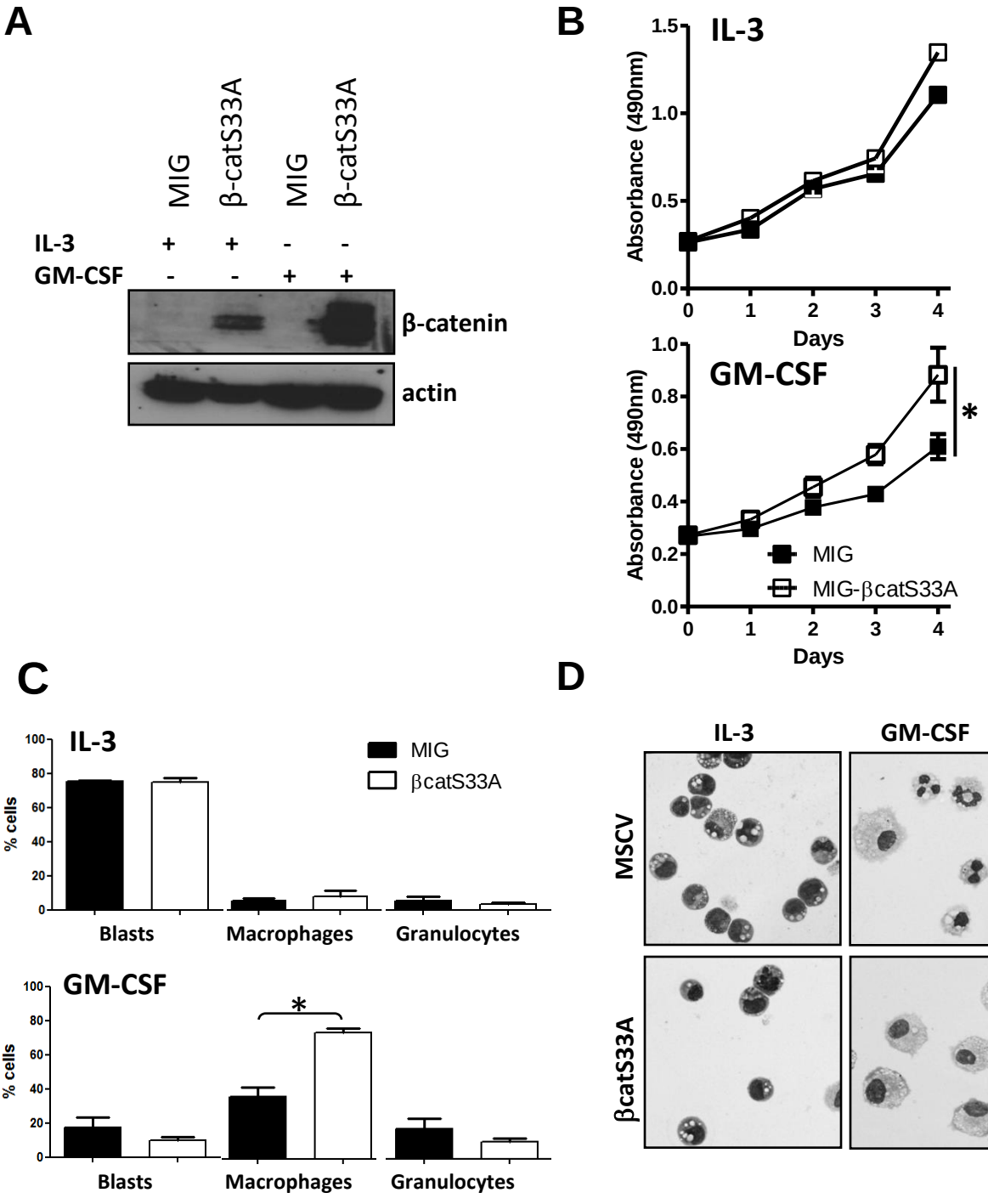

A
MIG
β-catS33A
MIG
β-catS33A
IL-3 + + - -
GM-CSF - - + +
β-catenin
actin
B
IL-3
Absorbance (490nm)
Days
GM-CSF
*
MIG
MIG-βcatS33A
C
IL-3
MIG
βcatS33A
% cells
Blasts
Macrophages
Granulocytes
GM-CSF
*
D
IL-3
GM-CSF
MSCV
βcatS33A

Fig. 5

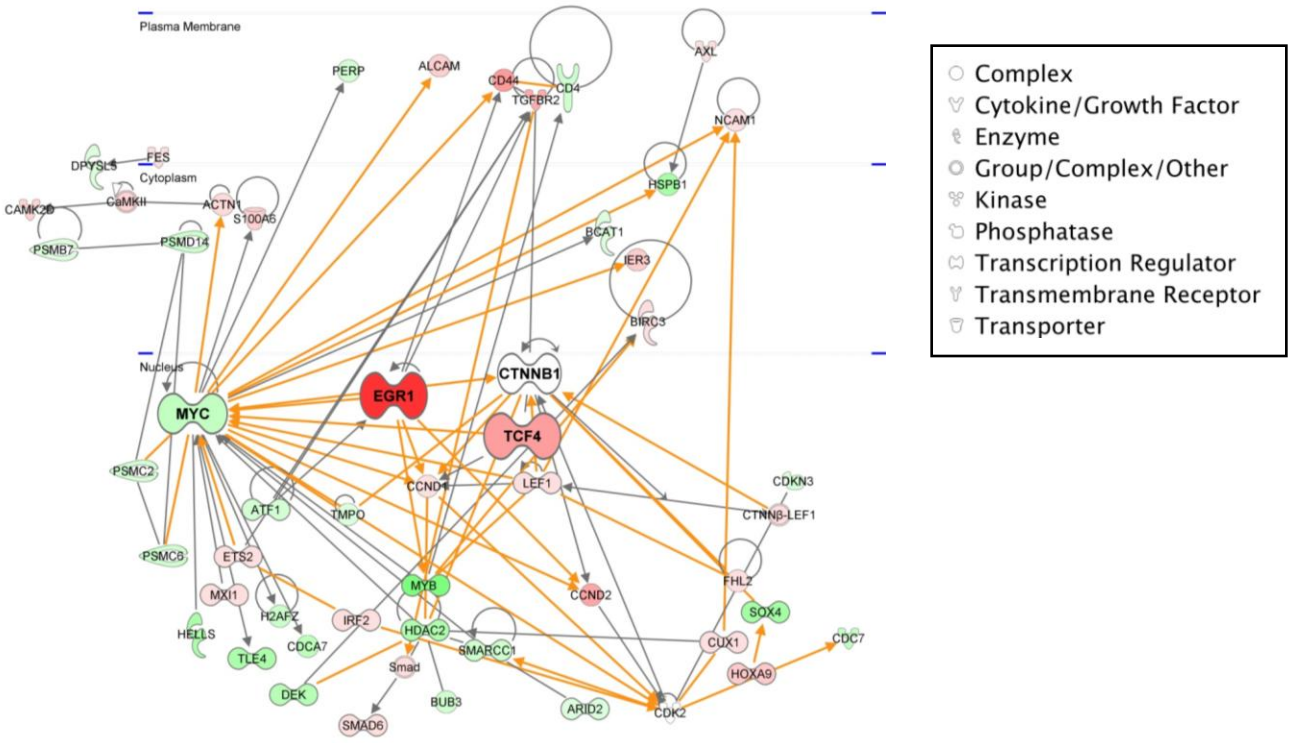
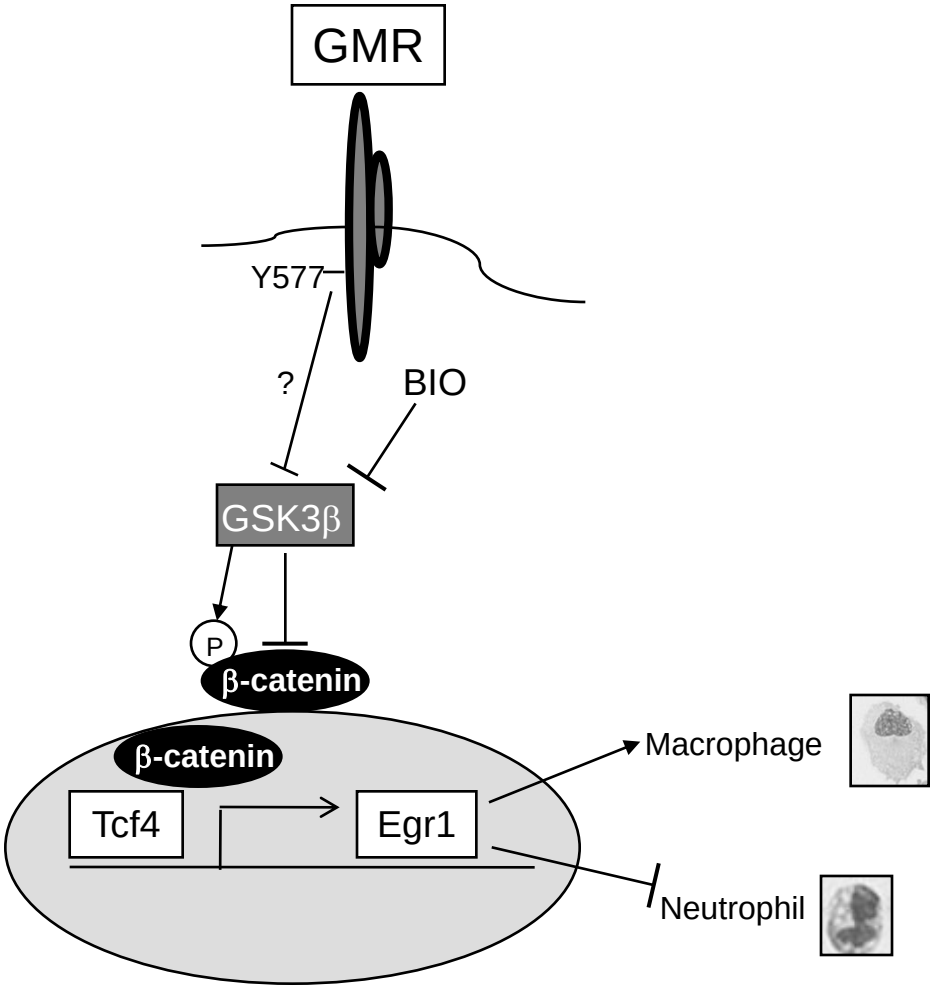
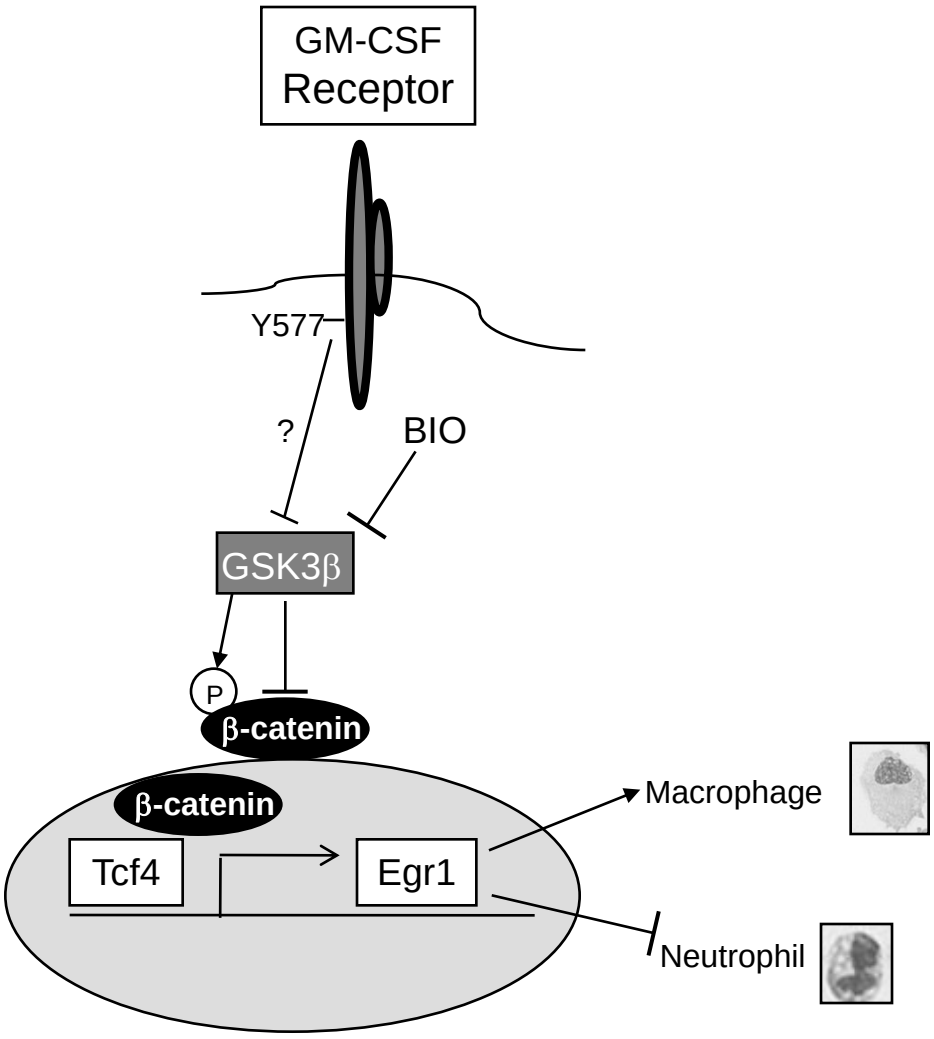

Plasma Membrane
Cytoplasm
Nucleus
PERP
ALCAM
CD44
CD4
TGFBR2
AXL
NCAM1
HSPB1
BCAT1
IER3
BIRC3
DPYSL5
FES
CAMK2D
CaMKII
ACTN1
S100A6
PSMB7
PSMD14
CTNNB1
EGR1
MYC
TCF4
PSMC2
CCND1
LEF1
CDKN3
CTNNB-LEF1
ATF1
TMPO
PSMC6
ETS2
MYB
CCND2
FHL2
MXI1
H2AFZ
SOX4
HELLS
IRF2
HDAC2
CUX1
CDC7
TLE4
CDCA7
SMARCC1
HOXA9
Smad
DEK
BUB3
ARID2
CDK2
SMAD6
Complex
Cytokine/Growth Factor
Enzyme
Group/Complex/Other
Kinase
Phosphatase
Transcription Regulator
Transmembrane Receptor
Transporter

Fig. 6


GMR
Y577
?
BIO
GSK3β
P
β-catenin
β-catenin
Tcf4
Egr1
Macrophage
Neutrophil

Graphical Abstract



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