Redox-Controlled Signaling in Normal Myeloid Cell Development and Leukemia

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Redox-Controlled Signaling in Normal Myeloid Cells and Leukemia

Redox gecontroleerde signaaltransductie in normale myeloide cellen en leukemie

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To my parents

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Manuscript in preparation for a review

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1.1 HEMATOPOIESIS

The word hematopoiesis is derived from the greek words, *haima* meaning blood and *poiesis* meaning to make. Hematopoiesis is a tightly regulated process which ensures production of appropriate amounts of different blood cell types. All blood cell types are derived from pluripotent hematopoietic stem cells (HSCs). The HSCs are capable of self renewal. Committed myeloid progenitor cells are derived from the HSCs. Committed myeloid progenitor cells are able to proliferate and differentiate into mature blood cells like, granulocytes (neutrophils, eosinophils, basophils), lymphocytes, erythrocytes, platelets, macrophages, natural killer cells, dendritic cells and mast cells. During embryogenesis HSCs develop in the aorta-gonad-mesonephros (AGM) region [1-3]. HSCs migrate to the fetal liver which is the main site of hematopoiesis until birth [4]. After birth, the bone marrow takes over as the primary site of hematopoiesis.

The process of hematopoiesis is largely dependent on the hematopoietic growth factors (HGFs). HGFs control proliferation and survival by activation of their respective receptors. Some HGFs also induce differentiation of cells. Major HGFs are erythropoietin (Epo), responsible for erythropoiesis; interleukins (IL's), involved in all lineages; colony-stimulating factors (CSFs) which are involved in myeloid cell development; thrombopoietin (TPO) involved in stem cells and megakaryocytic development; stem cell factor (SCF) and Flt3 which are growth factors for pluripotent stem cells [5]. The family of CSFs includes granulocyte-CSF (G-CSF), granulocyte- macrophage-CSF (GM-CSF), multipotential-CSF (multi-CSF or IL-3) and macrophage- CSF (CSF-1) [6-9].

Unlike other HGFs, G-CSF plays an important role not only in proliferation of early progenitor cells but also in induction of differentiation [9-11]. G-CSF regulates both, proliferation of myeloid progenitor cells as well as differentiation of myeloid progenitor cells into neutrophils [12-15]. Neutrophils have a limited life span of approximately 5.4 days [16]. They can ingest and kill microbes by phagocytosis. Each phagocytotic event results in the formation of a phagosome into which reactive oxygen species (ROS) and hydrolytic enzymes to kill the pathogens are released [17-18]. The rapid release of ROS into the phagosomes has been termed as the "respiratory burst". In the process of killing of microbes, the neutrophils die. It is important to maintain neutrophil levels as they form a barrier against infection. On an average about 50 million neutrophils are produced per minute in adults [5].

1.2. GRANULOCYTE COLONY STIMULATING FACTOR (G-CSF)

G-CSF is a member of the cytokine class I superfamily [19]. The best documented role of G-CSF is to maintain steady state levels of neutrophils *in vivo* by inducing differentiation of myeloid progenitor cells to form neutrophils [12-15, 20]. In mature neutrophils, G-CSF

enhances neutrophil effector functions like superoxide generation, production of leukocyte alkaline phosphatase, myeloperoxidase and release of arachidonic acid [21-23]. G-CSF deficient mice (*csf3*-/-) develop a severe neutropenia with blood neutrophil levels ranging from 15- 30% of wild type littermate controls [14]. These mice are especially susceptible to bacterial infections like *Listeria monocytogenes*. This is due to their inability to rapidly elevate neutrophil levels, a process known as 'emergency granulopoiesis', the second major function of G-CSF.

Because of its ability to induce neutrophil production, G-CSF is commonly used in treatment of severe congenital neutropenia (SCN), leukemia and anaemia. Administration of G-CSF to neutropenia patients, who have severely reduced neutrophil levels due to a block in myeloid differentiation, induces neutrophil production which helps in reducing the risk of bacterial infections [24-26]. Another important clinical application of G-CSF is for transplantation purposes, wherein G-CSF induces the release of hematopoietic stem cells and progenitor cells from the bone marrow into the peripheral blood, an important fraction of blood cells which is required for successful transplantations [27].

1.3 GRANULOCYTE COLONY STIMULATING FACTOR RECEPTOR (G-CSFR)

The effects of G-CSF are mediated via the G-CSFR. G-CSFR is also known as CSF3R and lacks intrinsic tyrosine kinase activity. G-CSFR is a transmembrane protein consisting of 813 amino acids with 603 amino acids being part of an extracellular domain [28-29]. The extracellular portion consists of an Immunoglobulin-like (Ig-like) domain, a cytokine receptor homology domain (CRH domain) and three fibronectin type III (FN III) modules. The CRH domain contains four highly conserved cysteine residues and a WSXWS motif that is required for ligand binding.

The cytoplasmic domain of the G-CSFR consists of box 1, box 2 and box 3 regions, involved either in transduction of proliferation signals (box 1 and box 2) or to be essential for ligand induced differentiation signals of myeloid progenitor cells (box 3) [30-32]. Besides the box regions, signaling from the G-CSFR is dependent on tyrosines (Y) and lysines (K) present in the cytoplasmic tail of the G-CSFR. The cytoplasmic tail contains four tyrosine residues located at positions 704, 729, 744 and 764. Upon activation of receptor, these tyrosines are phosphorylated and serve as docking sites for Src homology 2 (SH2) domain containing signaling proteins. Five conserved lysines at positions 632, 672, 681, 682 and 762 are also present in the cytoplasmic domain of G-CSFR. Both, the most membrane proximal lysine, K632, and the tyrosine, Y729, play a key role in ubiquitination and lysosomal routing of G-CSFR, as is discussed in section 1.5.2 [33-34]. A balance between phosphorylation and dephosphorylation of tyrosines and the ubiquitination status of lysines of the receptor ensures a regulated signaling response and routing of the G-CSFR

1.4 G-CSFR SIGNAL TRANSDUCTION

Activation of the G-CSFR leads to activation of several downstream signaling pathways including the Janus tyrosine kinases (Jak) and Signal transducer and activators of transcription (Stat) pathway, phosphatidyl ionositol-3 kinase (PI3K) pathway and the p21Ras/Raf/ mitogen activated protein kinase of Erk kinase (MEK) /mitogen activated protein kinase (MAPK) pathways (Figure 1). The mechanisms of activation of these pathways and their implications for G-CSFR signaling have been discussed below.

Figure 1

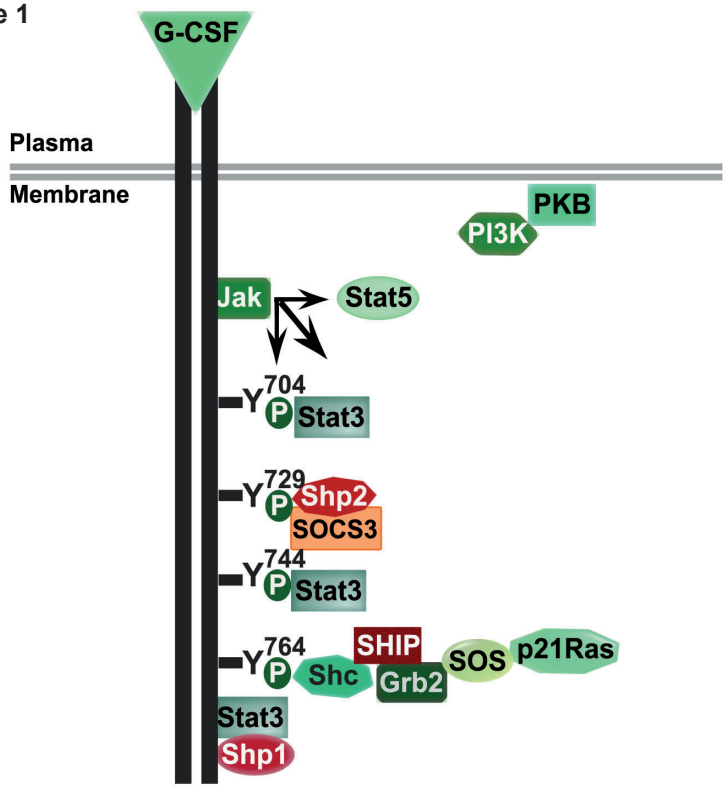

Figure 1: G-CSFR signaling
Activation of G-CSFR signaling is characterised by phosphorylation of tyrosines of the G-CSFR by Jaks. The phosphorylated tyrosines then act as docking sites for other molecules which either positively or negatively regulate signaling via the G-CSFR.

1.4.1 The Jak /Stat pathways

The G-CSFR lacks intrinsic tyrosine kinase activity and relies on the Janus tyrosine kinases (Jak) family for signal transduction. Activation of the G-CSFR leads to activation of 3 members of the Jak family, namely, Jak1, Jak2 and Tyk2 [35-39]. Phosphorylation-mediated-activation of Jak1, Jak2 and Tyk2 upon stimulation with G-CSF is dependent on tryptophan (W) 650 of the G-CSFR [37, 40]. Activated Jaks not only phosphorylate tyrosines of the G-CSFR but also those of Stat proteins. Phosphorylation of Stat proteins results in their homo/ heterodimerization [41]. The dimerized Stat molecules translocate to the nucleus where they activate transcription of target genes.

Upon stimulation with G-CSF, 2 members of the Stat family, Stat3 and Stat5 are activated. The role of Stat3 in negative regulation of G-CSFR signaling was elucidated in mice lacking Stat3 expression in hematopoietic progenitors. These mice developed neutrophilia due to hyper responsiveness to G-CSF because they fail to induce expression of suppressor of cytokine signaling 3 (SOCS3), a direct Stat3 target gene (see section 1.5.2) [42]. The activation of Stat3 occurs via its recruitment to phosphorylated Y704 and Y744 of the G-CSFR [43-44] (Figure 1). Stat3 plays a role in cell cycle exit by inducing expression of the cyclin dependent kinase (CDK) inhibitor, $p27^{Kip1}$ [45]. Stat3 binding sites have been identified in the $p27^{Kip1}$ promoter region. Introduction of a dominant negative form of Stat3 interferes with growth arrest and differentiation of 32D myeloid progenitor cells [45-46] mainly due to inhibition of $p27^{Kip1}$ expression upon G-CSF stimulation.

Unlike Stat3, Stat5 is not recruited directly to the tyrosines of G-CSFR. Its activation occurs by interaction with the activated Jaks [47]. Stat3 and Stat5 differ in their activation kinetics in response to G-CSF. While Stat3 activation by G-CSF is sustained for hours, Stat5 activation is transient and is reduced to basal levels 30 minutes after stimulation with G-CSF [48]. Stat5 is responsible for propagation of proliferation and survival signals via the G-CSFR [49]. In accordance with this, mice lacking both the isoforms of Stat5 show reduced colony forming capacity in response to G-CSF [50]. BA/F3 cells expressing a lysine-less G-CSFR show prolonged Stat5 activation indicating a role of ubiquitination of G-CSFR in Stat5 signal down regulation [34].

1.4.2 PI3K and RAS pathways

Phosphotidylinositol-3 kinase (PI3K) enzymes are a family of lipid kinases consisting of three distinct subclasses based on their structure and substrate specificity [51]. Phosphotidylinositol 4, 5 bisphosphate $\{PI(4,5)P_2\}$ or PIP_2 is the most common substrate for the PI3K class I members. PIP_2 can be phosphorylated upon extracellular stimuli to form phosphotidylinositol 3, 4, 5 trisphosphate $\{PI(3,4,5)P_3\}$ or PIP_3 [52]. The PIP_3 thus generated acts as a docking site for pleckstrin homology (PH) domain- containing proteins like protein kinase B (PKB/ c-AKT) [53].

The membrane proximal region of the G-CSFR is involved in activation of the PI3K/ PKB pathway [54]. The activation of PI3K pathway is dependant on c-Src activation, rather than Jak activation but this may be cell context dependent [54]. PI3K activation results in phosphorylation mediated activation of PKB. Myeloid 32D cells expressing the internalization defective Δ715-G-CSFR mutant show prolonged PKB activation due to increased activation of the upstream PI3K [54]. G-CSF induced activation of PI3K/ PKB pathway leads to increased cell survival brought about by inducing a block in apoptotic cascades. Pro-apoptotic protein Bad is activated by PKB. However the phosphorylated Bad is sequestered by 14-3-3 in the cytosol. This inhibits interaction between Bad and anti-apoptotic Bcl2 and Bcl-xl proteins resulting in increased cell survival [54-55].

Activation of the p21Ras/Raf/MEK/MAPK pathway via the G-CSFR is linked to the phosphorylation of Y764 of the G-CSFR [56-57]. Phosphorylated Y764 acts as a docking site for the SH2 domains of Shc and Grb2, the signaling intermediates of the p21Ras pathway [57-59]. Introduction of the Y764F-G-CSFR mutant results in reduced activation of the p21Ras pathway and accelerated neutrophil differentiation [57].

1.5 NEGATIVE REGULATION OF G-CSFR SIGNALING

Cytokine mediated activation of signaling is usually transient. Several negative regulatory pathways are responsible for switching-off signaling. Major mechanisms involved in signal attenuation are internalization of receptors, lysosomal degradation triggered by ubiquitination of proteins, Janus kinase inhibitory action of SOCS proteins and dephosphorylation of proteins by phosphatases. These mechanisms will be introduced below.

1.5.1 Trafficking of activated G-CSF receptors

Endocytosis of receptors involves internalization of receptors from the plasma membrane, trafficking to early endosomes, late endosomes, multi-vesicular bodies (MVBs) and then to lysosomes, where the receptors are degraded. Receptor internalization can occur either via clathrin-coated pits or via caveolae. The G-CSFR is known to undergo endocytosis via clathrin-coated pits [60]. A region of the G-CSFR that contains the dileucine motif (a.a 749-769) is essential for both ligand-induced and constitutive internalization. Besides the dileucine motif, the presence of tryptophan (W650, involved in Jak activation) and phosphorylation of serine (S749) are critical for ligand-induced internalization [60].

Upon internalization, the endocytosed G-CSFR vesicles form early endosomes. Early endosomes are considered to be sorting vesicles in which the fate of the internalized receptors is controlled. The receptors are sorted based on the ubiquitination of the lysines in the cytoplasmic tail. Ubiquitination of G-CSFR occurs via the SOCS box of SOCS3. The role SOCS3 plays in down modulation of G-CSFR signaling will be explained in section 1.5.2.

Receptors that have been ubiquitinated are targeted for degradation and further route to late endosomes/ lysosomes [61].

Fusion of early endosomes leads to the formation of late endosomes. Internalization of activated receptors and their routing to early endosomes occurs independent of ubiquitination of the G-CSFR [33-34]. However, the post-endocytotic routing of the G-CSFR to lysosomes is dependent on SOCS3-mediated ubiquitination of membrane proximal lysine, K632 of the G-CSFR [33-34]. By invaginations of the membrane of the late endosomes into its lumen are formed the multivesicular bodies (MVBs). The ESCRT (endosomal sorting complexes required for transport) machinery mediates efficient transport of the receptors into the MVB pathway [62]. The ESCRT complex interacts with the ubiquitinated receptors via the ubiquitin binding domains (UBDs) present in the proteins of the ESCRT complex [63]. Eventually the ubiquitinated receptors are de-ubiquitinated by de-ubiquitinating enzymes (DUBs) [63-65]. Finally the MVBs with the sorted and de-ubiquitinated receptors fuse with the lysosomes where the receptors are degraded. The lysosomes have an acidic pH (4.8) and degradative enzymes which are responsible for degradation of the receptor cargo.

1.5.2 Ubiquitination via SOCS3

In receptor-mediated signaling, a negative feedback loop exists as expression of SOCS3 is under direct control of Stat3 [66-69]. G-CSF stimulation is known to induce expression of SOCS3 [70-71]. *Stat3*-/- mice show reduced levels of SOCS3 expression upon stimulation with G-CSF, which suggests that SOCS3 is a target of Stat3 in G-CSF signaling [42]. In bone marrow cells, Stat3 is required for robust induction of SOCS3 in response to G-CSF stimulation [42, 48, 70]. SOCS3 is subsequently recruited to Y729 of the G-CSFR [70].

SOCS3 knock-out mice were shown to be embryonic lethal due to placental function defects [72-73]. Studies in SOCS3 conditional knockout mice have shown that SOCS3 is also a major negative regulator of G-CSF dependent neutrophil production. These mice demonstrate neutrophil leukocytosis after they reach adulthood and show infiltration of hematopoietic cells into various tissues [74-75]. When stimulated with G-CSF *in vitro*, the SOCS3 deficient cells exhibited prolonged Stat3 activation and enhanced cellular responses to G-CSF, including an increase in colony formation and proliferation. Consistent with the *in vitro* findings, SOCS3 mutant mice administrated with G-CSF displayed enhanced neutrophilia, indicating that loss of SOCS3 results in hypersensitivity towards G-CSF [74].

SOCS3 has been shown to inhibit G-CSF mediated signaling by different mechanisms. The first mechanism by which SOCS3 down regulates G-CSF signaling is by ubiquitination of the membrane proximal lysine K632 [33]. Although all the five lysines in cytoplasmic tail of G-CSFR are ubiquitinated, routing of G-CSFR from early endosomes to lysosomes is dependent on the ubiquitination of K632 by SOCS3 [33]. Loss of the SOCS3 recruitment site (Y729) of the G-CSFR, leads to decreased ubiquitination of the G-CSFR mutant in which K632 is the only lysine in its cytoplasmic tail.

In addition to the SH2 domain and the carboxy terminal SOCS box possessed by all of the SOCS family members, SOCS3 also possesses a kinase inhibitory region (KIR) [76-77]. The second mechanism, shown for other receptors is by inhibition of Jak activity via the KIR of SOCS3 bound to Y729 of G-CSFR [78-80]. Another mechanism by which SOCS3 inhibits cytokine receptor activation is by competing with Stat3 for binding the phosphorylated tyrosines of the G-CSFR; a process which is essential for activation for Stat3 [81-82].

1.5.3 De-phosphorylation via phosphatases

The human genome encodes for about 107 different protein phosphatases which recognize and remove phosphate moieties attached to tyrosine/ threonine/ serine from proteins. The PTP family consists of classical PTPs that dephosphorylate only tyrosine residues (pTyr) and dual specificity phosphatase (DUSPs) which dephosphorylate pSer, pThr and pTyr residues [83-85]. The PTPs which dephosphorylate tyrosines consist of two types, receptor protein tyrosine phosphatases (RPTPs) and non-transmembrane, cytoplasmic PTPs [86]. Oxidation of the catalytic cysteine residue of phosphatases renders the phosphatases inactive. However this process is reversible and antioxidants can recycle the oxidized inactive phosphatases to their active forms. The mechanism of oxidative inactivation of phosphatases and their reactivation by antioxidants will be discussed in detail in section 1.7.2 of this chapter. The role of some PTPs known to regulate G-CSF mediated signaling is discussed below.

- Shp1

The non-transmembrane protein tyrosine phosphatase, Shp1 (also known as Ptpn6), is expressed mostly in hematopoeitic cells and affects G-CSFR signaling [87-89]. Shp1 is mainly a cytosolic protein which translocates to the receptors at the plasma membrane upon activation [90]. Fractions of Shp1 have also been detected in the nucleus [91]. Mice carrying a loss of function mutation in the *Shp1* gene display myeloid as well as lymphoid abnormalities. Interaction of Shp1 with G-CSFR occurs independently of cytoplasmic tyrosines of the G-CSFR [92-94]. Protein levels of Shp1 are increased during G-CSF induced differentiation in 32D cells. While the overexpression of Shp1 in 32D cells inhibits proliferation and stimulates differentiation, the overexpression of a catalytically inactive Shp1 results in inhibition of differentiation and stimulation of proliferation in 32D cells [94]. Overexpression of Shp1 in 32D cells results in slightly decreased p-Stat5 levels while overexpression of catalytically inactive Shp1 mutant results in increased Stat5 activation [94].

- Shp2

Shp2, alternatively known as Ptpn11, has also been implicated in G-CSFR signal down regulation. Shp2, like Shp1, is predominantly a cytosolic protein which translocates to activated receptors at the plasma membrane [90]. Shp2 competes with SOCS3 for binding to the phosphorylated tyrosine Y729 of the G-CSFR [48]. However another C-terminal region of

the G-CSFR is also important for the interaction of Shp2 with G-CSFR [48]. Shp2 also binds to Y704 and Y764 of the G-CSFR along with Grb2 and/or Shc [59]. Because the tyrosines 704 and 764 are strongly involved in propagation of proliferation signals via G-CSFR, it is thought that Shp2, Grb2 and Shc mediate these responses. One of the proposed functions of Shp2 in G-CSFR signal regulation is dephosphorylation of Stat5 [95]. In the context of the epidermal growth factor receptor (EGFR), Shp2 prolongs Ras activation (GTP-Ras) [96]. Shp2 dephosphorylates the tyrosine (Y992) of the EGFR which acts as the docking site for the Ras GTPase activating protein (Ras-GAP). This leads to inability of Ras-GAP to translocate to the same micro domain as its substrate, GTP-Ras, thereby leading to increased levels of GTP-Ras. Whether Shp2 regulates GTP-Ras signaling in response to G-CSF remains unknown.

- SHIP1

Tyrosine, Y764 present in the C-terminal of the G-CSFR is required for activation of yet another cytosolic phosphatase, SHIP1 and for the formation of Shc/SHIP complexes [49, 97-98]. SHIP1 is a 5'-phosphatidylinositol (PtdIns) phosphatase that de-phosphorylates PIP3 at the 5 position. Numbers of neutrophils and monocytes/ macrophages are increased in *SHIP*1-/- mice [99-100]. Furthermore, neutrophils derived from *SHIP*1-/- mice show prolonged survival in response to apoptotic stimuli or to growth factor withdrawal [100]. Defects in hematopoietic signaling, particularly in the myeloid lineage have been observed in SHIP-deficient mice [99].

- Ptp1b

Protein tyrosine phosphatase 1b (Ptp1b) is localized to the ER with its C-terminal hydrophobic sequence anchored to the cytoplasmic face of the ER and a short tail extending into the lumen of the ER [101-102]. *Ptp1b*-/- mice are hypersensitive to insulin as well as leptin [103-105]. Ptp1b is known to modulate metabolic signaling in mammals [103, 106-107]. One of the important mechanisms by which Ptp1b negatively regulates signaling via EGFR, platelet derived growth factor receptor (PDGFR), insulin receptor and erythropoietin receptor (EPOR) is by dephosphorylation of tyrosines of the receptors [108-110]. Besides dephosphorylation of the tyrosines of several receptors, Ptp1b was shown to physically interact with Jak2 and negatively regulate the activity of both Jak2 and Stat3 in case of the leptin receptor signaling [105, 111] and Stat6 in case of interleukin 4 (IL-4Rα) signaling [112]. MEFs and splenocytes lacking Ptp1b expression also showed increased ROS levels in response to IL-4 stimulation as compared to wild type controls.

Although the role of Ptp1b in negative regulation of signaling via RTKs and cytokine receptors is clear, how could an endoplasmic reticulum (ER) localized phosphatase negatively regulate signaling from activated receptors. However, data from Haj et al showed that activated EGFRs routed via the ER where the receptors were dephosphorylated by Ptp1b when the EGFR-containing early endosomal vesicles were in the vicinity of the ER [113].

More recent findings show that the interaction between Ptp1b and EGFRs occurs via direct membrane contacts between the outer membrane of the multivesicular bodies (MVBs), containing endocytosed EGFR vesicles and the endoplasmic reticulum, onto which is present Ptp1b [114]. Loss of Ptp1b leads to decreased formation of internal vesicles within the MVB suggesting that the dephosphorylation of EGFRs occurs prior to their internalization within the MVBs and that dephosphorylation is essential for routing to MVBs [114].

Ptp1b is also thought to regulate the endocytotic pathway. This was proposed based on the findings that Ptp1b dephosphorylates STAM2, a member of the ESCRT complex, thereby increasing its affinity for binding ubiquitinated proteins resulting in lysosomal degradation of EGFR [115-116]. Taken together, it is evident that Ptp1b plays a key role in negative regulation of receptor signaling. Whether Ptp1b down modulates G-CSFR mediated signaling and its routing has not been examined so far.

Attenuation of G-CSFR mediated signal transduction likely involves dephosphorylation of G-CSFR tyrosines. Ptp1b has been shown to be essential for dephosphorylation of tyrosines of activated receptors and also attenuation of downstream signaling initiated by activated receptors.
We examined whether Ptp1b played a role in down modulation of signaling and proliferation in response to G-CSF. This could be either by means of dephosphorylation of tyrosines of G-CSFR and/or by means of dephosphorylation of other downstream signaling molecules involved in G-CSFR signal transduction.
These questions have been addressed in Chapter 2 of this thesis.

1.6 ABNORMAL G-CSF SIGNALING IN MYELOID DISORDERS

Acute myeloid leukemia (AML) is characterised by accumulation of blasts in the bone marrow due to a block in differentiation and enhanced proliferation and survival of myeloid progenitor cells. As mentioned before, G-CSF is known to induce differentiation of myeloid progenitor cells to neutrophils. However administration of G-CSF to blasts from AML patients leads to enhanced proliferation of blasts and marginal/ no differentiation [117]. This difference in response to G-CSF has been linked to the increased expression of a different isoform of the *G-CSFR* (Class IV) as compared to normal immature myeloid cells which express Class I isoform [118]. The Class IV isoform of the *G-CSFR* was shown to be defective in differentiation [119].

A novel splice variant of *G-CSFR* was described by Awaya and colleagues [120]. This splice variant results in deletion of three nucleotides in the juxtamembrane region of the *G-CSFR* resulting in the conversion of aspargine and arginine (N630 R631) residues to lysine

(K630). Although the splice variant was also detected in normal bone marrow cells with a frequency of 2%, it was found at an increased frequency of 8% in myeloid progenitor cells in myelodysplastic syndrome (MDS). This modification in the juxtamembrane region leads to increased proliferation in response to G-CSF compared to the wild type G-CSFR. However the role of this modification in MDS remains unknown.

Another polymorphism in the *G-CSFR* distal carboxyl region leading to the conversion of glutamic acid (E785) to lysine (K785) has been associated with high risk MDS patients [121]. Expression of this variant in myeloid progenitor cells leads to decreased proliferation of progenitor cells compared to the wild type controls. This finding can be explained based on the established role of lysines in ubiquitination mediated proteasomal degradation of substrates.

A sub class of leukemia, acute promyelocytic leukemia (APL) characterised by t(15;17) is hyper proliferative in response to G-CSF treatment [122]. In case of cells derived from APLs, G-CSF is unable to induce differentiation indicative of defects in signaling via the G-CSFR.

Mutations in *G-CSFR* are rare in de-novo AMLs and myelodysplasia (MDS). In contrast, mutations in *G-CSFR* commonly occur in patients with severe congenital neutropenia (SCN) [123]. Most frequently occurring mutations in the *G-CSFR* in SCN patients are nonsense mutations introducing stop codons in the cytoplasmic domain of the *G-CSFR* [123-124]. Expression of truncated forms of the *G-CSFR* in SCN patients is commonly associated with progression towards development of AML [123-125].

Expression of the mutant *G-CSFR* (Δ715-G-CSFR) in mice results in hyper proliferation in response to G-CSF [126-127]. Signaling via the Δ715-G-CSFR (PI3K/PKB and p-Stat5) is prolonged as compared to the wt-G-CSFR due to loss of binding sites of the negative inhibitory molecules like SOCS3, SHIP1 and Shp1 [33, 49, 92, 94, 128]. Internalization of the Δ715-G-CSFR is also affected because of loss of internalization motifs present in the C-terminal region [60]. Expression of the Δ715–G-CSFR truncation mutant in 32D cells results in an increased production of reactive oxygen species (ROS) upon stimulation with G-CSF as compared to wt-G-CSFR expressing cells [129].

1.7 REDOX SIGNALING

Redox signaling is the process in which free radicals or ROS act as messengers in biological systems. ROS are commonly produced as by-products of metabolism in cells. If produced in large amounts ROS are toxic to cells as they can induce damage to DNA, proteins, carbohydrates and lipids [130-131]. ROS levels are deregulated in ageing and a variety of chronic diseases, such as cardiovascular diseases and cancer [132-135]. However, in small amounts ROS have been shown to have a major impact on cellular signaling. The importance of ROS mediated signaling and its regulation is discussed in the following sections.

1.7.1 Production of reactive oxygen species

ROS have for long been considered to be unwanted by-products of metabolism in cells, with the exception of phagocytic cells of the immune system, in which ROS are produced in large amounts [136-137]. The high level of ROS (H_2O_2) produced in these short-lived cells acts as an oxidative burst which is a major weapon in host defence against bacteria. In non-phagocytic cells, ROS such as H_2O_2 or superoxide are generated by different pathways in cells mostly in the mitochondria and in the endoplasmic reticulum. Besides being the normal end-products of metabolism in cells, ROS are produced when cells are activated by extracellular stimuli. ROS act as second messengers which activate signaling pathways in cells by transient deregulation of the activity of redox sensitive enzymes, in particular protein tyrosine and lipid phosphatases [130]. The mechanism by which ROS regulate the activity of phosphatases has been discussed in section 1.7.2 of this chapter.

The NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (Nox) system plays a major role in production of ROS. The Nox system is involved not only in ROS production upon receptor activation but also in the production of an oxidative burst in phagocytic cells. In phagocytic cells, the Nox system comprises of the membrane bound cytochrome b (b558) consisting of the gp91phox (also known as Nox2) and p22phox subunits. Generation of ROS by the catalytic Nox2 subunit requires the recruitment of the organiser p47phox, activator p67phox subunits and Rho guanosine triphosphatase (GTPase)-Rac1 [138-140]. In the non-phagocytic cells homologs of Nox2- Nox1, Nox3, Nox4 and Nox5 have been identified [141]. These homologues of Nox2 require different subunits for their activation.

Components of the Nox complex are localized within specific subcellular compartments. While Nox1 is localized within the nucleus and at the plasma membrane, specifically in the caveolae [142-143], Nox2 is detected on the plasma membrane but translocates to endosomes in IL-1 stimulated cells [144]. Under overexpression conditions, Nox3 was detected in the cytoplasm [145]. Nox4 is localized in the perinuclear regions, endoplasmic reticulum, at the plasma membrane and also within the mitochondria [146-148]. Under overexpression conditions, Nox5 was also detected at the plasma membrane [149-150]. The differential subcellular localization of the Nox subunits is important for the local production of ROS, necessary for signal transduction.

The local role of the Nox sytem has been elucidated in the context of the interleukin-1 receptor (IL-1R) [144]. It was demonstrated that Nox2 controls the binding of Traf6 to the IL-1R/MyD88 complex in early endosomes (Figure 2). Upon assembly of the Nox2 machinery at the early endosomes at the IL-1R/MyD88 complex, ROS production occurred locally in the early endosome compartment [144]. The ROS produced locally diffused through the membrane of the endosomes and this led to recruitment of Traf6 to the IL-1R complex at the early endosomes. Also in case of the IL-4R, Nox1 and Nox5L were shown to be involved in ROS production [112].

Figure 2

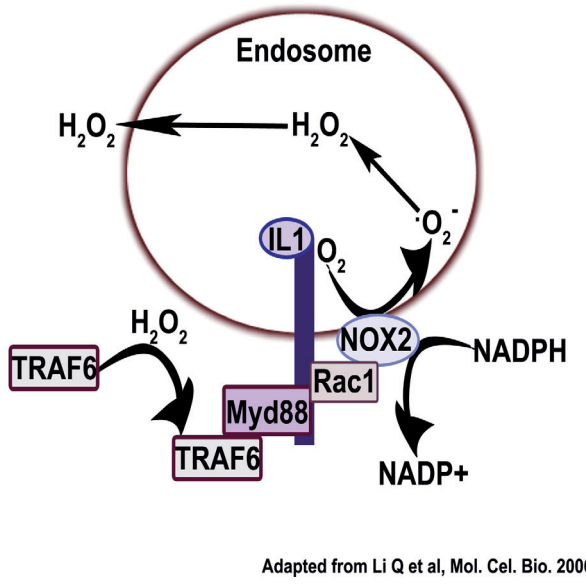

Figure 2: Assembly of the Nox2 complex in early endosomes
Activation of IL-1 receptors leads to internalization of the IL-1 receptor along with membrane localized- Nox2, Rac1 and Myd88. The assembly of the Nox complex occurs at the endosomes where IL-1 mediated production of ROS occurs. The ROS produced are capable of diffusing through the membrane of the endosomes and this brings about the recruitment of Traf6 to the IL-1-Nox2 complex.

Activation of transmembrane receptors, such as platelet-derived growth factor receptor (PDGFR), interleukin 3 receptor (IL-3R) and granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR), results in increased intracellular ROS levels which enhances the signaling function of these receptors, conceivably by down regulation of phosphatase activities [129, 151-153].Taking into account the toxic effects of ROS, the timely inactivation of Nox and also the maintenance of ROS levels produced are critical processes.

1.7.2 Oxidative inactivation of phosphatases

All phosphatases contain the amino acid cysteine in their catalytic site. The catalytic cysteines of phosphatases have a very low pK_a [154]. The low pK_a of the cysteine is necessary for the process of catalysis, whereby a phosphate group is accepted by the thiol group of the cysteine residue. But the low pK_a also makes the cysteine extremely susceptible to undergo oxidative inactivation upon exposure to ROS [155-156]. ROS selectively inactivate tyrosine phosphatases while they have no effect on serine or threonine phosphatases [157].

Figure 3

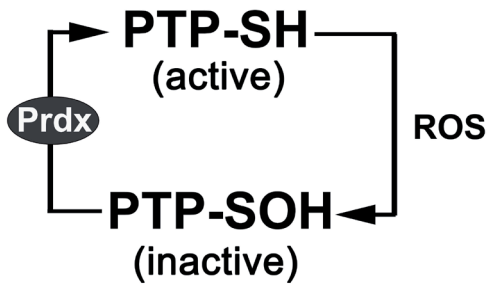

Figure 3: Inactivation of phosphatases by ROS
The amino acid cysteine in the catalytic site of protein tyrosine phosphatases (PTPs) is especially sensitive to oxidation by ROS. Upon oxidation, the thiol group is converted into a sulfenic acid derivative which renders the phosphatase inactive. The inactive phosphatase is then reactivated by peroxiredoxins by reduction of the sulfenic acid derivative to thiol group.

The amino acid cysteine consists of a thiol group (SH) in its side chain. Upon exposure to hydrogen peroxide, the hydroxyl group of hydrogen peroxide covalently interacts with the thiol side chain of the catalytic cysteine (Figure 3). This leads to formation of a sulfenic acid (S-OH) derivative which renders the phosphatase inactive [157]. The sulfenic stage of oxidation of phosphatases can be reversed by antioxidants leading to the formation of a thiol group thereby generating a catalytically active cysteine. However excessive oxidation of the thiol group could also occur leading to the formation of catalytically inactive sulfinic acid (SO_2H) or sulphonic acid (SO_3H) derivatives, which cannot be readily reversed back to their active forms.

Under physiological conditions, only reversible oxidation of PTP occurs. Several reports show oxidation of PTPs in response to various stimuli. For eg. Ptp1b oxidation has been reported in response to stimulation with EGF or insulin [155, 158-159]. It has been shown for the EGF receptor that the ROS production coincides with inhibition of Ptp1b activity [160]. Nox4 activation upon stimulation with IL4 also leads to inhibition of Ptp1b activity [112]. While TC-PTP was shown to be oxidized in response to insulin [159], Shp2 was shown to be oxidized in response to PDGF stimulation [156]. The sensitivity of each PTP to oxidation might depend on a couple of factors. Firstly, the structure of the catalytic domain of the PTP as well as its regulatory domain might influence its sensitivity to oxidation. Another key factor could be the relative distance of the PTP from the site of ROS generation

1.7.3 Role of antioxidants in signaling

Antioxidant enzymes protect the cells against the damaging effects of oxidants. Antioxidants play distinct roles in the different metabolic pathways of cells. Factors like variable sensitivity to different levels of ROS and also the sub-cellular localization of these antioxidants define the roles of these antioxidants in the diverse metabolic processes.

1.7.3.1 Localization of antioxidants

Although, ROS can be produced locally, they can easily diffuse throughout the cytosol and through the membranes of cell organelles [136, 144, 148]. To protect the cells from oxidative damage, it is important to have antioxidant systems at the site of ROS production. Antioxidants have a distinct sub-cellular localization. Some antioxidants are localized in the cytoplasm, like peroxiredoxin isoforms- Prdx1, Prdx2 and Prdx6 [161]. Other peroxiredoxins are localised either in the mitochondria (Prdx3) or within the ER (Prdx4) or within peroxisomes (Prdx5), which are the major sites of ROS production in cells [161]. Other antioxidants like glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) are also present at the major sites of ROS production in the mitochondria or peroxisomes.

1.7.3.2 Sensitivity of antioxidants to oxidative inactivation

Cells express a variety of antioxidant enzymes like catalase, superoxide dismutase, thioredoxin (Trx), glutathione peroxidases and peroxiredoxins. Each of these antioxidants vary in their sensitivity to quantities of ROS. The enzymatic activities of GPx and catalase are maximal under 5mM and 30mM H_2O_2 concentrations respectively [162-163]. At such high concentrations of hydrogen peroxide, peroxiredoxins are irreversibly inactivated. The amount of ROS produced upon receptor activation ranges between 10μM and 1mM [153]. The concentration of peroxide required to achieve half maximal activity for peroxiredoxins has been reported to be less than 20μM [164]. Most likely because of the high sensitivity of peroxiredoxins to small concentrations of ROS produced, they are more critical for signaling in cells. The other antioxidants which are less sensitive to small changes in ROS might be responsible for protection of cells under conditions of oxidative stress.

1.8 PEROXIREDOXINS AND THEIR ROLE IN SIGNALING

The peroxiredoxin family of antioxidants consists of 6 members (Prdx1-6). The peroxiredoxins are classified into typical 2 Cys-peroxiredoxins (Prdx 1 to 4), atypical 2-Cys peroxiredoxin (Prdx5) and 1-Cys peroxiredoxin (Prdx6), based on the number and the position of cysteine residues involved in their redox activities [161]. The N-terminal cysteine takes part in the peroxidase activity. In presence of peroxide the thiol group of Prdx undergoes oxidation to form a sulfenic acid (Figure 4). This oxidized state is easily reversed back to the active state by another antioxidant, thioredoxin (Trx). Trx itself also plays an important role in signaling (section 1.9). The sulfenic acid derivative can undergo further oxidation to form a sulfinic acid derivative. The sulfinic acid derivative can also be recycled back to the active thiol group by sulfiredoxin (Srx). Further oxidation of the sulfinic acid under conditions of oxidative stress leads to the formation of sulphonic acid derivative. This super oxidized state of peroxiredoxins is irreversible.

Figure 4

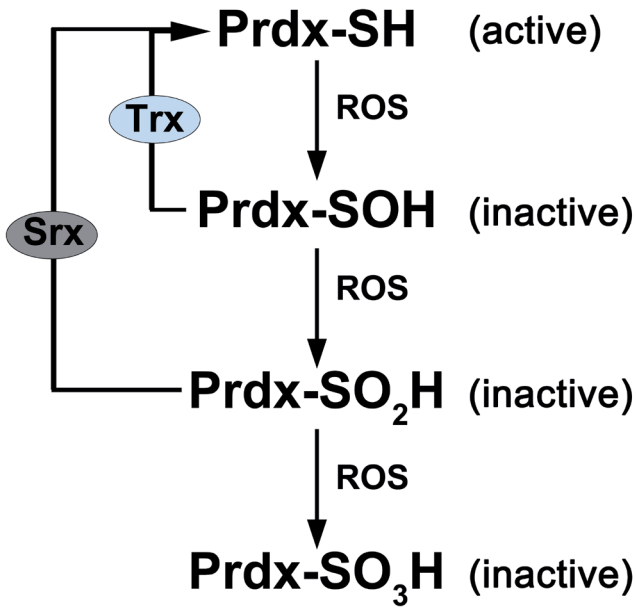

Figure 4: Inactivation of peroxiredoxins by ROS
Upon oxidation, the cysteine in the catalytic site of antioxidants is converted into sulfenic acid thereby leading to inactivation of peroxiredoxins. The sulfenic acid derivative can be easily reactivated by thioredoxin. Sulfenic acid can be further oxidized to form sulfinic acid which can be further oxidized under conditions of oxidative stress to sulphonic acid. The sulphonic acid form is an irreversibly oxidized form. However the sulfinic acid derivative can be reactivated by reduction by sulfiredoxin (Srx).

In response to growth factors, Prdx1 and Prdx2 are transiently inactivated by different mechanisms. Prdx1 undergoes Y194 phosphorylation-mediated-inactivation upon stimulation with EGF/ PDGF. Only the fraction of Prdx1 located in direct proximity of the plasma membrane underwent tyrosine phosphorylation-mediated-inactivation upon EGF/ PDGF treatment as compared to cytosolic Prdx1 [165]. Prdx2 is inactivated by hyperoxidation of the catalytic cysteine upon stimulation with PDGF or EGF [165]. This allows transient signaling in cells in response to EGF and PDGF.

As mentioned in section 1.7.3.1, different peroxiredoxins are distributed over different cellular compartments like cytosol, mitochondria, peroxisomes, endoplasmic reticulum [161]. The localization of Prdx4 is still subject to controversy, with some studies suggesting that Prdx4 is both a cytoplasmic and a secreted protein, whereas others suggest that Prdx4 is predominantly retained in the endoplasmic reticulum (ER) [166-168].

Peroxiredoxins are involved in recycling phosphatases implicated in signal transduction from an oxidized, inactive state back to their reduced, active, form as described in the previous sections of this chapter [131]. Peroxiredoxins also down modulate receptor medi-

ated signaling. Direct evidence for the role of Prdx proteins in PDGFR-β signaling comes from studies performed in *Prdx2-/-* MEFS by Choi and others [151]. Prdx2 was shown to be recruited to the PDGFR-β where it indirectly stimulated dephosphorylation of the tyrosines of the PDGFR-β by means of activation of a phosphatase. In case of the EGF receptor, over-expression of Prdx2 leads to a decrease in PKB activation upon stimulation with EGF, suggestive of a role for Prdx2 in activation of the PTEN phosphatase [169]. It has been shown that over-expression of Prdx2 in cells leads to a block in the activation of NF-κB upon stimulation with TNFα [170]. Furthermore, the production of H_2O_2 upon stimulation with TNFα was also blocked in cells over-expressing Prdx2 [170]. In A431 cells that express EGF receptors abundantly, the over expression of Prdx4 decreased ROS production upon stimulation with EGF, as compared to controls [171]. Besides, Prdx4 not only interacted with thromboxane A2 receptor (TPβ) but also regulated the membrane levels of expression of TPβ under conditions of oxidative stress [166].

Increased amounts of ROS are produced in cells expressing the truncated Δ715-CSFR as compared to those expressing the wild type G-CSFR. This suggests that a mechanism involved in negative regulation of ROS is linked to the C-terminus of G-CSFR, a region that is lacking in the Δ715-G-CSFR.
As antioxidants are responsible for down modulation of ROS levels, we investigated whether any antioxidants interact with the C-terminus of G-CSFR and if so, whether they regulate redox signaling pathways coupled to G-CSFR activation.
We studied the interaction between G-CSFR and peroxiredoxins and the effect peroxiredoxins have on G-CSFR mediated signaling and proliferation, which is reported in Chapter 2.

Diseases that involve abnormal inflammatory and metabolic processes such as heart dysfunction, cancer, diabetes mellitus and neural disorders usually involve abnormal ROS generation or their regulation. *PRDX4* transcript as well as protein levels decrease upon treatment of cancer cell lines with TNF-apoptosis inducing ligand (TRAIL) [172]. In MEFs, Ndy1, induces expression of antioxidant proteins including Prdx4 [173]. Ndy1, a histone H3 demethylase, is also known to protect cells from oxidative damage. Methylation of lysine 36 of histone H3 (K36me2) of promoter leads to repression of transcription [174]. Ndy1 binds to specific sites in the *Prdx4* promoter and demethylates H3K36-me2 associated with the promoter of *Prdx4* thereby inducing its expression. A unique case of AML showing involvement of P*PRDX4* locus in a fusion with AML1 (RUNX1) carrying the t(X;21)(p22;q22) has been reported [175].

A class of leukemia, namely the acute promyelocytic leukemia (APL) bearing the t(15;17) translocation is associated with hyper responsiveness to G-CSF treatment.
We examined whether the expression of peroxiredoxins involved in down regulation of G-CSFR signaling is suppressed in APL thereby leading to hyper responsiveness to G-CSF treatment.
Chapter 3 of this thesis addresses these studies.

1.9 THIOREDOXIN AND THIOREDOXIN INTERACTING PROTEIN

As mentioned in section 1.8, the antioxidant thioredoxin (Trx) is responsible for reducing the sulfenic acid derivative of inactive peroxiredoxins to the thiol groups thereby resulting in their activation. Besides peroxiredoxins, thioredoxins catalyse the reduction of disulphide bonds in many proteins. In the process of reducing the disulphide bonds of substrates, Trx itself gets inactivated because of oxidation of cysteines in its catalytic site. Inactivated thioredoxin is reactivated by the thioredoxin reductase (TrxR) and NADPH [176-177].

Besides its oxido-reductase function, Trx carries out other functions via its binding partners. In its reduced state, Trx binds to transcription factors like AP-1 and NF-κB and modulates their DNA binding activity [178-180]. The best studied interaction of Trx is that with apoptosis signaling-regulating kinase 1 (Ask1) (Figure 5) [181]. Only reduced Trx is able to bind Ask1 and inhibit the activity of Ask1. Txnip (Thioredoxin-interacting protein) also known as Vdup1 (vitamin D3 upregulated protein 1) or Tbp2 (thioredoxin binding protein 2) is another protein that binds Trx. *Txnip* was originally identified in HL-60 cells as the gene upregulated upon treatment of these cells with 1α,25-dihydroxyvitamin D3 [182]. Txnip was found to negatively regulate Trx function as well as its expression [183]. Txnip interaction with Trx requires the same domian of Trx as required for interaction of Trx with Ask1 [184-185]. Thus Txnip and Ask1 compete with each other for interaction with Trx. The inhibitory effect of Txnip on Trx causes increased ROS levels thereby leading to oxidative stress in cells [184].

1.9.1 Role of Txnip in disease and tumorigenesis

The gene encoding Txnip was identified as a common virus integration site (VIS) in murine myeloid leukemias induced by Graffi 1.4 and Cas-Br murine leukemia viruses [186]. *Txnip*-/- mice show severely reduced numbers of natural killer (NK) cells as compared to wild type controls [187]. Also the activity of NK cells was severely reduced in *Txnip* knockout mice. Studies done in the *Txnip* knockout mice also suggest that Txnip plays a role in Krebs cycle mediated fatty acid utilization [188].

Figure 5

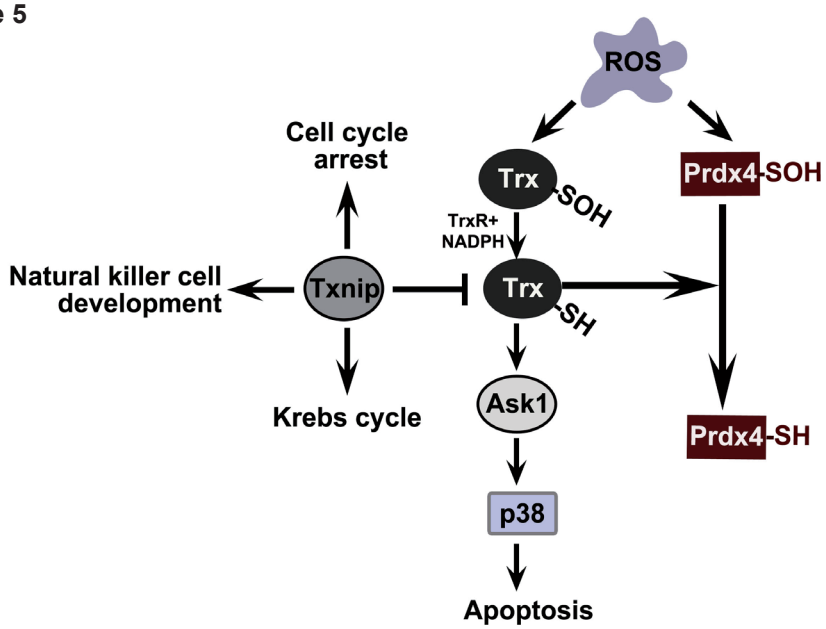

Figure 5: Role of Txnip
The major role of Txnip is to inhibit the activity of antioxidant protein Trx by physically interacting with Trx. Besides, inhibition of Trx function, Txnip also plays a role in other processes, like inducing cell cycle arrest, Krebs cycle and NK development. Txnip not only inhibits the activity of Trx but indirectly also that of Prdx.

Txnip has been suggested to act as a tumor suppressor [189-190]. For instance, increased expression of Txnip leads to decreased proliferation of stomach cancer cell lines and a pro-myelocytic leukemia cell line [190] and also prevents metastasis in transplantation models [189]. Enforced expression of Txnip resulted in a cell cycle arrest [190]. Txnip was shown to induce a cell cycle arrest by affecting several cell cycle regulators like $p16^{Ink4a}$ [191] and $p27^{Kip1}$ [192]. The stability of $p27^{kip1}$, the cell cycle inhibitor, was found to be regulated by Txnip-Jab1 [192]. c-Jun activation domain-binding protein-1 (Jab1) controls the stability and intracellular distribution of $p27^{Kip1}$ [193]. Binding of Txnip to Jab1 prevents translocation of $p27^{kip1}$ to the cytoplasm. In HTLV infected T cells with reduced $p16^{Ink4a}$ expression, the loss of Txnip expression was required for progression towards adult T cell leukemia (ATL) [191]. Txnip, via interaction with thioredoxin, not only inhibits the activity of thioredoxin but also that of peroxiredoxins. Blocking activities of two different antioxidant enzymes might result in increased ROS production thereby resulting in oxidative stress and DNA damage in cells.

Injection of Graffi 1.4 murine leukemia virus causes AML in mice. Integration of the virus in the genome disrupts normal gene expression in cells. *Txnip* was identified as a common integration site in the insertional mutagenesis screens performed.
We investigated whether viral integrations in *Txnip*, deregulate *Txnip* expression and whether these integrations play an important role in leukemogenesis. In chapter 4 of this thesis we address these questions.
These studies are reported in Chapter 4.

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CHAPTER 2

Peroxiredoxin-controlled G-CSF signaling at the endoplasmic reticulum-early endosome interface

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ABSTRACT

Reactive oxygen species (ROS) regulate growth factor receptor signaling at least in part by inhibiting oxidation sensitive phosphatases. An emerging concept is that ROS act locally to affect signal transduction in different subcellular compartments and that ROS levels are regulated by antioxidant proteins at the same local level. Here, we show that the ER-resident antioxidant peroxiredoxin 4 (Prdx4) interacts with the cytoplasmic domain of the G-CSF receptor (G-CSFR). This interaction occurs when the activated G-CSFR resides in early endosomes. Prdx4 inhibits G-CSF-induced signaling and proliferation in myeloid progenitors, depending on its redox-active cysteine core. The protein tyrosine phosphatase 1b (Ptp1b) appears to be a major down stream effector controlling these responses. Conversely, Ptp1b may keep Prdx4 active by reducing its phosphorylation. These findings unveil a new signal transduction regulatory circuitry involving redox-controlled processes in the ER and activated cytokine receptors in endosomes.

INTRODUCTION

Reactive oxygen species (ROS) are generated by nicotinamide adenine dinucleotide phosphate oxidase (Nox) complexes. In phagocytes the Nox system mainly serves to produce high levels of H_2O_2 for the so-called oxidative burst, a major weapon in host defence against bacteria. In non-phagocytic cells, H_2O_2 has long been considered as an unwanted by-product of cell metabolism, potentially hazardous because of the damaging effects on proteins, lipids, carbohydrates and nucleic acids [1-2]. There is increasing evidence that moderate levels of ROS are pivotal for many cellular processes, including the control of cell proliferation, survival and differentiation. For example, H_2O_2 inactivates enzymes, in particular protein tyrosine and lipid phosphatases, involved in growth factor signaling [1]. As a consequence, the signal magnitude and duration from cell surface receptors, such as platelet-derived growth factor receptor (PDGFR), interleukin 3 receptor (IL-3R), granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) and G-CSFR is increased [3-5]. An important new insight that emerges is that ROS are produced in specific subcellular compartments and act locally to modulate signaling responses in different organelles [1,6].

In view of the local actions of ROS, an attractive hypothesis is that their levels are being controlled by nearby antioxidants, but evidence supporting this concept remains limited. Proteins of the peroxiredoxin (Prdx) family are major candidates for such local antioxidant activities [7]. Prdx proteins contain a core of usually 2 cysteines responsible for their redox activity [7]. Prdx1 and Prdx2 were found to associate with PDGFR and to modulate signaling by controlling H_2O_2 levels at the plasma membrane [8-9]. Importantly, PDGFR-induced tyrosine phosphorylation of Prdx1 temporarily reduced its antioxidant activity, thereby allowing a transient accumulation of H_2O_2 and inhibition of phosphatase activity in the vicinity of the plasma membrane [9]. Among the 6 mammalian Prdx proteins [7], Prdx1, 2, and 6 are cytosolic, Prdx3 mitochondrial and Prdx5 peroxisomal. The exact localization of Prdx4 has been somewhat ambiguous, but recent studies suggest that Prdx4 resides mainly in the endoplasmic reticulum (ER) [10-12].

Granulocyte colony-stimulating factor (G-CSF) is the major hematopoietic growth factor involved in the production of neutrophils [13-15]. G-CSF induces the proliferation, survival and neutrophilic differentiation of myeloid progenitor cells, cellular responses that require a balanced activation and subsequent attenuation of signaling pathways linked to the G-CSFR [15], a member of the cytokine receptor class I superfamily [16]. Signal attenuation of the G-CSFR is severely compromised by mutations causing truncations in the cytoplasmic domain of the G-CSFR, as observed in severe congenital neutropenia (SCN) patients showing disease progression to acute myeloid leukemia (AML) [17-19]. A major mechanism implicated in the perturbed signaling functions of these truncated G-CSFR mutants is the loss of appropriate receptor endocytosis and lysosomal routing. These processes are controlled by a dileucine-based internalization motif [20-21] and by receptor ubiquitination involving the suppressor of cytokine signaling protein SOCS3 [22-26].

Here, we investigated whether Prdx proteins control signaling from G-CSFR and in which subcellular compartment this takes place. In contrast to PDGFR, G-CSFR does not bind Prdx1 and Prdx2, but exclusively interacts with Prdx4. This interaction takes place during retrograde routing, when the activated G-CSFR resides in early endosomes. Prdx4 attenuates G-CSF-induced STAT activation and proliferation, and this depends on the integrity of its redox active cysteine core. The tyrosine phosphatase Ptp1b, known to reside in the ER [27-28], appears the major target for Prdx4-controlled modulation of G-CSF responses. In its turn, Ptp1b reduces Prdx4 phosphorylation, which by analogy to Prdx1 may keep Prdx4 in an active state. These findings identify the ER as an important signaling organelle controlling G-CSF responses of myeloid progenitors and provide insight into the complex interplay between redox-controlled processes and receptor signaling at the boundary between ER and retrograde endocytotic vesicles.

MATERIALS AND METHODS

PCR primers

Primers used for the preparation of constructs are listed in Table S1. All PCR products were checked for correct nucleotide sequences.

G-CSFR expression constructs

The G-CSFR WT and K5R expression constructs have been described previously [25]. To create G-CSFR-Prdx4 fusions, the G-CSFR part was amplified using primers Fw7 G-CSFR and Δ73Prdx4-Δ795GR Rv (Table S1). A glycine-glycine-serine (GGS) flexible linker was introduced between G-CSFR and Prdx4. For amplification of Δ73 Prdx4, primers Δ73Prdx4 Δ795GR Fw and Rv Prdx4 EcoRV were used. These fragments were used as a template for the fusion PCR, performed with primers Fw7 G-CSFR and Rv Prdx4 EcoRV. The fusion product was cloned as an EcoRV-HpaI fragment into the pBABE-wt-G-CSFR vector. Multisite-directed mutagenesis kit from Stratagene (Huissen, The Netherlands) was used to mutate both cysteines in the active site of Prdx4, using Δ795-G-CSFR- Δ73Prdx4 as a template.

Cells, retroviral transduction and transfection

Mouse bone marrow progenitor cells were isolated as described [29] and transduced with pBABE virus generated in Phoenix E cells expressing the different G-CSFR constructs. Cells were pre-cultured for two days in CellGro medium supplemented with IL-3 (10 ng/mL), Flt3-ligand (FL) (50 ng/mL), stem cell factor (SCF) (10 ng/mL) and thrombopoietin (Tpo) (10 ng/mL) [23]. 32D cells were transduced with the same retroviral constructs, as described [29]. For each construct, multiple independent clones were expanded for further analysis. Mouse embryonic fibroblasts were transduced with virus generated by transfection of Phoenix E cells with pBABE/ wt-G-CSFR. Cells expressing wt-GCSFR were selected us-

ing puromycin (1.5μg/mL) selection. HekT cells were transiently transfected using calcium phosphate precipitation, and HeLa cells were transfected using lipofectamine (Invitrogen, Breda, The Netherlands).

Mammalian Protein-Protein Interaction Trap (MAPPIT) assay

Bait Constructs. G-CSFR fragments were cloned in-frame with the MAPPIT bait receptor, consisting of the extracellular domain of the EPO receptor and the cytoplasmic domain of leptin receptor lacking Stat3-binding sites, as described [29].

Prey Constructs. Prdx1, 2, 4 and 6 sequences were amplified from HL60 cells using forward primers with a 5`EcoRI site, followed by the respective Prdx sequence. The reverse primers were designed with an XhoI restriction site 3'of the STOP codon. The Prdx fragments were cloned into the pMG2 prey vector [30], thus generating the FLAG-tagged Prdx-gp130 fusion constructs. Prey-bait interactions were quantified in Stat3 luciferase assays as described [29-30]. In brief, HekT cells (2 x 10^5) were transfected with bait and prey constructs along with a luciferase reporter (pXP2d2-rPAP-Luci). 48 hours after transfection, the chimeric bait receptors were activated with Epo (0.5 U/ml) for 24 hours or left unstimulated. Luciferase activity from Stat3 luciferase reporter was determined using the Steady Glo luciferase assay system (Promega, Leiden, The Netherlands).

Antibodies and fluorescent reagents

Prdx4 rabbit polyclonal (Ab15574), Prdx4 mouse monoclonal (Ab16943) and GRP94 rabbit polyclonal (Ab3674) antibodies were purchased from Abcam (Cambridge, UK). Goat polyclonal antibodies against EEA1 (sc6414) and β-actin (sc1616) were purchased from Santa Cruz Biotechnology Inc, (Santa Cruz, CA, USA). Mouse anti-human G-CSFR (CD114) was from Becton Dickinson (Franklin Lakes, NJ, USA); p-Stat3 (Tyr 705 and Ser 727), p-Akt and phospho-Jak2 (Y1007/1008) antibodies were from Cell Signaling Inc (Danvers, MA, USA) and anti phosphotyrosine 4G10 and 4G10-biotin antibodies from Millipore (Billerica, MA, USA). The latter were visualized on Western blots using Streptavidin-IRDye800CW (LI-COR, Lincoln, NE, USA). Secondary donkey anti- rabbit Cy3 and donkey anti-mouse Cy5 antibodies used for confocal imaging were from Jackson Immunoresearch (Suffolk, UK). ERGIC-53 rabbit polyclonal antibody (E1031) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Immune precipitations, Bio-G-CSF pull-downs and western blotting

HekT cells transfected with pBABE/G-CSFR were placed for 4 hrs in DMEM without FCS and then stimulated with G-CSF (100ng/mL). After stimulation, cells were lysed at 4°C in lysis buffer (50mM Tris-HCL pH 7.4, 50mM NaCl, 10% glycerol, 1% NP40, 0.5% Na-deoxycholate, 20mM NaF) containing a cocktail of protease inhibitors. Lysates were incubated overnight at 4°C with pre-washed Protein G Dynabeads (Invitrogen/ DYNAL) coated with Prdx4 mouse

monoclonal antibody purchased from Abcam. Immune complexes were visualized by western blotting. To study tyrosine phosphorylation of the G-CSFR, pull-down of G-CSFR using biotinylated G-CSF and streptavidin-coated beads was performed as described [26].

Confocal microscopy scanning microscopy (CLSM)

HeLa cells transiently transfected with pLNCX2/G-CSFR (WT) were used for CLSM. Forty-eight hours after transfection, HeLa cells were growth factor- and serum- deprived by incubation for 4 hrs in DMEM. G-CSFR antibody (2.5μg/mL) was added to the medium and incubated at room temperature for 20 min to allow binding of the antibody to the extracellular domain of the G-CSFR. Excess antibody was washed off and cells were stimulated with G-CSF for different time periods. Immune-staining was performed as described previously [25]. Cells were imaged using the multi-track detection mode on a Zeiss LSM 519 confocal microscope equipped with Argon/HeNe lasers using a 63x Planochromat oil immersion objective.

Colony assays

Retrovirally transduced Csf3r$^{-/-}$ bone marrow cells. Forty-eight hours after retroviral infection, cells were harvested and placed in methocult (M3231, Stem Cell Technologies Inc., Vancouver, Canada) containing puromycin (1.5 μg/mL) and either human recombinant G-CSF (100 ng/mL, Amgen, Breda, The Netherlands) or mouse GM-CSF (20 U/mL, Peprotech Inc., USA). All cultures were done in triplicate and colonies were counted on day 7 of culture.

Prdx4$^{-/-}$ *and Ptp1b$^{-/-}$ bone marrow cells.* Femurs, tibiae and sterna from *Prdx4$^{-/-}$* [31], *Ptp1b$^{-/-}$* and age and sex matched control mice [32] were shipped on ice from the Fujii and Neel labs. Bone marrow mononuclear cells were obtained as described [33] and cultured in colony assays with different concentrations of G-CSF or one standard concentration of GM-CSF (10 ng/mL).

RESULTS

Prdx4 interacts with the G-CSFR

In an earlier yeast two hybrid screen, we identified peroxiredoxins as putative G-CSFR-interacting proteins. To assess whether these interactions occur in mammalian cells, we used the mammalian protein-protein interaction trap (MAPPIT) assay [30]. Bait and prey constructs are shown in Figure 1A. Prdx3 and Prdx5 were excluded from this analysis because of their mitochondrial and peroxisomal localizations, respectively. After a series of standard positive and negative controls to assure specificity of the system (Figure S1), we performed experiments with the Prdx prey constructs and found that Prdx4, but not Prdx1, 2, or 6, interacted with the G-CSFR C-terminus (Figure 1B). Using additional bait constructs, we identified the distal region spanning amino acids 792-813 as the major PRDX4 binding site (Figure 1C). This region does not show sequence homology with other cytokine receptors. To assess whether the integrity of the cysteine core of Prdx4 is required for its interaction with G-CSFR, we generated Prdx4 prey constructs in which both cysteines in the active site were changed into serines (Prdx4mut). Prdx4 and Prdx4mut prey constructs showed comparable binding to the G-CSFR baits, implying that the redox status of Prdx4 does not affect this interaction (Figure 1D). Prdx4 differs from the other family members in that its N-terminus has a 73 amino acid extension, including a predicted signal peptide of 37 amino acids. Deletion of the N-terminal region abolished MAPPIT activity, suggesting that this region is predominantly responsible for Prdx4 binding to the G-CSFR (Figure 1E). Conversely, a prey construct consisting of only the N-terminal 73 amino acids (N73a.a.-Prdx4) interacted with the G-CSFR bait, confirming the importance of the Prdx4 N-terminus for G-CSFR binding (Figure 1F). In immune precipitation experiments, G-CSFR co-precipitated with endogenous Prdx4 from lysates of HEK293 cells ectopically expressing WT G-CSFR, confirming the results from the MAPPIT assay. However, under these conditions basal Prdx4 binding to G-CSFR was seen, indicating that the physical interaction between G-CSFR and Prdx4 proteins *per se* does not depend on receptor activation (Figure S2).

Figure 1

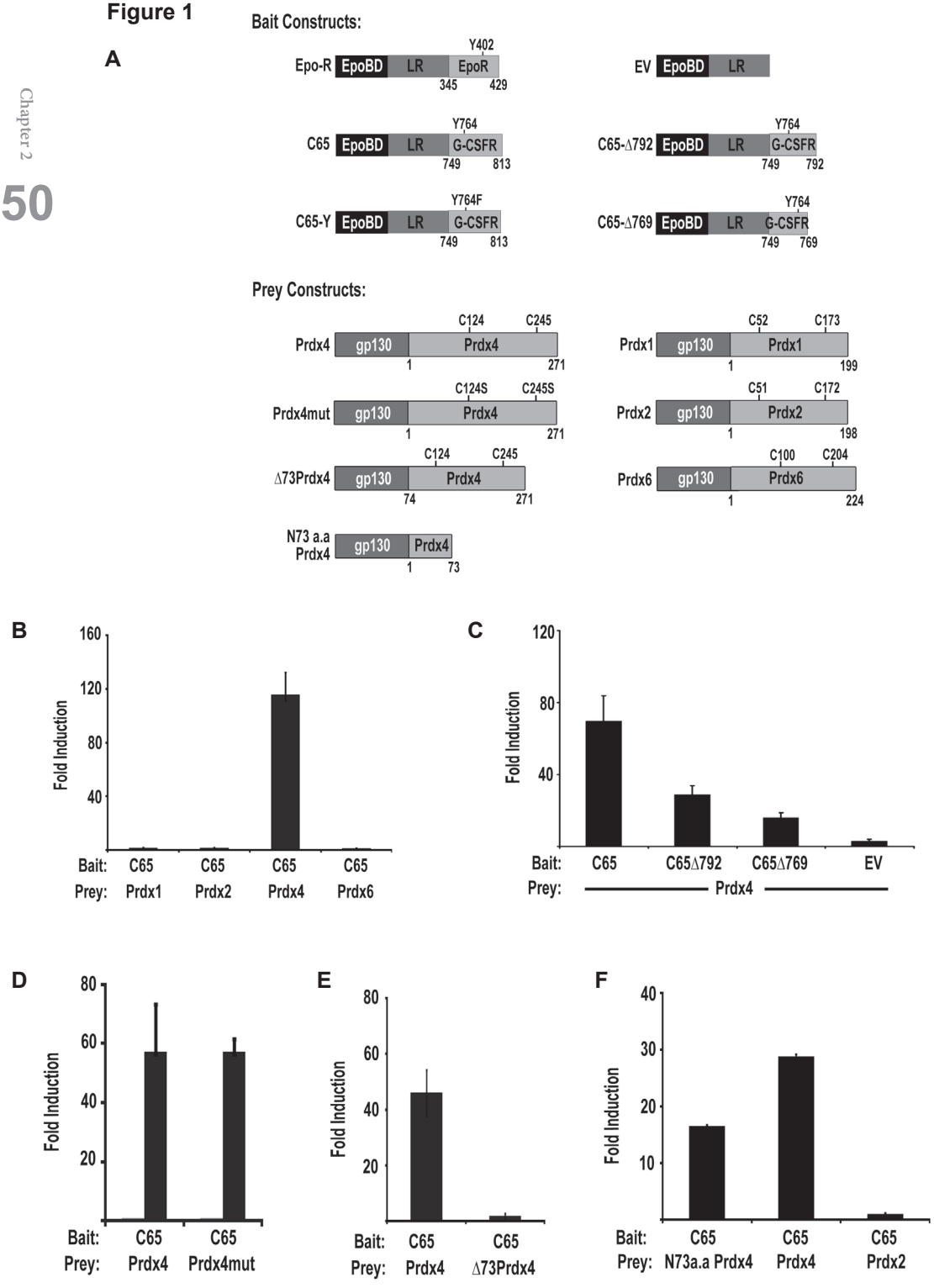
A
Bait Constructs:
Epo-R EpoBD LR EpoR Y402 345 429
EV EpoBD LR
C65 EpoBD LR G-CSFR Y764 749 813
C65-Δ792 EpoBD LR G-CSFR Y764 749 792
C65-Y EpoBD LR G-CSFR Y764F 749 813
C65-Δ769 EpoBD LR G-CSFR Y764 749 769
Prey Constructs:
Prdx4 gp130 Prdx4 C124 C245 1 271
Prdx1 gp130 Prdx1 C52 C173 1 199
Prdx4mut gp130 Prdx4 C124S C245S 1 271
Prdx2 gp130 Prdx2 C51 C172 1 198
Δ73Prdx4 gp130 Prdx4 C124 C245 74 271
Prdx6 gp130 Prdx6 C100 C204 1 224
N73 a.a Prdx4 gp130 Prdx4 1 73
B
Fold Induction
160
120
80
40
Bait: C65 C65 C65 C65
Prey: Prdx1 Prdx2 Prdx4 Prdx6
C
Fold Induction
120
80
40
Bait: C65 C65Δ792 C65Δ769 EV
Prey: Prdx4
D
Fold Induction
80
60
40
20
Bait: C65 C65
Prey: Prdx4 Prdx4mut
E
Fold Induction
80
60
40
20
Bait: C65 C65
Prey: Prdx4 Δ73Prdx4
F
Fold Induction
40
30
20
10
Bait: C65 C65 C65
Prey: N73a.a Prdx4 Prdx4 Prdx2

Figure 1. MAPPIT assay of Prdx/G-CSFR interactions
(A) Schematic representation of bait and prey MAPPIT constructs. **(B)** MAPPIT with G-CSFR-C65 (a.a. 749-813) bait and Prdx1, Prdx2, Prdx4 and Prdx6 prey constructs showing specific interaction with Prdx4. **(C)** Mapping of domains of the G-CSFR involved in Prdx4 binding. FL: full length G-CSFR cytoplasmic domain; 686-735 and 736-774 represent isolated amino acid stretches within the G-CSFR cytoplasmic domain; C65Δ mutants represent C-terminal deletions of C-CSFR-C65 bait; EV: empty bait vector control. **(D)** MAPPIT showing that cysteines in the active sites of Prdx4 (Cys 124 and Cys 245) are not involved in interaction of Prdx4 with G-CSFR-C65. **(E)** Loss of Prdx4 binding to G-CSFR upon deletion of the specific extended N-terminus of Prdx4 (Δ73Prdx4). **(F)** Binding of the N-terminal region of Prdx4 (N73 a.a Prdx4) to C65-G-CSFR. Full length Prdx4 and Prdx2-containing preys were included as positive and negative controls, respectively.

Co-localization of endocytosed G-CSFR with Prdx4 residing in the ER/ERGIC

We subsequently used confocal microscopy to study where Prdx4 and G-CSFR interact in intact cells. Prdx4 did not co-localize with G-CSFR at the cell surface. Thirty minutes after G-CSF treatment, when G-CSFR resided in EEA1 positive early endosomes, co-localization with Prdx4 was maximal and declined after 1 hr, when the G-CSFR was present in late endosomes and lysosomes (Figure 2A and Figure S3). In contrast, co-localization of the lysosomal routing defective G-CSFR mutant K5R persisted at 60 min after G-CSF stimulation, confirming that Prdx4 interacts with G-CSFR localized in early endosomes [25-26] (Figure S4a). Quantification of these data is shown in Figure S4b. As expected based on the MAPPIT experiments, no significant co-localization of Prdx2 and G-CSFR was detectable, neither at the plasma membrane nor in endocytotic vesicles (Figure S5). Because Prdx4 was detected mainly in the ER and ER-Golgi intermediate compartment (ERGIC), with only a minor fraction in the Golgi (Figure S6), these findings imply that the interaction between G-CSFR and Prdx4 takes place when the endocytosed G-CSFR complexes are in proximity of the ER/ERGIC. Supporting this, G-CSFR also co-stained with ER marker Grp94 and with ERGIC marker ERGIC-53 after ligand-induced internalization (Figure 2B).

Prdx4 inhibition of G-CSF-induced proliferation of myeloid progenitor cells requires the integrity of its cysteine core

To directly study the functional implications of Prdx4 binding on G-CSFR signaling and to determine the role of the redox-active cysteines herein, we fused the catalytic domain of Prdx4 and the mutant domain missing the critical cysteines directly to G-CSFR-Δ795. G-CSFR-Δ795 was chosen because it lacks a major C-terminal domain responsible for endogenous Prdx4 binding as identified by MAPPIT (Figure 3A). Although a drawback of this approach is that the interaction with Prdx4 is constitutive, rather than temporal and dependent on endocytotic routing, routing of the G-CSFR towards the ER/ERGIC was not affected by the Prdx4 fusion (Figure S7A). Hence, Prdx4 is still able to function in this setting at the ER-early endosome interface. We first transduced 32D cells with these constructs and selected clones with comparable expression of WT G-CSFR, G-CSFR-Δ795, G-CSFR-Δ795-Prdx4 and G-CSFR-Δ795-Prdx4mut at the plasma membrane (Figure S7B), allowing comparisons of their signaling abilities at equal receptor densities. Relative to 32D/WT, 32D/Δ795 clones

Figure 2

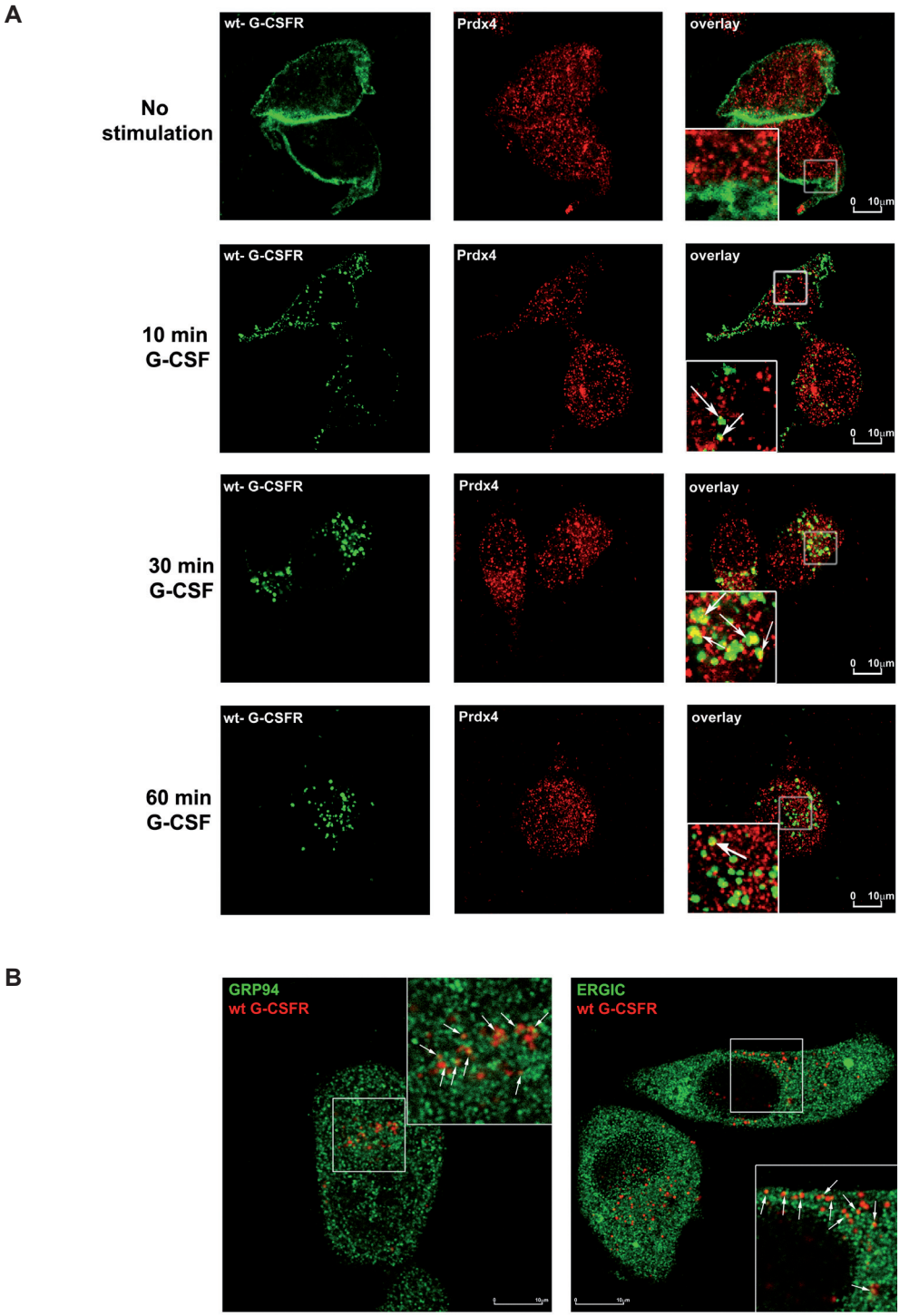
A
wt- G-CSFR
Prdx4
overlay
No stimulation
10 min G-CSF
30 min G-CSF
60 min G-CSF
0 10µm
B
GRP94
wt G-CSFR
ERGIC
wt G-CSFR

Figure 2. G-CSFR and Prdx4 interaction and co-localization
(A) HeLa cells ectopically expressing WT G-CSFR were growth factor-deprived for 4 hrs. Surface membrane G-CSFRs were labelled with anti-G-CSFR antibody prior to stimulation with G-CSF for different time points. Cells were permeabilised, fixed and stained with anti-Prdx4 antibody, followed by secondary and anti-rabbit Cy3 and anti-mouse Cy5 antibodies, and analyzed by CLSM. **(B)** Co-localization of endocytosed G-CSFR with Grp94-stained ER and with ERGIC 30 min. after ligand stimulation. Experimental conditions were the same as under B.

showed increased proliferation in response to G-CSF, whereas the proliferation rate of 32D/Δ795-Prdx4 clones was similar to 32D/WT clones (Figure 3B). It contrast, 32D/Δ795-Prdx4mut clones showed the same elevated proliferation rate as and 32D/Δ795 clones, indicating that the redox-active thiol group of Prdx4 confers growth inhibition (Figure 3B). No differences in proliferation rates of these clones were seen in interleukin 3-containing cultures (not shown). To extend these observations to primary myeloid progenitors, we transduced bone marrow cells from *Csf3r*$^{-/-}$ mice with the above-mentioned G-CSFR constructs using the BABE retroviral vector (conferring puromycin resistance), and cultured these cells in colony cultures supplemented with G-CSF or GM-CSF and puromycin. GM-CSF-induced colonies did not differ significantly between the constructs, indicative of comparable transduction efficiencies, (Figure 3C). In line with the findings in 32D cells, G-CSFR-Δ795-expressing BM cells produced higher numbers and greater-sized colonies, whereas G-CSFR-Δ795-Prdx4-expressing BM cells generated significantly fewer and smaller colonies in response to G-CSF compared to wt G-CSFR-expressing BM cells (Figure 3C). As in 32D cells, this effect of Prdx4 depended on the integrity of the redox active cysteines.

Prdx4 attenuates G-CSF-induced STAT3 activation

To identify the signaling pathways that are modulated by Prdx4, lysates of 32D clones expressing the different G-CSFR forms were subjected to western blotting with phospho-specific antibodies to detect activation of Stat3, Stat5 and Akt. In experiments in which cells were first growth factor and serum deprived and then stimulated with G-CSF for up to 120 min, fusion of active Prdx4, but not the inactive mutant, inhibited Stat3 phosphorylation (Figure 4A). In long-term cultures, in which cells were switched from IL3- to G-CSF-containing culture medium and lysates made every 2 days, p-Stat3 levels were again markedly reduced in 32D/Δ795-Prdx4 clones compared to 32D/Δ795-Prdx4mut (Figure 4B). In contrast, no significant differences were seen in Stat5 and Akt phosphorylation (not shown). A possible explanation for this difference is that G-CSF-induced Stat3 activation requires recruitment of Stat3 to phosphotyrosines in the G-CSFR membrane distal cytoplasmic region, whereas Stat5 and Akt activation occurs through the membrane proximal G-CSFR region without the involvement of receptor tyrosines [34-36]. Moreover, Akt phosphorylation is already downregulated early in G-CSFR endocytosis, i.e., before endosome-ER contacts take place and Prdx4 modulates G-CSFR signaling[25].

Figure 3

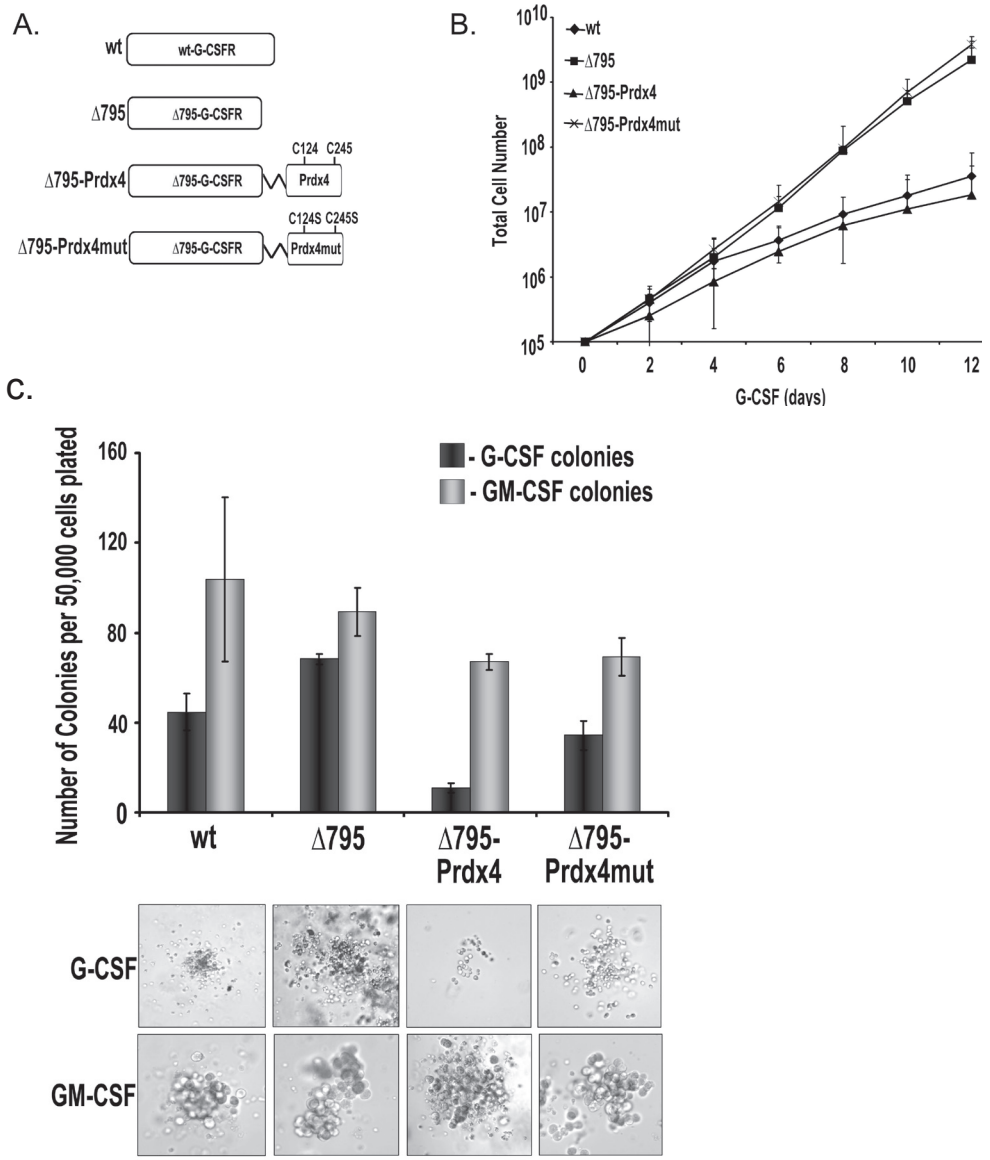

Figure 3. Prdx4 attenuates G-CSF-induced proliferation in myeloid progenitors depending on its active cysteine core

(A) Schematic representation of G-CSFR-Prdx4 fusion constructs. For details see Material and Methods. **(B)** Proliferation of 32D cells expressing constructs shown in panel A. Cells were cultured in 10ng/ml G-CSF. Data represent the means ± standard deviation of 5 independent clones for each transfectant. **(C)** Colony assay of *csf3r*-/- bone marrow cells transduced with constructs shown in panel A. Transduced bone marrow cells (50,000) were plated in triplicate in medium containing either G-CSF or GM-CSF and puromycin as a selection marker. Colonies were counted on day 7 of culture (upper panel). Data are expressed as the mean of triplicate cultures ± s.d. Lower panel: representative photomicrographs showing differences in colony size.

Figure 4

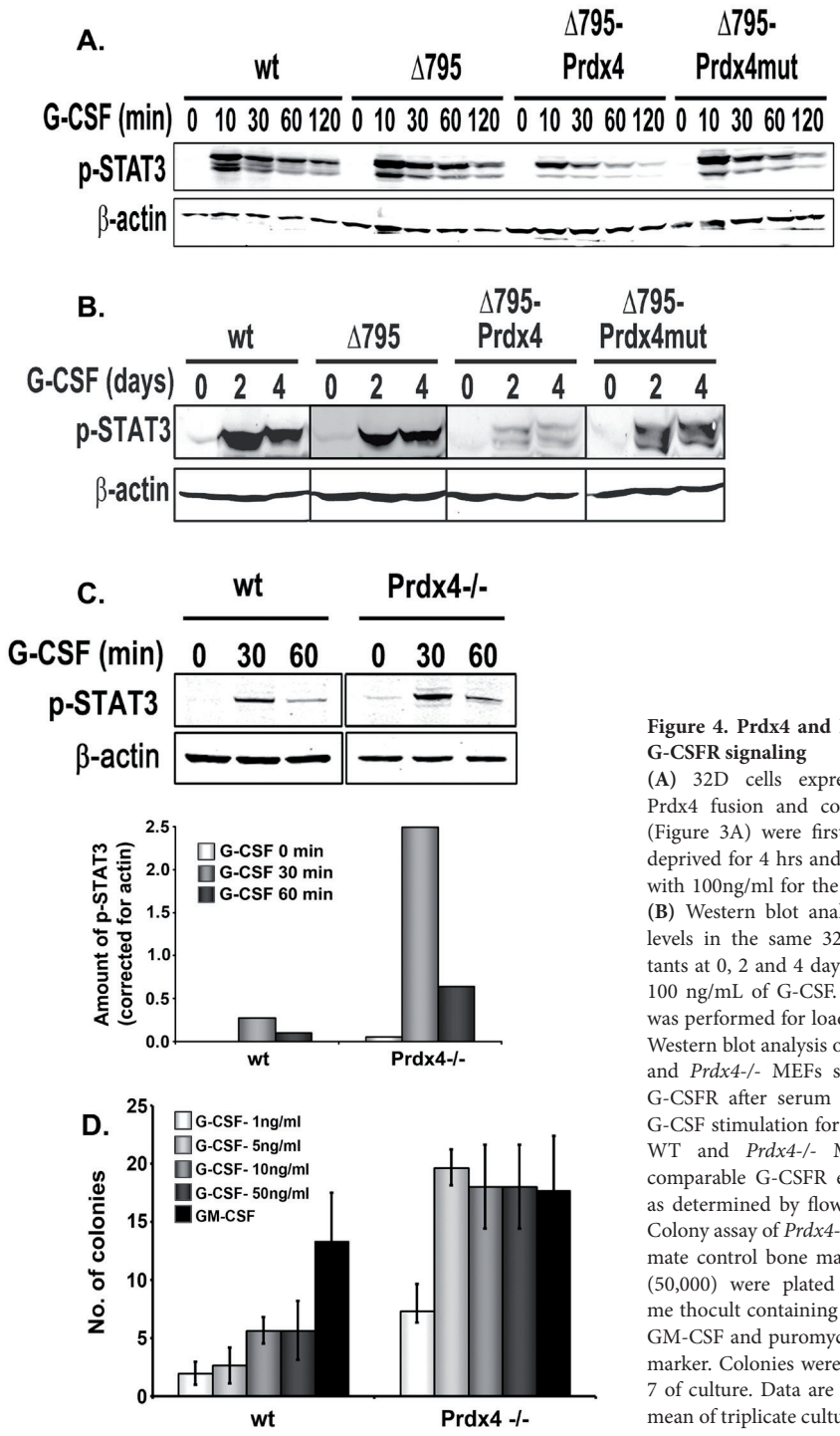

Figure 4. Prdx4 and Ptp1b attenuate G-CSFR signaling

(A) 32D cells expressing G-CSFR-Prdx4 fusion and control constructs (Figure 3A) were first growth factor-deprived for 4 hrs and then stimulated with 100ng/ml for the indicated times. **(B)** Western blot analysis of p- Stat3 levels in the same 32D cell transfectants at 0, 2 and 4 days of culture with 100 ng/mL of G-CSF. b-actin staining was performed for loading control. **(C)** Western blot analysis of p- Stat3 in WT and *Prdx4-/-* MEFs stably expressing G-CSFR after serum deprivation and G-CSF stimulation for indicated times. WT and *Prdx4-/-* MEFs expressed comparable G-CSFR expression levels as determined by flow cytometry. **(D)** Colony assay of *Prdx4-/-* and WT littermate control bone marrow cells. Cells (50,000) were plated in triplicate in me thocult containing either G-CSF or GM-CSF and puromycin as a selection marker. Colonies were counted on day 7 of culture. Data are expressed as the mean of triplicate cultures ± s.d.

Increased G-CSF-induced Stat3 activation and myeloid colony formation in *Prdx4*$^{-/-}$ cells

Having shown that enforced interaction of Prdx4 with G-CSFR attenuates G-CSF signaling in myeloid progenitors, we investigated how depletion of Prdx4 affects G-CSF signaling. To this end, we transduced *Prdx4*$^{-/-}$ and wild type mouse embryonic fibroblasts (MEFs) with the G-CSFR and assessed phosphorylation of Stat3, Stat5 and Akt. Phospho-Stat3 levels were significantly increased in *Prdx4*$^{-/-}$ cells relative to wild type controls (Figure 4C). Again, phospho-Akt levels did not significantly differ between *Prdx4* deficient versus proficient cells, while phospho-Stat5 was below detection level in these cells (data not shown). To address whether *Prdx4* deficiency affects G-CSF signaling in primary hematopoietic cells, we performed colony cultures with increasing concentrations of G-CSF. *Prdx4*$^{-/-}$ bone marrow cells yielded significantly higher numbers of colonies relative to wild type littermate control cells at different concentrations of G-CSF (Figure 4D). In contrast, GM-CSF-induced colony growth did not differ between *Prdx4*-deficient and wild type bone marrow cells, showing that G-CSF, but not GM-CSF, signaling is controlled by Prdx4.

Ptp1b inhibits G-CSF-induced signaling and proliferation of myeloid progenitors

Ptp1b resides at the ER [28, 37], is highly sensitive to oxidation [38] and is therefore a likely down stream effector of Prdx4-modulated signaling. We confirmed the co-localization of Ptp1b and Prdx4 in the ER and ERGIC compartments in primary bone marrow cells (Figure S8). Biotinylated G-CSF pull-down showed that Ptp1b interacts with G-CSFR (Figure 5A). Western blot analysis in *Ptp1b*$^{-/-}$ and *Ptp1b* reconstituted MEFs transduced with G-CSFR showed significantly elevated G-CSF-induced Jak2 and Stat3 phosphorylation in the *Ptp1b*$^{-/-}$cells (Figure 5B). Similar to *Prdx4*-deficient cells, phospho-Akt levels were unaffected by *Ptp1b* depletion (Figure 5B). Colony cultures with *Ptp1b*$^{-/-}$ bone marrow cells also yielded results comparable to those obtained with *Prdx4*-deficient cells, showing elevated numbers and significantly greater-sized colonies in response to G-CSF, with GM-CSF-induced colony formation unchanged relative to *Ptp1b*$^{+/+}$ controls (Figure 5C).

Ptp1b reduces phosphorylation of both G-CSFR and Prdx4

We then asked whether Ptp1b controls ligand-induced G-CSFR phosphorylation. For this, we performed off-rate experiments, in which cells were stimulated for 10 min with G-CSF, where after the cells were washed and further cultured without growth factor. Tyrosine phosphorylation of G-CSFR was elevated and prolonged in *Ptp1b*-/- MEFs compared to the reconstituted control cells (Figure 6A). Supporting the notion that Ptp1b reduces G-CSFR phosphorylation in early endosomes, this difference was even more pronounced in cells expressing G-CSFR mutant K5R (Figure 6B). Finally, because phosphorylation of Prdx1 has been reported to decrease its activity [9], we investigated whether Ptp1b controls the phosphorylation status of Prdx4. Prdx4 was clearly hyperphosphorylated in *Ptp1b* deficient MEFs compared to reconstituted cells (Figure 6C). This was also evident in the absence of G-CSF stimulation (Figure 6C), indicating that receptor signaling is not required for Ptp1b-controlled phosphorylation of Prdx4.

Figure 5

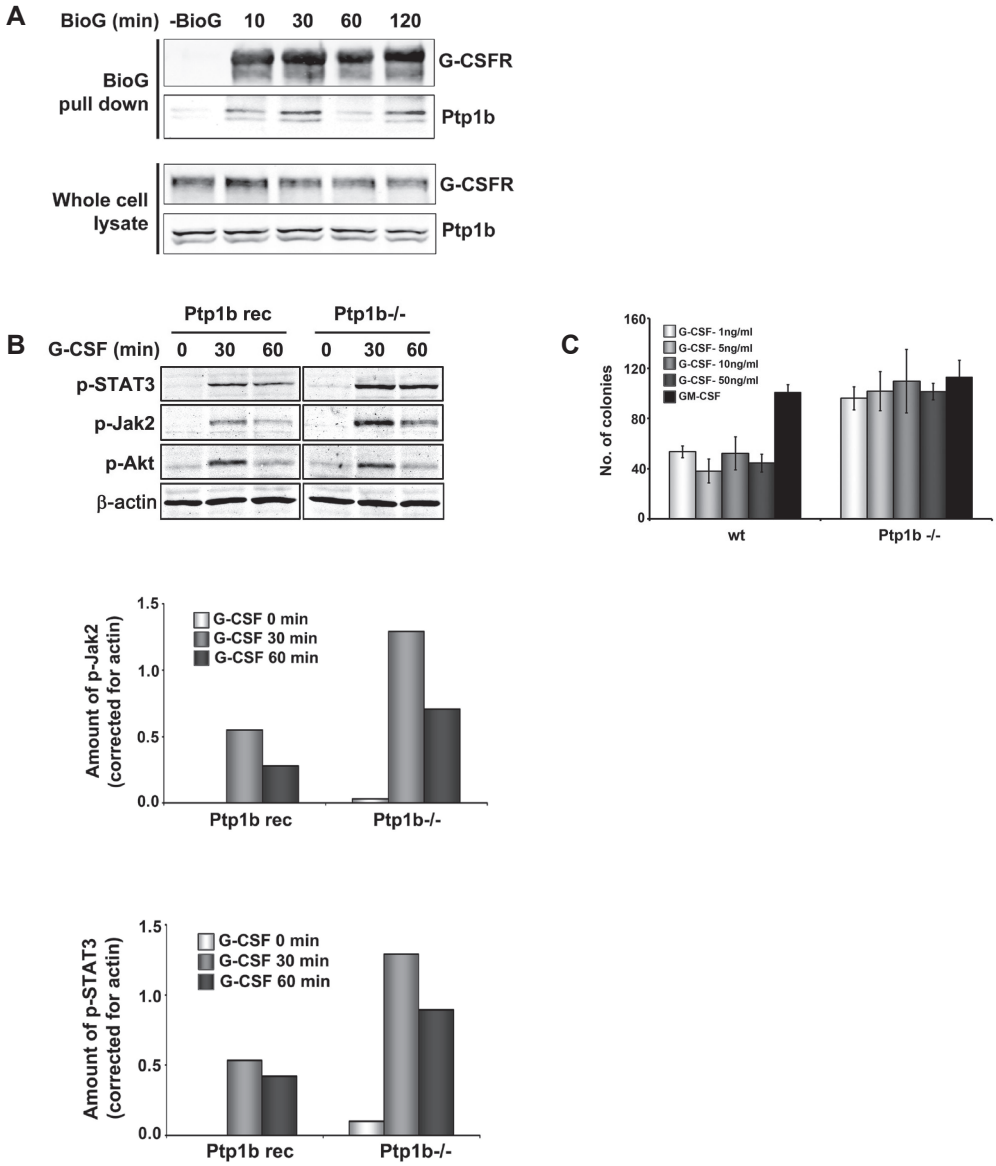

Figure 5. Ptp1b interacts with G-CSFR and attenuates signaling
(A) Ptp1b immunoprecipitations from Hek cells transfected with lysine-less pBABE-K5R-G-CSFR and pJ3H-Ptp1b-HA constructs. Cells were growth factor-deprived for 4 hrs, followed by stimulation with biotinylated G-CSF for the indicated times. Precipitates were collected on streptavidin-coated beads. Blots were stained for G-CSFR and Ptp1b. TCL: total cell lysate. **(B)** Western blot analysis of p-Stat3, p-Jak2 and p-Akt in *Ptp1b-/-* and reconstituted MEFs stably expressing G-CSFR; stimulation conditions as in Figure 4C. b-actin staining served as loading control in all experiments. *Ptp1b-/-* and reconstituted MEFs expressed comparable G-CSFR expression levels as determined by flow cytometry. **(C)** Colony assays of *Ptp1b-/-* and WT littermate control bone marrow cells. Culture conditions were similar to those described in panel A. Photomicrographs in lower panel show differences in colony size from cultures of panel B.

Figure 6

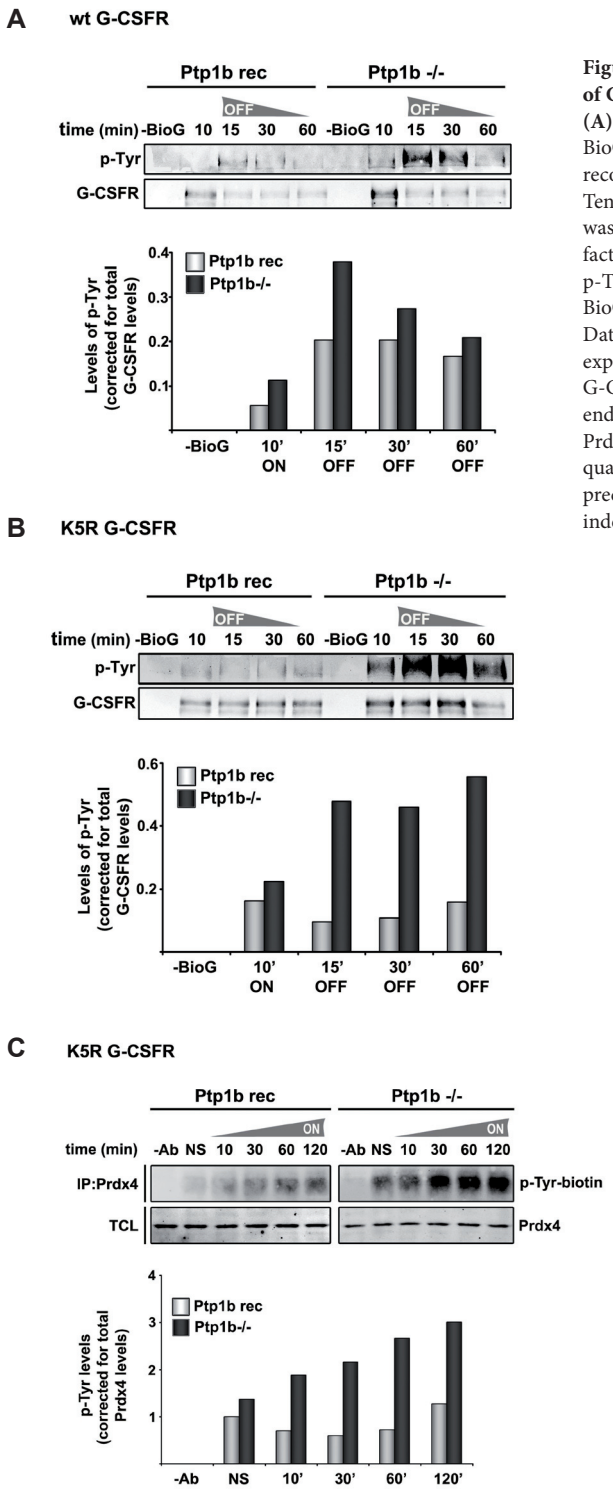

Figure 6. Increased tyrosine phosphorylation of G-CSFR and Prdx4 in Ptp1b deficient cells
(A) Phosphotyrosine analysis of G-CSFR after BioG-CSF pull down in *Ptp1b-/-* or *Ptp1b* reconstituted MEFs expressing wt-G-CSFR. Ten min after addition of Bio-G-CSF, cells were washed and further cultured without growth factor. Histograms show quantifications of p-Tyr levels relative to total G-CSFR protein in BioG-CSF pull downs at the indicated times. Data shown are representative of 3 independent experiments. **(B)** Similar to (A) with lysine-less G-CSFR mutant K5R that accumulates in early endosomes. **(C)** Phosphotyrosine analysis of Prdx4 immunoprecipitates. Histograms show quantifications of p-Tyr levels relative immunoprecipitated Prdx4. Data are representative of 2 independent experiments. TCL: total cell lysate.

DISCUSSION

The key finding reported here is that the antioxidant protein Prdx4, localized in the ER, attenuates G-CSFR signaling from early endosomes. In addition, we provided evidence to suggest that this is achieved by preventing the loss of activity of the ER-resident tyrosine phosphatase Ptp1b. Recent studies have shown that different Nox complexes allocate to specific subcellular compartments [39-43] and act in spatially restricted microdomains, which is thought to be essential for specificity of ROS-mediated signaling [6]. For instance, the local action of Nox2 in a signaling module was demonstrated for interleukin-1 (IL-1) signaling: after activation of the IL-1 receptor, Nox2 controlled the binding of TRAF6 to the IL1R/MyD88 complex in early endosomes [44]. Nox4, on the other hand has been shown to drive ROS signaling from the ER [43, 45]. A recent study on interleukin-4 (IL-4) receptor showed that ROS promotes IL-4 signaling by inhibition of Ptp1b [46]. Furthermore, Nox4 was shown to be responsible for ROS production in the ER and to be critical for the regulation Ptp1b, further illustrating specificity of intracellular ROS-controlled signaling depending on the localization of Nox isoforms within particular subcellular compartments [45]. Based on our findings and these recent reports, we propose a model in which Prdx4 negatively controls G-CSF signaling by neutralizing ROS produced by Nox4 in the ER, thereby keeping Ptp1b in an active state (Figure 7). Conversely, Ptp1b inhibits tyrosine phosphorylation of Prdx4, which by analogy to Prdx1, may increase its activity [9]. Whether G-CSFR and Prdx4 are direct substrates of Ptp1b or whether Ptp1b acts mainly through dephosphorylation and inhibition of Jaks (Figure 5B and shown previously for interferon and leptin signaling [47-48]) is presently unknown. The latter appears the most likely, given that neither the G-CSFR cytoplasmic domain nor Prdx4 contains the Ptp1b recognition motif E/D-pY-pY-R/K present in Jak2 and Tyk2 [47, 49] Another still open question is whether Prdx4, in addition to its antioxidant function, acts as a tethering molecule, thereby stabilizing the interaction between G-CSFR and Ptp1b at the ER-early endosome interface.

Our observation that Prdx4 co-localizes with the ER/ERGIC corroborates with an earlier report [12]. Based on structure prediction and resistance to proteinase K digestion, this study also suggested that Prdx4 resides inside the ER lumen [12]. Although this would be in apparent conflict with an interaction of Prdx4 with the G-CSFR cytoplasmic domain, proteins residing in the ER may retrotranslocate from the lumen and enter the cytoplasm. For instance this was demonstrated for the protein chaperone calreticulin [37, 50]. A similar process could explain how Prdx4 exits the ER lumen and binds to the cytosolic tail of G-CSFR. Possibly, an interaction with the ER lipid bilayer or with binding proteins located herein would keep Prdx4 in proximity of the ER. Notably, in immunoprecipitations, G-CSFR also co-precipitated with Prdx4 in the absence of growth factor (Figure S2). This interaction might either occur after constitutive retrograde routing of G-CSFR, between Prdx4 and G-CSFR residing in forward-routing vesicles or both, but whether this has any functional significance is presently unclear.

In conclusion, our study suggests that the antioxidant protein Prdx4 negatively controls G-CSF signaling in the early endosome compartment by reducing ROS levels in proximity of the ER, thereby keeping Ptp1b active. As a consequence, the tyrosine-based Stat3 docking sites in the G-CSFR are dephosphorylated, resulting in attenuation of Stat3 activation. Intriguingly, the G-CSFR truncation mutants found in SCN/AML respond differentially to SOCS3 in terms of Stat3 versus Stat5 inhibition: whereas SOCS3-mediated inhibition of Stat5 activation is abolished as a result of the G-CSFR truncation, inhibition of Stat3 remained largely intact [51]. This discrepancy relates to the fact that SOCS3-induced Stat5 inhibition entirely depends on SOCS box-mediated ubiquitination of G-CSFR, whereas SOCS3-induced Stat3 inhibition is less dependent on this process [25-26, 51]. Together with these previous results our current data suggest that, whereas attenuation of Stat5 mainly depends on lysosomal degradation of the activated G-CSFR, Stat3 inhibition is mediated mainly by dephosphorylation of Stat3-binding tyrosine motifs controlled by the kinase inhibitory region of SOCS3 and by Ptp1b, when the G-CSFR resides in the early endosome. These findings support the idea that signal diversification from certain growth factor receptors is to a major extent determined by routing dynamics, a concept that becomes increasingly attractive to explain how growth factor receptors exerts specific functions, while activating largely overlapping signaling pathways. The dynamic interplay between ER-localized enzyme systems and activated receptors in endocytotic vesicles turns out to be a key event in this process.

Deregulation of redox-controlled signaling pathways is increasingly implicated in a variety of diseases, including leukemia. For instance, the gene encoding Thioredoxin inhibitory protein (Txnip), a common target for retroviral integration in murine leukemia virus-induced mouse leukemia, appeared to be significantly upregulated in a subgroup of human AML patients [52]. A case of AML with a t(X;21)(p22;q22) has been reported in which the *PRDX4* gene located on Xp22 was fused to *RUNX1* at 21q22, resulting in a *RUNX1-PRDX4* fusion transcript [53]. We screened 65 MDS patients and 113 AML patients for possible mutations, but no mutations in the *PRDX4* coding region were detected, suggesting that genomic aberrations affecting *PRDX4* are rare in MDS/AML [54]. Intriguingly, this study also showed that *PRDX4* expression is significantly down regulated in acute promyelocytic leukemia (APL) cells, involving H3K27 tri-methylation as a mechanism of histone-mediated gene silencing [54]. Although the role of PRDX4 down regulation in primary APL stem and progenitor cells remains to be established, predictably the loss of PRDX4 may lead to reduced ER-linked PTP1B activity, providing an explanation for the increased responsiveness of APL clonogenic precursors to G-CSF [55].

Figure 7

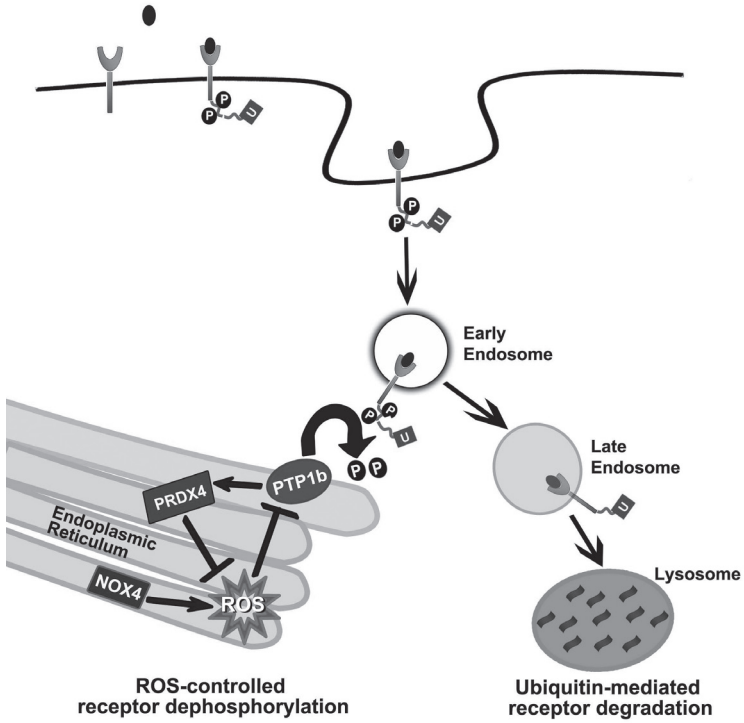

Figure 7. Model of Prdx4-controlled G-CSF signaling
Activation of G-CSFR leads to internalization and entry in early endosomes. G-CSFR dephosphorylation is then mediated by Ptp1b, which requires endosomal trafficking of the G-CSFR towards the ER, where Ptp1b resides. Ptp1b activity is inhibited by ROS, which is locally produced in the ER by Nox4 [45]. ER-resident Prdx4 reduces ROS, thereby elevating Ptp1b activity. After or during dephosphorylation in early endosomes, G-CSFR ubiquitination takes place [26], which triggers routing to late endosomes and lysosomes, where G-CSFR are degraded. P: phosphate; U: ubiquitin.

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SUPPLEMENTARY DATA

S1

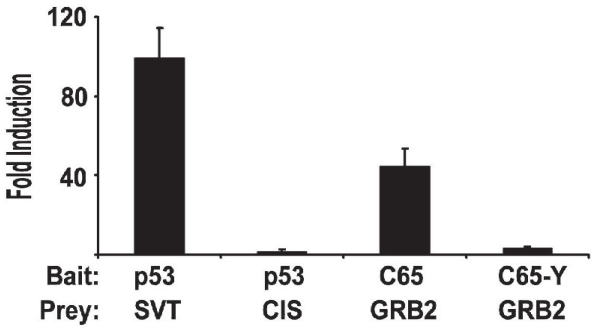

Figure S1: MAPPIT with control bait and prey constructs to demonstrate specific interactions. Bait-prey combinations p53-SVT and p53-CIS are general positive and negative MAPPIT controls, respectively. Interactions of G-CSFR C65 with Grb2, and C-Y (lacking Grb2-binding Tyr764 of the G-CSFR) with Grb2 are shown to support binding specificity in the context of G-CSFR.

S2

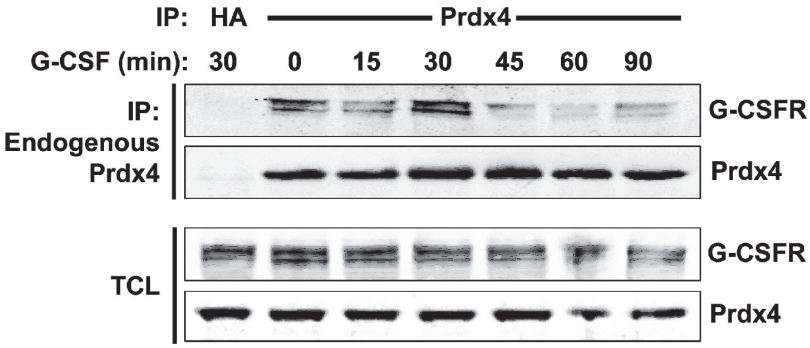

Figure S2: Prdx4 immunoprecipitations from Hek cells transfected with pBABE-G-CSFR or pBABE-ev constructs. Cells were growth factor-deprived for 4 hrs, followed by stimulation with G-CSF for the indicated time points. Blots were stained for G-CSFR and Prdx4.

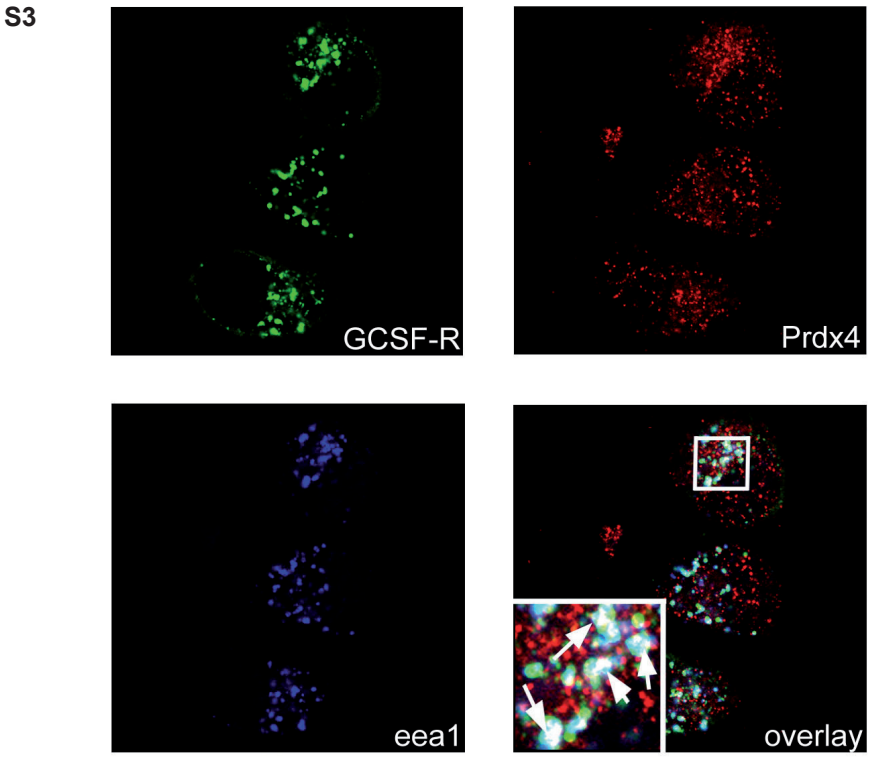

Figure S3: HeLa cells ectopically expressing wt-G-CSFR were growth factor deprived for 4 hrs. Surface membrane G-CSFRs were labeled with anti-G-CSFR antibody prior to stimulation with G-CSF for different time points. Cells were permeabilised, fixed and stained with anti-Prdx4 and eea1 antibodies followed by secondary anti-goat 488, anti-rabbit Cy3 and anti-mouse Cy5 antibodies antibodies and analyzed by CLSM.

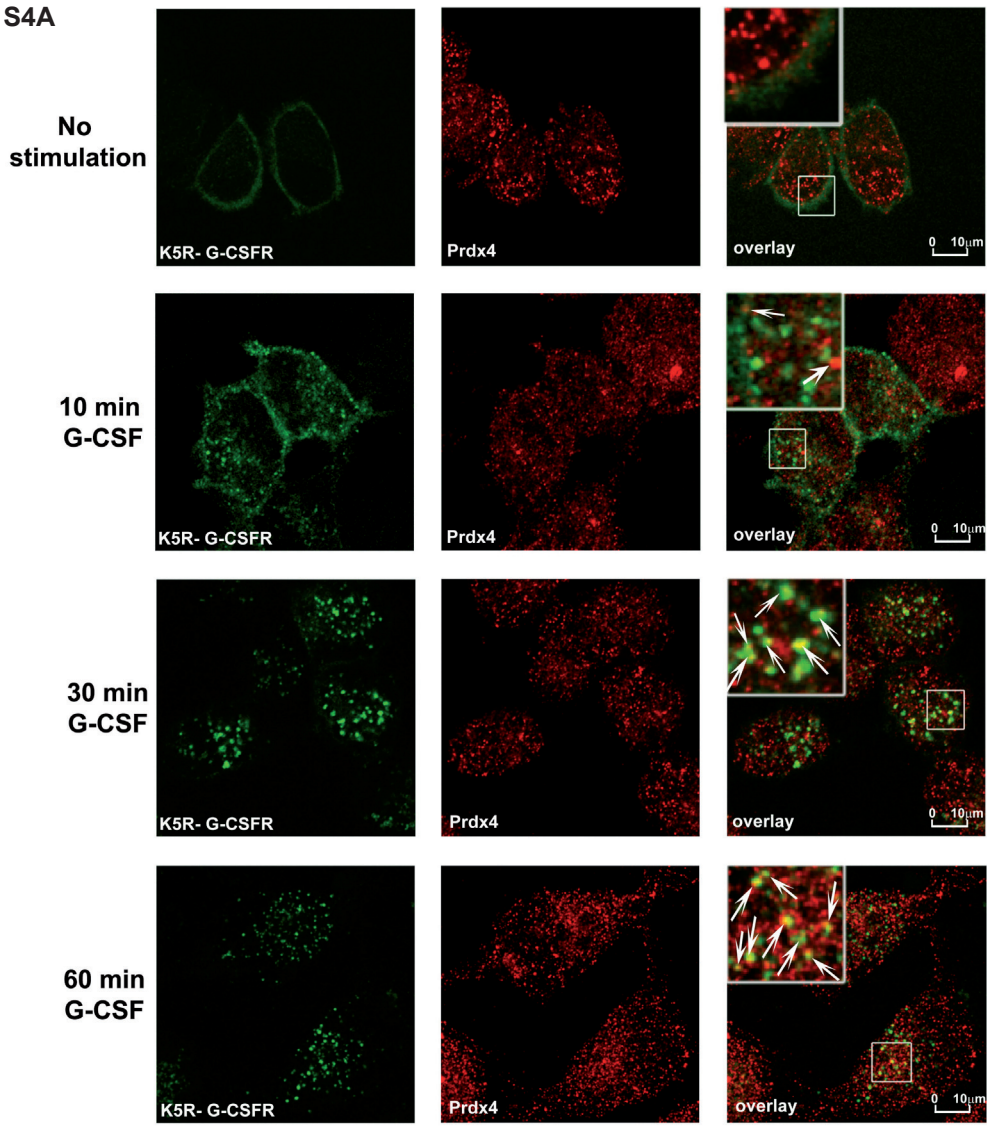

Figure S4a: HeLa cells ectopically expressing K5R-G-CSFR were growth factor deprived for 4 hrs. Surface membrane G-CSFRs were labeled with anti-G-CSFR antibody prior to stimulation with G-CSF for different time points. Cells were permeabilised, fixed and stained with anti-Prdx4 antibody followed by secondary and anti-rabbit Cy3 and anti-mouse Cy5 antibodies antibodies and analyzed by CLSM.

S4B

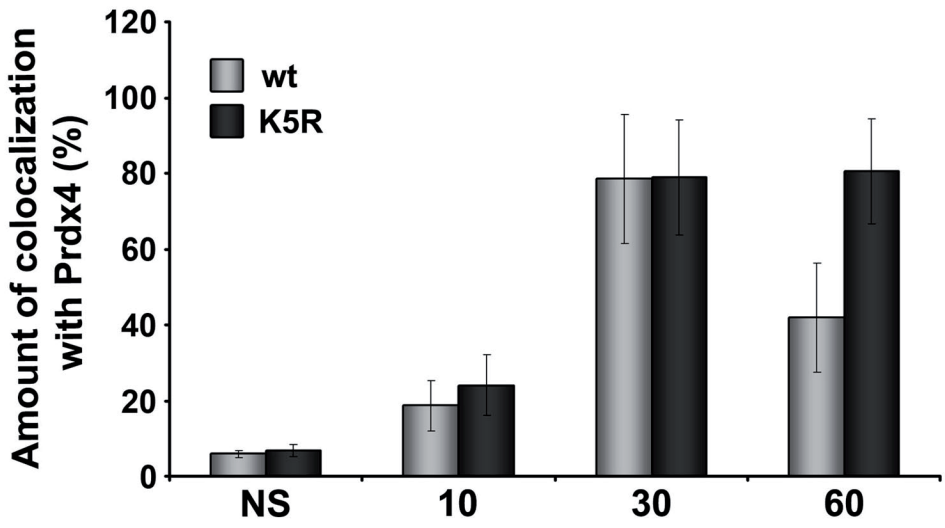

Figure S4b: Quantification of co-localizing wt-G-CSFR (grey bars) or K5R-G-CSFR vesicles (black bars) with endogenous Prdx4 upon stimulation with G-CSF.

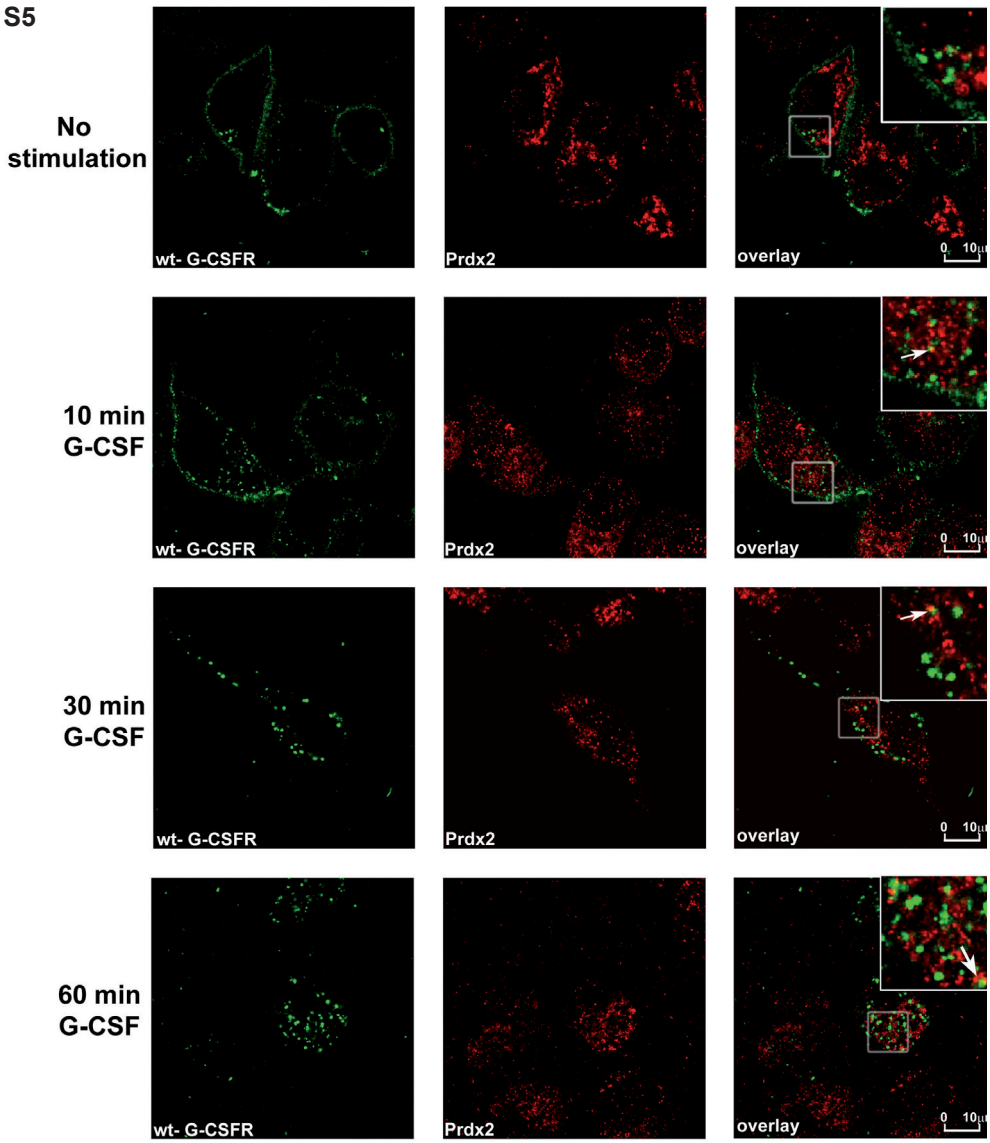

Figure S5: HeLa cells ectopically expressing wt-G-CSFR were growth factor deprived for 4 hrs. Surface membrane G-CSFRs were labeled with anti-G-CSFR antibody prior to stimulation with G-CSF for different time points. Cells were permeabilised, fixed and stained with anti-Prdx2 antibody followed by secondary and anti-rabbit Cy3 and anti-mouse Cy5 antibodies antibodies and analyzed by CLSM.

S6

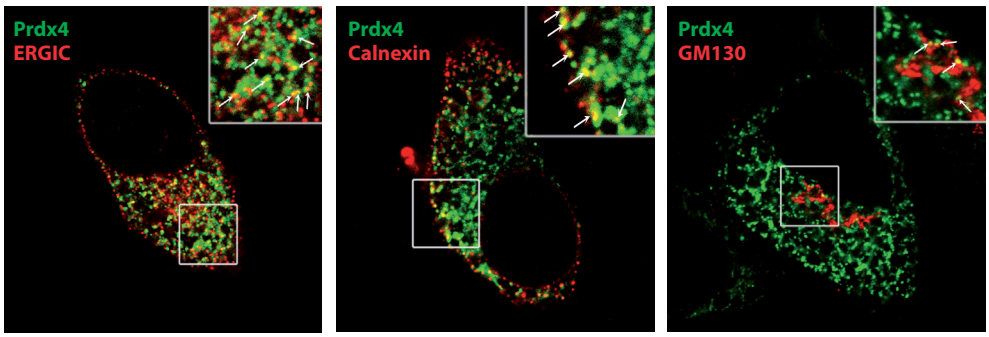

Figure S6: CLSM showing the co-localization of Prdx4 with markers for various sub-cellular compartments in HekT cells. The ER-Golgi-Intermediate Compartment was stained using an antibody against ERGIC, the ER with anti-calnexin and the Golgi with anti-GM130.

S7A

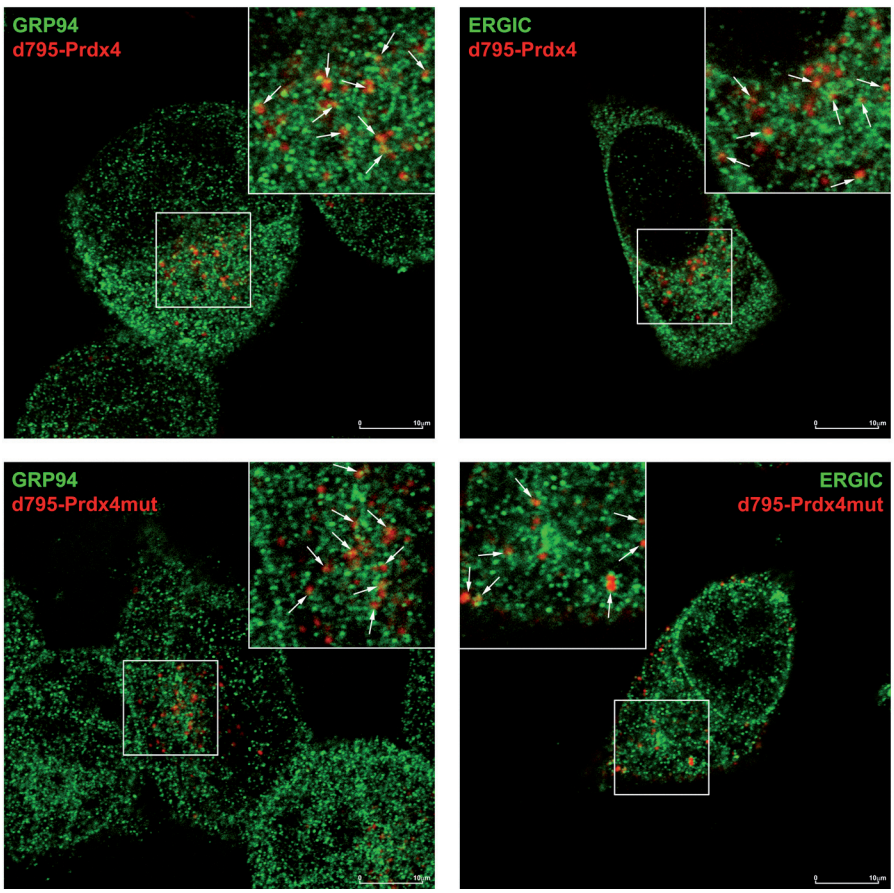

Figure S7: (A) CLSM showing routing of G-CSFR-Prdx4 fusion protein towards ER and ERGIC comparable to WT G-CSFR

S7B

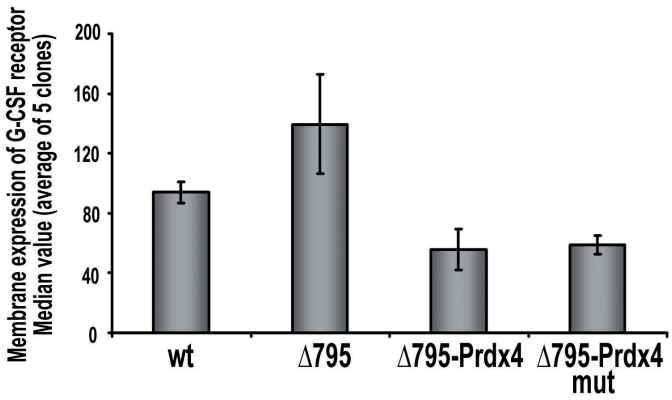

Figure S7: (B) Flow cytometric analysis of cell surface expression of G-CSFR constructs in 32D cells

S8

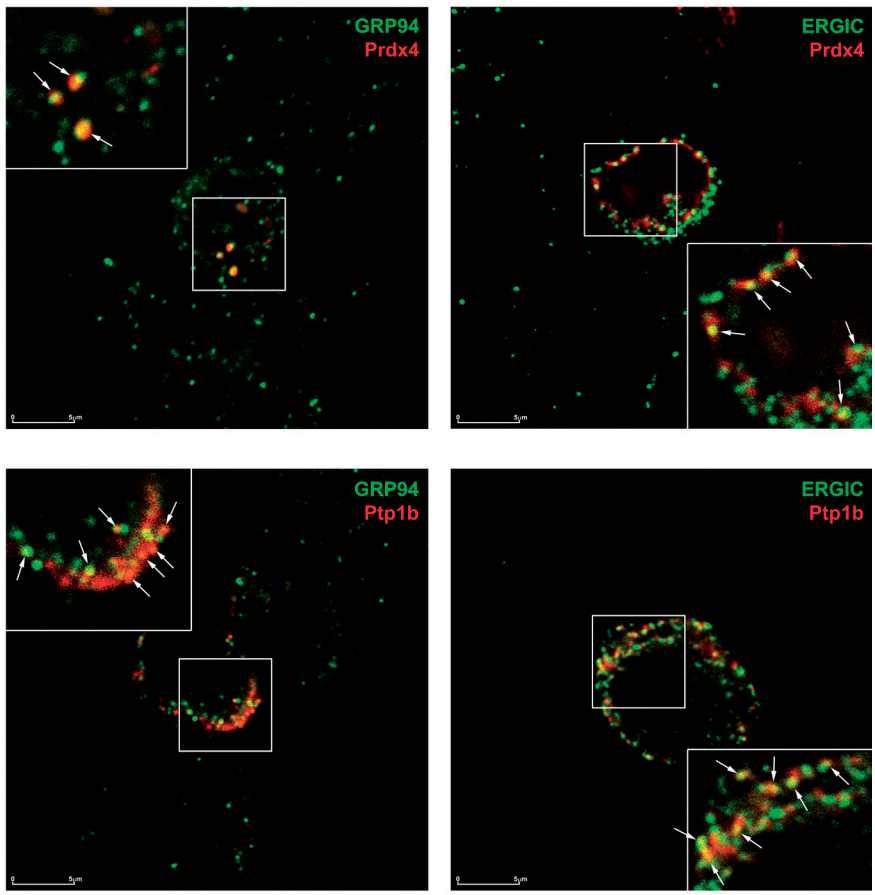

Figure S8: CLSM showing co-localization of Prdx4 or Ptp1b with ER (Grp94) or ERGIC markers in mouse bone marrow cells.

Table S1

Name of the primer	Sequence (5`-3`)
PRDX1 MAPPIT Fwd	CGGAATTCATGTCTTCAGGAAATGCT
PRDX1 MAPPIT Rv	GACTCCGAGTCACTTCTGCTTGGAGAA
PRDX2 MAPPIT Fwd	CGGAATTCATGGCCTCCGGTAACGCG
PRDX2 MAPPIT Rv	GACTCGAGCTAATTGTGTTTGGAGAA
PRDX4 MAPPIT Fwd	CGGAATTCATGGAGGCGCTGCCGCTG
PRDX4 MAPPIT Rv	GACTCGAGTCAATTCAGTTTATCGAAATAC
PRDX6 MAPPIT Fwd	CGGAATTCATGCCCGGAGGTCTGCTT
PRDX6 MAPPIT Rv	GACTCGAGTTAAGGCTGGGGTGTGAGCG
Fw7 G-CSFR	GTCCTCACCCTGATGACC
Δ73Prdx4 Δ795GR Rv	TTAGGTGCAGTGATGATCCTCCGTTGAGCAGTGGCCCAAAGA
Δ73Prdx4 Δ795GR Fwd	ACTGCTCAACGGAGGATCACTGCACCTAAGCAAAGCGAA
Rv Prdx4 EcoRV	CCGATATCTCAATTCAGTTTATCGAA
PRDX4mut R1	AATTTCAGTTGGAGACACAAATGTGAA
PRDX4mut R2	CCAGCCAGCAGGGGAGACTTCTCC

CHAPTER 3

The antioxidant protein peroxiredoxin 4 is epigenetically down regulated in acute promyelocytic leukemia

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ABSTRACT

The antioxidant peroxiredoxin (PRDX) protein family comprises 6 members, which are implicated in a variety of cellular responses, including growth factor signal transduction. PRDX4 resides in the endoplasmic reticulum (ER), where it locally controls oxidative stress by reducing H_2O_2 levels. We recently provided evidence for a regulatory function of PRDX4 in signal transduction from a myeloid growth factor receptor, the granulocyte colony-stimulating factor receptor (G-CSFR). Upon activation, the ligand-induced G-CSFR undergoes endocytosis and routes via the early endosomes where it physically interacts with ER-resident PRDX4. PRDX4 negatively regulates G-CSFR mediated signaling. Here, we investigated whether *PRDX4* is affected in acute myeloid leukemia (AML); genomic alterations and expression levels of *PRDX4* were investigated. We show that genomic abnormalities involving *PRDX4* are rare in AML. However, we find a strong reduction in *PRDX4* expression levels in acute promyelocytic leukemia (APL) compared to normal promyelocytes and different molecular subtypes of AML. Subsequently, the possible role of DNA methylation and histone modifications in silencing of *PRDX4* in APLs was investigated. We show that the reduced expression is not due to methylation of the CpG island in the promoter region of *PRDX4* but correlates with increased trimethylation of histone 3 lysine residue 27 (H3K27me3) and lysine residue 4 (H3K4me3) at the transcriptional start site (TSS) of *PRDX4*, indicative of a bivalent histone code involved in transcriptional silencing. These findings suggest that the control of G-CSF responses by the antioxidant protein PRDX4 may be perturbed in APL.

INTRODUCTION

G-CSF induces the proliferation, survival and neutrophilic differentiation of myeloid progenitor cells [1]. This response depends on the balanced activation and subsequent attenuation of signaling pathways linked to the G-CSFR, which is to a major extent controlled by lysosomal routing of the activated G-CSFR complex [2-3]. Signal attenuation of the G-CSFR is compromised by mutations causing truncations in the cytoplasmic domain of the receptor, found in severe congenital neutropenia (SCN) patients showing disease progression to acute myeloid leukemia (AML) [4-6]. These truncated G-CSFR forms are defective in internalization and lysosomal routing, which plays a major role in the abnormal signaling function of these receptor mutants.

Another important feature of the G-CSFR truncation mutants is that their activation results in elevated levels of reactive oxygen species (ROS) relative to the wild type G-CSFR [7]. ROS have been shown to modulate growth factor signal transduction, due to inactivation of oxidation sensitive protein and lipid phosphatases [8-9]. Increased concentrations of intracellular ROS, however, have been implicated in DNA damage as well as in damage to proteins and lipids. Hence it is of importance to maintain steady state levels of ROS. PRDX4 is an antioxidant protein which resides in the endoplasmic reticulum. It is not only responsible for maintaining steady state levels of ROS but also for reactivation of oxidized phosphatases [10-11]. Our recent findings suggest that PRDX4 interacts with a C-terminal region of the G-CSFR, a region lacking in the truncated G-CSFR found in SCN/AML. PRDX4 was shown to attenuate G-CSFR signaling dependent on the integrity of its redox active thiol group (Palande et al, manuscript submitted). Supporting a possible involvement of *PRDX4* in leukemogenesis, a chromosomal translocation, t(X;21)(p22;q22) has been reported in a case of AML resulting in a *PRDX4-RUNX1* fusion transcript [12].

We have investigated whether the *PRDX4* locus is associated with translocations and mutations in AML and whether its expression levels are altered. We report that chromosomal translocations involving the *PRDX4* locus are rare in AML. In addition, we did not detect single nucleotide variations in the *PRDX4* coding region in a large panel of AML and MDS patients. On the other hand, we found that *PRDX4* expression is significantly decreased in APL, a subset of leukemias that is characterized by hyper responsiveness to G-CSF [13]. Reduced *PRDX4* expression in APL is associated with a bivalent histone methylation mark, i.e., the combination of repressive histone methylation mark H3K27me3 and the activating histone methylation mark H3K4me3, at the TSS of *PRDX4* but not with DNA methylation of CpG islands in its promoter region.

MATERIALS AND METHODS

Human cell samples

All human cell samples were obtained after written informed consent and stored anonymously in a biobank. The study was performed under the permission of the Institutional Review Board of the Erasmus MC, registration number MEC-2008-387. Preparation of leukemia cell samples has been described previously [14]. Normal myeloblasts, promyelocytes and neutrophils were isolated from normal bone marrow samples using fluorescence activated cell sorting (FACS). Erythrocytes were removed prior to FACS sorting, by hypotonic lysis (15 min.), followed by a wash with phosphate buffer saline (PBS). The cells were then resuspended in PBS and incubated with fluorescent dye conjugated antibodies CD10-APC, CD11b-APC-Cy7, CD34-Pe-Cy7 and CD117-PE (Becton Dickinson, NJ) dead cells were excluded using a combination of forward and side scatter and DAPI staining. Cells were sorted with a FACSAria (Becton Dickinson, Becton Dickinson and Company, NJ) on the following criteria: CD34- and CD117+ for promyelocytes; CD34+ and CD117+ for myeloblasts; CD10+ and CD11b+ for neutrophils.

Mutation analysis of the *PRDX4* coding region

A WAVE device (Transgenomics, Omaha, NE, USA) was used to screen for mutations in the *PRDX4* coding region. cDNA from 113 AML and 65 MDS patients was analyzed. The coding region of *PRDX4* was PCR amplified in 4 separate parts, the length of each part being approximately 300 base pairs with an overlap of at least 60 base pairs between two consecutive fragments. The first part of the PCR was amplified using primers *PRDX4* F1 (5'-CCAAGGGACGTGTTTCTGCG-3') and primer *PRDX4* R1 WAVE (5'-CTGGCTTGGAAATCTTCGC-3'). The second part was amplified using primers *PRDX4* F2 WAVE (5'-GAGGAGTGCCACTTCTACGCG-3') and *PRDX4* R2 WAVE (5'-CTGTGAATCAACAGAGCATG-3'). The third part of *PRDX4* was amplified using primers *PRDX4* F3 WAVE (5'-GGTTTTCTTCTTCTACCCACT-3') and *PRDX4* R3 WAVE (5'-CATTCAGAGTAATTTGTCTTAG-3'). The last part of *PRDX4* was amplified using primers *PRDX4* F4 WAVE (5'-GGACTATGGTGTATACCTAG-3') and *PRDX4* R1 (5'-CCGTGAACTTTATTGAGAACTTTC-3').

FISH analysis

A locus-specific break apart FISH probeset was designed using the BAC clones CTD-2114P24 (chrX: 23457583- 23659210) and CTD-2594J24 (chrX:23638596-23807502). Clone isolation and labeling were performed using biotin-16-dUTP and digoxigenin-11-dUTP (Roche Diagnostics Belgium, Vilvoorde, Belgium) according to the manufacturer's protocol. The FISH analysis was performed as previously described on metaphase preparations. For this, patient samples were cultured and harvested according to standard cytogenetic protocols.

Hybridized preparations were digitally imaged and analyzed using Isis (MetaSystems, Altlussheim, Germany). A minimum of 100 interphase nuclei and 5 metaphases were analyzed [15].

Gene expression analysis

To determine expression of different peroxiredoxin family members (*PRDX1-6*), data of 439 de novo AML and 22 APL samples were used [16]. For each gene, probe sets were determined and the average expression levels in AML and APL were calculated. Significant differences within these two groups were determined with a Wilcoxon test. Multiple testing correction was performed using the Benjamini-Hochberg algorithm. Probesets with an FDR below 0.05 were considered to be differentially expressed in APL compared to AML. To examine a possible inverse correlation between expression of *PRDX4* and *TRAIL*, Pearson correlation coefficients were calculated separately in the APL cases.

Western blotting and antibodies

Cells were lysed in lysis buffer (20mM Tris HCl pH 8.0, 137mM NaCl, 10mM EDTA, 100mM NaF, 1% NP40, 10% glycerol, 2mM Na3VO4 and 1mM Pefablock SC). SDS-polyacrylamide gel electrophoresis was performed using precast 4-12% bis-Tris gradient gels (Invitrogen, Breda, the Netherlands). Prdx4 mouse monoclonal antibody was purchased from Abcam, (Cambridge, UK), β-actin antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Bisulfite sequencing

Bisulfite treatment of genomic DNA from APL or AML samples was performed using the EpiTect bisulphite kit (Qiagen, Hilden, Germany). PCR was performed on bisulfite treated DNA using methylation insensitive primers *PRDX4* bisulfite seq Fw1 (5'-TT-GTTTTTATAGAGTTGGGTAA-3') and *PRDX4* bisulfite seq Rv1 (5'-AAACCTCTCCTC-TATCTCC-3'). A nested PCR was performed on this PCR product using methylation insensitive primers *PRDX4* bisulfite seq Fw2 (5'- TAAATGTAGGTTTGGGATGG-3') and *PRDX4* bisulfite seq Rv2 (5'- CCAACCCTACACAACTCCAA-3'). For sequencing, the PCR product was sub-cloned into TA cloning vector (Invitrogen, Breda, the Netherlands). DNA was isolated from individual colonies and sequenced using the M13Fw (5'-GTAAAACGAC-GGCCAG-3') and the M13Rv (5'-CAGGAAACAGCTATGAC-3') primers.

Chromatin isolation

Ten million cells were suspended in 10 ml culture medium and cross-linked with 1% formaldehyde for 10 min at RT. The reaction was quenched with 130 mM glycine. Cells were washed twice with cold PBS and subsequently incubated on ice for 10 min with cold nuclei lysis buffer (50 mM Tris HCl, 10mM EDTA, 1% SDS) to which 1 mM protease inhibitors (Sigmafast, Sigma Aldrich, St Louis, MO) and 4 mM PMSF (Sigma Aldrich, St Louis, MO)

was freshly added. Lysed cells were subjected to 6 cycles of sonication for 20 sec to obtain fragments of 200-1000 bp (Sanyo Soniprep 150). After centrifugation (10 minutes, 10.000 g) at 4 °C to remove cell debris, the chromatin-containing supernatant fraction was pre-cleared by incubation with 50µg pre-immune serum (IgG from rabbit serum I8140, Sigma-Aldrich, St Louis, MO) for 30 min at 4 °C followed by addition of 100µl protein A magnetic beads (Invitrogen, Breda, the Netherlands) for 30 min at RT.

Chromatin immunoprecipitation (ChIP)

For immunoprecipitation, protein A dynabeads (Invitrogen, Breda, the Netherlands) were pre-incubated with antibodies against H3K27me3 (07-449, Upstate Biotechnology Inc, Charlottesville, VA) or IgG pre-immune serum as control (Sigma Aldrich, St Louis, MO). Twenty-five µl beads and 2.5µg antibody were incubated for 30 min at room temperature. Beads pre-coupled to respective antibodies were then added to the pre-cleared chromatin and incubated for 2 hrs at 4 °C. Magnetic beads containing the chromatin complexes were collected with a magnet and sequentially washed (5x) with 500 µl: 1x low salt wash for 1 min (20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1mM PMSF), 1x high salt wash for 1 min (20 mM Tris-HCl, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF), 1x LiCl wash for 5 min (10 mM Tris-HCl, 0.25M LiCl, 1 mM EDTA, 1% IGEPAL, 1% deoxycholate, 1 mM PMSF) and 2x TE buffer for 1 min each (10 mM Tris HCl, 1 mM EDTA). ChIP samples were eluted with 200 µl elution buffer (25 mM Tris HCl, 10 mM EDTA, 0.5 % SDS) at 65 °C for one hr, subsequently 3.75mM NaCl was added and incubated overnight at 65°C to reverse the cross-linking. After de-cross-linking, 2.4µg proteinase K (Roche, Basel, Switzerland) was added for protein digestion. DNA was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). For PML-RARα ChIP experiments, HEK cells were transiently transfected with FLAG-tagged PML-RAR-pCMV or empty vector (ev-pCMV) constructs [24]. Chromatin isolation and precipitation was performed as described [24].

Quantitative PCR of ChIP-enriched sequences was performed using SYBR Green PCR Master Mix (Applied Biosystems, Weiterstadt, Germany). The *MYT1* gene served as a positive control in H3K27me3 ChIP [17]. For *PRDX4*, a primer set for the predicted TSS was used: Fw (5'-CAAATGCAGGCTTGGGATGG-3') and Rv (5'-CAGCGCCTCCATGACCACG-3').

For the region 1kb downstream from the TSS: Fw (CAGGAATGACACGTCAGACG) and Rv (5'-CACTGAATACTGGCATGGAAC-3'); For the region 5kb downstream from the TSS: Fw (5'- GTTATGGTTATTGTGGGGTTTC-3') and Rv (5'-GACATAACTCTTTTTG-GTCTCTTC-3'). *RARβ* primers used were the same as described in [24].

RESULTS

Genetic abnormalities affecting the *PRDX4* coding region are rare in MDS and AML

Previously, a case of AML with a t(X;21)(p22;q22) has been reported in which the *PRDX4* gene located on Xp22 was fused to *RUNX1* at 21q22, resulting in a *RUNX1-PRDX4* fusion transcript [12]. In a cohort of AML and MDS patients karyotypically analyzed in our institution between 1990 and 2008, we found 9 cases with chromosomal abnormalities involving Xp21/p22 by standard banding techniques. These cases were further studied for possible rearrangements in the *PRDX4* locus using FISH with a *PRDX4* break apart probeset, but no translocations, deletions or other gross rearrangements were detected. Subsequently, we screened cDNA from 65 MDS patients and 113 AML patients for possible mutations or polymorphisms, but no mutations in the *PRDX4* coding region were detected in these samples. Thus, our data confirms that genomic aberrations affecting the *PRDX4* coding region are rare in MDS/AML and so far confined to the one reported case [12].

Figure 1

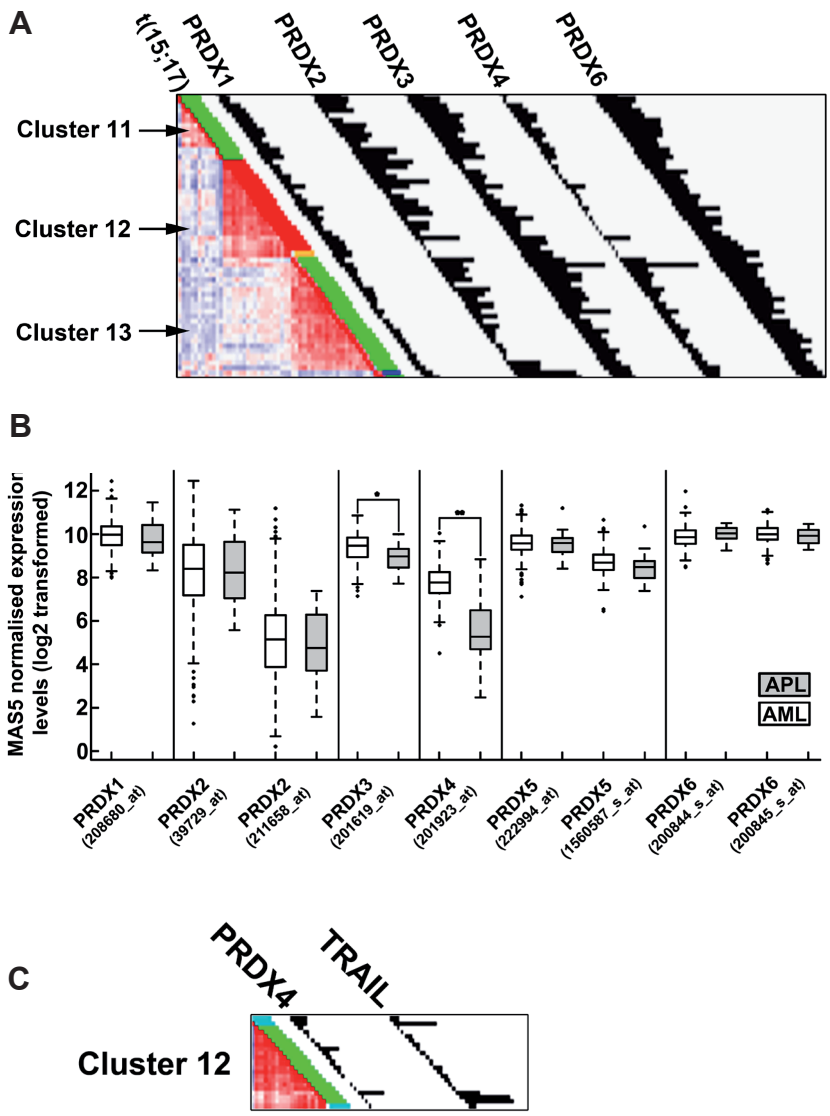

Figure 1: Expression of *PRDX* transcripts in AML and APL

(A) Graphical representation of expression of *PRDX* family members in APL patients clustered based on expression of ~2000 genes as described [14]. Cluster 12 is exclusively formed by APL patients, as indicated by the red bars indicating the presence of t(15;17). Cluster 11 comprises AML patients with normal karyotype and an underlying NPM1 mutation. Cluster 13 is formed by AML patients with t(8;21). Histograms represent MAS5-normalized expression values. **(B)** Expression levels of the peroxiredoxin gene family in APL (n=22) were compared to transcript levels in AML (n=439). Significant differences were calculated using a Wilcoxon test. * = p-value < 0.001, ** = p-value <0.0001. **(C)** APLs are not associated with high expression of *TRAIL* ligand excluding the possibility that low expression of *PRDX4* could be attributed to high levels of *TRAIL*.

PRDX4 is down regulated in APL

Next, we analyzed *PRDX4* expression levels in 461 myeloid leukemia patients measured on gene expression arrays [16]. In the majority of APL patients, PRDX4 levels are below detection levels; on average, *PRDX4* transcript levels were 4 to 5 times lower in APL, relative to other AML samples (Figure 1A and 1B). In contrast, expression of the other *PRDX* family members was not (*PRDX1*, *2*, *5* and *6*) or only marginally (*PRDX3*) reduced in the APL patient cluster (Figure 1A and B). A recent study showed that TNFα-related Apoptosis Inducing Ligand (TRAIL) suppresses the expression of *PRDX4* [18]. We therefore first investigated whether low levels of *PRDX4* expression in APL are associated with high expression of *TRAIL*, but we did not find evidence for such an inverse correlation (Pearson correlation coefficient 0.26-0.35 for 3 different *TRAIL* probe set comparisons, figure 1C). Western blot analysis showed that the PRDX4 protein, while readily detectable in AML blast cells with high transcript levels, is low/ undetectable in APL cells correlating with the gene expression profiling data (Figure 2A). In contrast, PRDX4 protein levels were not reduced in normal bone marrow-derived myeloblasts, promyelocytes and myelocytes (Figure 2B, upper panel), suggesting that the down regulation of PRDX4 is specific for leukemic promyelocytes. The purity of the sorted fractions of cells used for Western blotting as mentioned above was assessed using cytospins of the individual fractions stained with May Grünwald Giemsa (Figure 2B, lower panel).

Figure 2

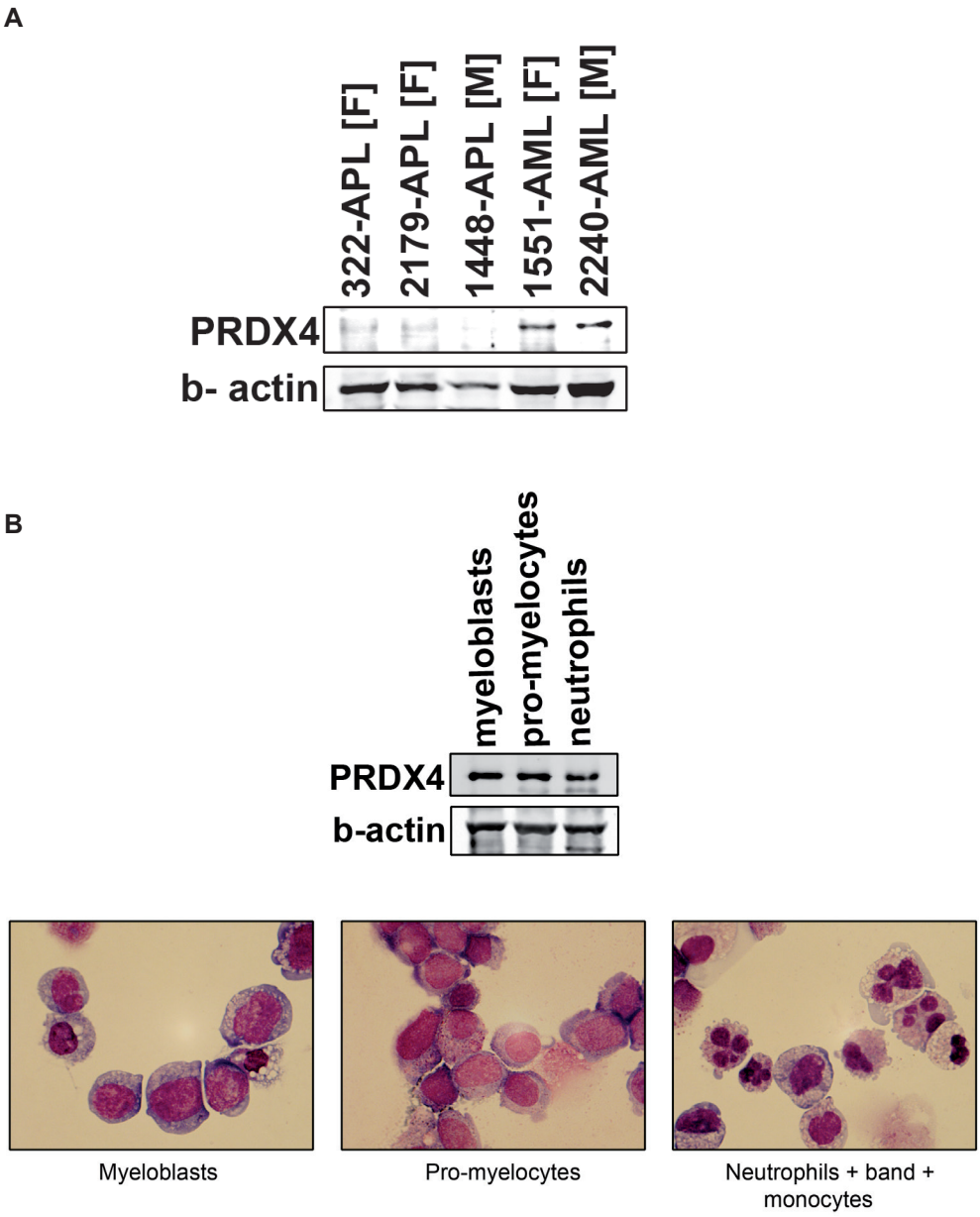

Figure 2: Reduced PRDX4 protein levels in APL

(A) Lysates of APL and AML samples were analyzed for PRDX4 protein levels. Because human *PRDX4* gene is located on the X-chromosome, male samples and female samples were compared with male and female AML control samples respectively to rule out the possibility of X-inactivation involvement. Patient number followed by F indicates female sample while patient number followed by M indicates male sample.

(B) Upper panel: Western blot analysis of PRDX4 expression in different normal bone marrow fractions (myeloblasts, promyelocytes, neutrophils). **Lower panel:** Micrographs of May Grunwald Giemsa stained fractions of cells used for Western blotting.

Reduced *PRDX4* expression in APL is not due to DNA methylation of CpG islands in the *PRDX4* promoter

PML-RARα recruits DNA methyltransferases such as DNMT1 and DNMT3a and is thereby potentially able to methylate CpG islands in target genes, leading to transcriptional silencing [19]. We asked whether *PRDX4* expression in APLs might be silenced by DNA methylation. Because *PRDX4* is located on the X chromosome, one allele is methylated in female cells. CpG methylation on both alleles in female samples or on one allele in the male APL samples was not observed (data not shown). Thus, no evidence was obtained indicating that DNA methylation of CpG islands within the promoter region contributes to silencing of *PRDX4* in APL cells.

APL cells have increased levels of H3K27me3 and H3K4me3 at the TSS of *PRDX4*

PML-RARα recruits the polycomb repressor complex 2 (PRC2) that contains EZH2 as a major effector protein [20]. EZH2 represses gene expression through H3K27me3, leading to an inactive chromatin configuration [21]. ChIPs performed on primary APL cells showed an increased occupation of H3K27me3 at the TSS of *PRDX4* (Figure 3A), which was not observed in AML samples expressing *PRDX4*. Unlike the H3K27me3 ChIPs, results from H3K4me3 ChIPs showed occupation of the TSS of *PRDX4* by the H3K4me3 methylation mark in both, APLs as well as AMLs (Figure 3B).

To study a possible direct involvement of PML-RARα in *PRDX4* silencing, we performed ChIP experiments on HEK cells expressing a FLAG-tagged form of PML-RARα. This set up was chosen because of a lack of antibodies suitable for PML-RARα specific ChIP. The RARβ locus was used as a positive control in these experiments [24]. PML-RARα interacted with the TSS and even more strongly with a region 1kb downstream of the TSS of *PRDX4* as compared to the IgG controls (Figure 4). In contrast, the region 5kb downstream of the *PRDX4* TSS showed no binding of PML-RARα.

Figure 3

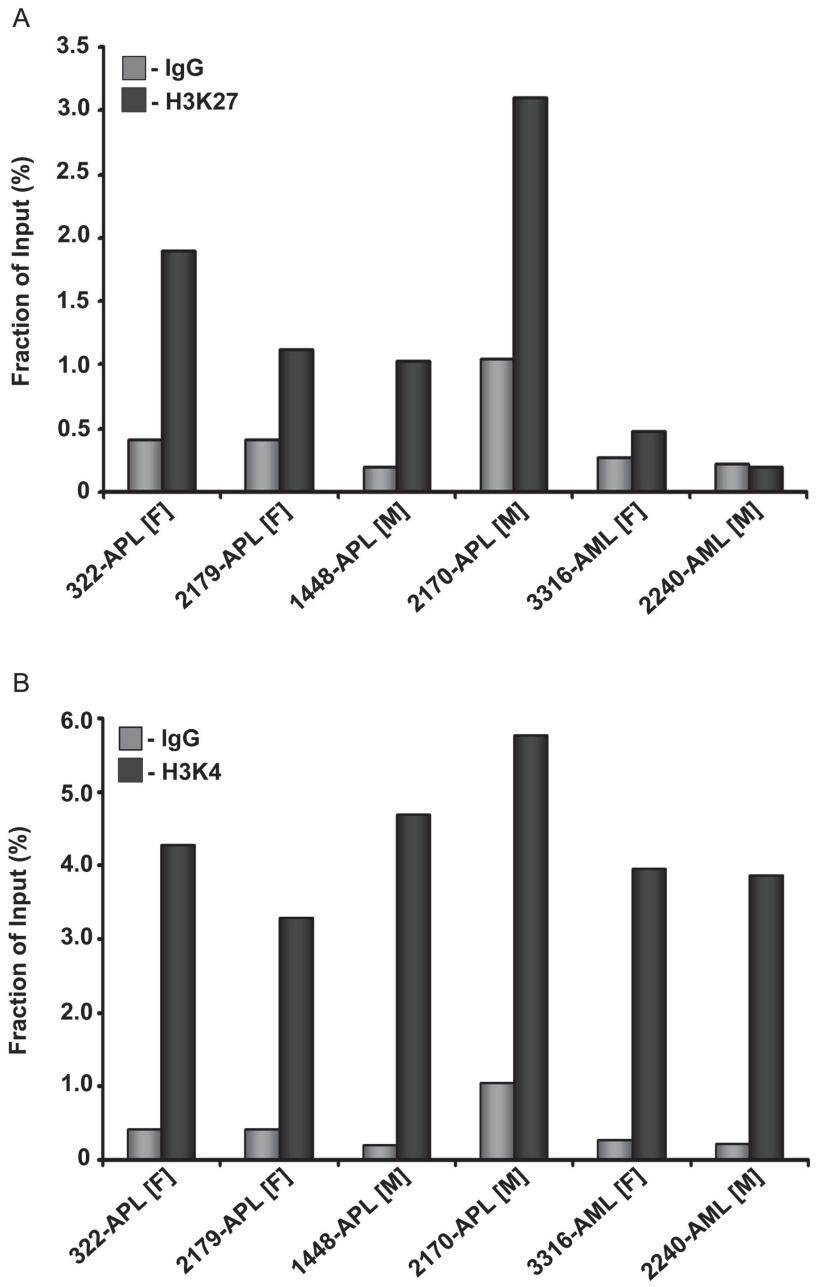

Figure 3: Bivalent H3K27me3 and H3K4me3 marks are present at the TSS of *PRDX4*
ChIP using αH3K27me3 and an IgG control (A) and αH3K4me3 and an IgG control (B) showing an enrichment of both H3K27me3 and H3K4me3 at the TSS of *PRDX4* in APL cells. To rule out a possible involvement of X-inactivation, and thereby H3K27me3, in silencing of *PRDX4* expression, male APL samples are compared to a male AML control and female APL samples are compared to a female AML control.

Figure 4

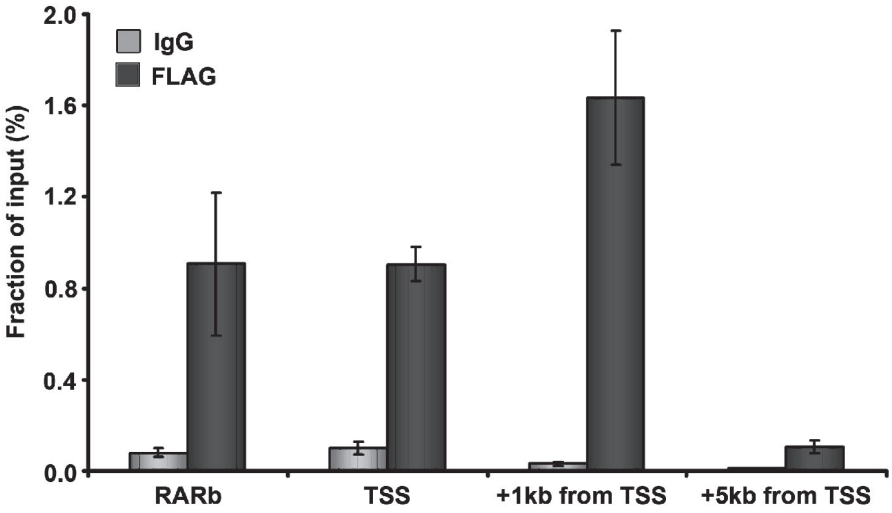

Figure 4: PML-RARα binds the promoter of *PRDX4*
Chromatin IPs on HEK cells expressing FLAG-tagged PML-RARα fusion protein using anti-FLAG and IgG control antibodies. Binding of PML-RAR occurs to the TSS of *PRDX4* and a region 1kb (1kb) of the TSS but not to a region 5kb downstream of the TSS (+5kb). *RARβ* is used as a positive control.

DISCUSSION

The major finding reported here is that expression of the antioxidant protein PRDX4 is repressed in t(15;17) APL harboring the PML-RARα fusion protein. Our data indicate that transcriptional regulation of *PRDX4* is perturbed in APLs because normal promyelocytes do not show reduced PRDX4 protein levels. This reduced expression is linked to a bivalent histone mark, i.e., increased H3K27me3 and H3K4me3, at the TSS of *PRDX4*. Bivalent histone marks provide a switch mechanism shown to be involved in transcriptional regulation of developmental genes; loss of the H3K27me3 mark leads to transcriptional activation whereas loss of H3K4me3 results in permanent silencing [22]. Our data suggest that reduced *PRDX4* expression in APLs is caused by maintenance of H3K27me3, which perturbs the bivalent switch. We hypothesized that PML-RARα might be involved in repressing *PRDX4* expression, especially because PML-RARα recruits the polycomb repressor complex (PRC2) that contains the histone methyltransferase EZH2 responsible for H3K27me3 [20]. Although PML-RARα indeed binds in the vicinity (1kb downstream) of the *PRDX4* TSS, further studies are required to support the direct involvement of PML-RARα in *PRDX4* downregulation.

Wild type retinoic acid receptors (RARs) are known to bind to specific DNA sequences called RA responsive elements (RAREs) and are able to repress transcription by recruiting co-repressor complexes such as SMRT/NCoR/ HDAC [23]. The promoter region of *PRDX4* however does not contain any retinoic acid responsive element (RARE) that may be bound by RARα/RXR directly. Another mechanism shown to be involved in suppression of genes via PML-RARα is the modulation of the activity of Sp1 target genes via binding to the transcription factor Sp1 [24]. Six Sp1 sites are present within the region of -1kb to +1kb of the TSS of *PRDX4*, to which the PML-RARα fusion protein may bind in a RARE independent manner [24].

To support the hypothesis of PML-RARα-mediated recruitment of PRC2/EZH2 to the *PRDX4* TSS, leading to H3K27me3 repression of the gene, we have attempted to restore Prdx4 expression in primary APL. To achieve this, APL cells were incubated with (combinations of) pharmacological compounds that included the "de-repressing" agents ATRA, the histone deacetylase inhibitor valproic acid and EZH2 inhibitor DZNep, which reduces EZH2 protein levels and thereby the level of H3K27me3 at transcriptional start sites of certain genes [25]. However, down regulation of EZH2 by DZNep, as previously shown by Jiang et al in colorectal cancer cells, was not seen in the APL samples used in our studies. Further studies are required to support a direct involvement of PML-RARα in *PRDX4* down regulation.

Irrespective of the molecular mechanism of transcriptional silencing, a lack of PRDX4 may have a major impact on cellular responses of myeloid cells and contribute to leukemogenesis. PRDX4 resides mainly in the endoplasmic reticulum (ER), where it is thought to neutralize the oxidizing effects of locally produced ROS [26]. This would keep for instance oxidation sensitive ER-resident phosphatases, such as protein tyrosine phosphatase 1b

(PTP1B), in an active state. PTP1B has been shown to play a role in down modulation of G-CSFR mediated signaling by dephosphorylating Jak2, Stat3 and the tyrosines of the G-CSFR (Palande et al, manuscript submitted). Predictably, loss of PRDX4 could lead to a reduction of phosphatase activity, providing an explanation for the increased responsiveness of APL clonogenic precursors to G-CSF.

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CHAPTER 4

The gene encoding for TXNIP is a frequent virus integration site in virus induced murine leukemias and is overexpressed in a subset of AMLs

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ABSTRACT

Thioredoxin-interacting protein (TXNIP) is involved in reactive oxygen species-induced stress responses. In a screen for novel disease genes in murine leukemia virus (MLV)-induced mouse leukemias, we identified *Txnip* as a frequent target for proviral integration. Ectopic TXNIP expression inhibited the proliferation of myeloid progenitor cells. TXNIP transcript and protein levels were significantly elevated in human AML blasts of certain patients, particularly those harboring translocation t(8;21). Nucleotide sequencing revealed no abnormalities in the *TXNIP* coding region in AML. These findings suggest that deregulated TXNIP expression contributes to MLV-induced murine leukemia as well as human AML.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by the uncontrolled outgrowth of malignant immature myeloid cells in the bone marrow (BM) and peripheral blood. It is now generally accepted that leukemogenesis is a multistep process, requiring defects in multiple regulatory genes that may be caused by chromosomal translocations and deletions or by mutations. In addition, increasing evidence suggests that inadequate control of intracellular levels of reactive oxygen species (ROS) may contribute to AML development, mechanisms being implicated including different anti-oxidant systems, such as superoxide dismutase, glutathione and peroxiredoxin/thioredoxin systems [1-2]. Thioredoxin-interacting protein (TXNIP) is a stress response protein implicated in the control of cancer [3]. Based on its growth suppressive effects and the finding that *TXNIP* expression is down regulated in certain tumors, TXNIP has been classified as a tumor suppressor [3]. TXNIP is also known as Vitamin D Upregulated Protein 1 [4]. In a retroviral screen with Graffi 1.4 (Gr-1.4) MLV, we previously found *Txnip* as a target for viral integration in myeloid leukemia [5]. This finding suggests a role for *TXNIP* in murine and human leukemias. To address this hypothesis, we mapped the provirus integrations in *Txnip* in Gr-1.4 and CasBrM MLV-induced leukemias and studied the consequences of these integrations for the regulation of *Txnip* transcription. In addition, we have studied the effects of enforced expression of *Txnip* in myeloid 32D cells and primary BM progenitors. Finally, we have performed an extensive mutation analysis of the human *TXNIP* gene and investigated TXNIP protein levels in human AML samples.

MATERIAL AND METHODS

Locus specific nested PCR

The PCR strategy and sequence protocols to identify provirus integrations in specific loci in mouse leukemia cells has been described in detail [5-6].

PCR primers and probes

Primers and probes used in this study are listed in supplementary Table 1.

Cell culture and retroviral transduction

Txnip cDNA was amplified with primers SmaIF and SmaIR and cloned in pBABE and R780 (SF91-I-eGFP-PRE, a kind gift of Dr. C. Stocking) using standard procedures. BM cells were harvested from the femurs and tibiae of 8 to 12 weeks old FVB mice, cultured and co-infected with equal amounts of BABE virus together with recombinant R780-Txnip or empty R780 virus as previously described [6]. EGFP expression was measured by flow cytometry. Equivalent numbers of GFP positive cells were plated in triplicate in methocult™ M3231 (Stem

Cell Technologies Inc, Vancouver, Canada) and selected with puromycin (1.5 μg/ml). The cells were plated with human (h) granulocyte colony stimulating factor (GCSF) (10 ng/ml), granulocyte macrophage (GM)-CSF (20 U/mL), or with a cocktail of murine (m) interleukin-3 (mIL-3) (10 ng/mL), hIL-6 (100 ng/mL), and stem cell factor (mSCF) (100 ng/mL).

Promoter constructs

To generate pGL3-V5, a 1448 nucleotide region upstream of the major ATG of *Txnip* was amplified by PCR using primers F1 and R2, and inserted into the MluI and BglII sites of pGL3-basic (Promega, Leiden, The Netherlands). The 3'UTR of *Txnip* was amplified with primers F3 and R5, and ligated into the XbaI site of pGL3. The Gr-1.4 LTR was PCR amplified with primers Graffi-LTR1 and Graffi-LTR2 and inserted into the DraI site in the *Txnip* promoter region and in the NcoI site of the *Txnip* 3'UTR.

Luciferase reporter assay

HEK293 were seeded and transfected by standard CaPO4 methods with pcDNA3.1/mycHis/LacZ (Invitrogen, The Netherlands), encoding LacZ as an internal control. Cells were lysed and assayed as described [6].

Human AML samples

Human AML samples were obtained following informed consent and prepared as described [7].

Western blotting

Lysates of BM blast cells from AML patients [7] were prepared and subjected to Western blotting as described [6] using mouse monoclonal anti-TXNIP1 antibody Yat315 (a kind gift of Drs. Junji Yodoi and Hiroshi Masutani [8] to visualize TXNIP and Goat anti-ACTIN (I-19; Santa Cruz Biotechnology, CA) as control.

TXNIP sequence analysis

TXNIP cDNA from AML patients and normal individuals was amplified with primers TXNIP-forward-2 and TXNIP-reverse-2. For sequencing, the same primers were used in addition to primer seq-R1 and seq-R2. SNP analysis was performed using probes VIC and FAM (Applied Biosystems) as described previously [9].

RESULTS

Identification of virus integration sites in *Txnip*

The first two Gr-1.4 integration sites in the *Txnip* locus identified by inverse PCR were located 1.1 kb upstream of the transcriptional start site and 0.35 kb downstream of *Txnip* [5]. To determine the frequency of integrations in these regions, we performed directed PCR on genomic DNA isolated from 14 independent leukemias. Gr-1.4 integration sites in the *Txnip* locus are mainly located between 0.2 to 1.8 kb upstream of the transcriptional start site, or dispersed within the 3'UTR of exon 8. All samples contained multiple virus integrations in the 5' promoter region or in the 3' UTR of *Txnip* and occurred in both orientations with equal frequencies (Fig. 1A and B). Directed PCR on genomic DNA from 24 CasBrM-induced myeloid leukemias gave comparable results, but with a lower frequency (58%) (data not shown). Interestingly, retroviral insertions in the *Txnip* locus were not found in B- and T-cell leukemia screens, except for one mapped integration in the AkxD leukemia model (http://rtcgd.abcc.ncifcrf.gov), suggesting a specific involvement in murine myeloid leukemia.

Integrations of Gr-1.4 LTR deregulates *Txnip* expression

Integrations in the 5' region are thought to increase the expression of the flanking gene through the transcriptional enhancer activity of the LTR. In agreement with this, insertion of Gr-1.4 LTR sequences in the 5' region resulted in a 2-fold increased activity in a luciferase reporter assay (Fig. 1D and E). LTR sequences in the 3' region had a weaker effect. Possibly, 3' virus integrations interfere with mRNA stability or normal regulation of translation. Finally, we observed elevated Txnip protein levels in some of the MLV-induced leukemia samples compared to normal BM in Western blot analysis (Figure 1C). Together, these data suggest that virus insertions in the *Txnip* locus result in enhanced protein expression.

Figure 1

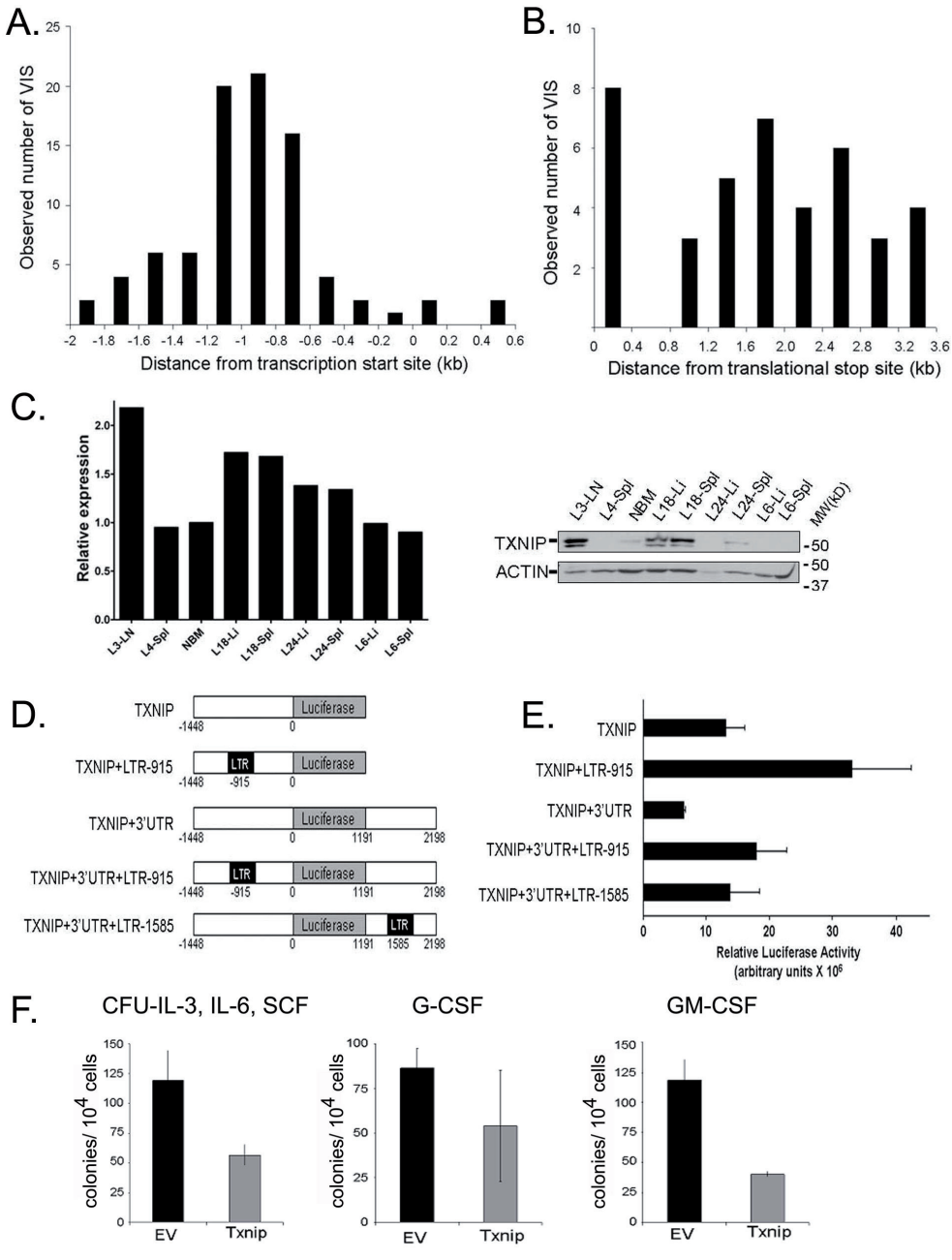
A.
Observed number of VIS
Distance from transcription start site (kb)
B.
Observed number of VIS
Distance from translational stop site (kb)
C.
Relative expression
L3-LN
L4-Spl
NBM
L18-Li
L18-Spl
L24-Li
L24-Spl
L6-Li
L6-Spl
MW(kD)
TXNIP
ACTIN
D.
TXNIP
TXNIP+LTR-915
TXNIP+3'UTR
TXNIP+3'UTR+LTR-915
TXNIP+3'UTR+LTR-1585
Luciferase
LTR
E.
Relative Luciferase Activity
(arbitrary units X 10⁶
F.
CFU-IL-3, IL-6, SCF
G-CSF
GM-CSF
colonies/ 10⁴ cells
EV
Txnip

Figure 1. Viral integrations in *Txnip*

(A) Virus integrations in Gr-1.4 MLV-induced leukemias in the 5' UTR of *Txnip*. **(B)** Virus integrations in Gr-1.4 MLV-induced leukemias in the 3' UTR of *Txnip*. Data are compiled from 14 leukemia samples. **(C)** Western blot analysis of MLV-induced leukemia samples and normal bone marrow (NBM) with anti-Txnip antibodies (Yat315). Actin was stained for loading control. Txnip expression normalized to actin levels and relative to NBM is plotted. **(D)** Reporter plasmids containing different promoter and 3'UTR sequences were generated as depicted. **(E)** Luciferase assay results in HEK293 cells. Cells were transfected with the indicated reporter plasmids and a LacZ plasmid as an internal control. Forty hours post transfection, cells were lysed and assayed for luciferase activity. Luciferase activity values were normalized against β-galactosidase activity. Values are the mean of three independent experiments, each performed in triplicate. Error bars represent standard deviation. **(F)** CFU assay of primary BM progenitor cells following transduction with R780-Txnip and R780 control virus (EV). Equivalent numbers of GFP-positive BM cells were plated in triplicate at densities of 105 cells (CFU-G), 5x104 cells (CFU-GM), or 104 cells (CFU-IL-3, IL-6, SCF) per dish in 1 ml methylcellulose medium containing G-CSF, GMCSF, or a cocktail of Il-3, Il-6 and SCF. Colonies of more than 50 cells were counted on day 7 of culture. Values are the mean of two independent experiments, each performed in triplicate. Error bars represent standard deviation.

Ectopic expression of Txnip inhibits primary BM cell growth

Next, we investigated how increased Txnip expression affects the outgrowth of primary BM progenitors. To this end, we retrovirally transduced mouse BM cells with R780-FLAG-Txnip or control virus and plated these cells in colony assays with different growth factors. Colony formation by Txnip overexpressing BM cells was significantly reduced under all stimulatory conditions tested (Fig. 1F). However, ectopic Txnip expression did not affect the differentiation, nor did it increase apoptosis (data not shown). Similar results were observed in the myeloid cell line 32D (data not shown). These data suggest that elevated Txnip expression predominantly causes a growth arrest, rather than cell death in myeloid progenitor cells. Similar findings in 293 cells have been reported previously [4].

TXNIP expression in human AML

To extend the observations in mice to patients, we studied *TXNIP* expression in purified human AML samples [7, 10]. We determined *TXNIP* transcript levels in different classes of adult and pediatric AML patients [7, 10]. Differential expression between classes of patients was performed for all probe sets on the Affymetrix HGU133A GeneChip using significance analysis of microarrays (SAM) [11]. This method tests a class of AML patients for significantly differentially expressed genes compared to a second class of AML patients, in this case the remaining samples. *TXNIP* mRNA levels were lower in cluster 6 compared to the average of all 285 AML samples measured. In contrast, *TXNIP* expression was significantly higher by SAM analyses in cluster 13 of human adult AML (fold change value of 1.53) and in a cluster of pediatric AML (fold change value of 1.83), both characterized by t(8;21) (Figure 2A). Quantitative PCR confirmed expression values determined by micro array analysis (data not shown). Next, we examined TXNIP protein expression by Western blot in 42 purified human AML blast samples [7] compared to normal CD34+ blast samples. Representative Western blots are shown in Figure 2B. In 33 cases, TXNIP protein levels were significantly elevated compared to normal CD34+cells. Notably, increased TXNIP protein levels were not exclusively observed in AML t(8;21) cases, suggesting that post transcriptional mechanisms may also contribute to elevated TXNIP expression.

Figure 2

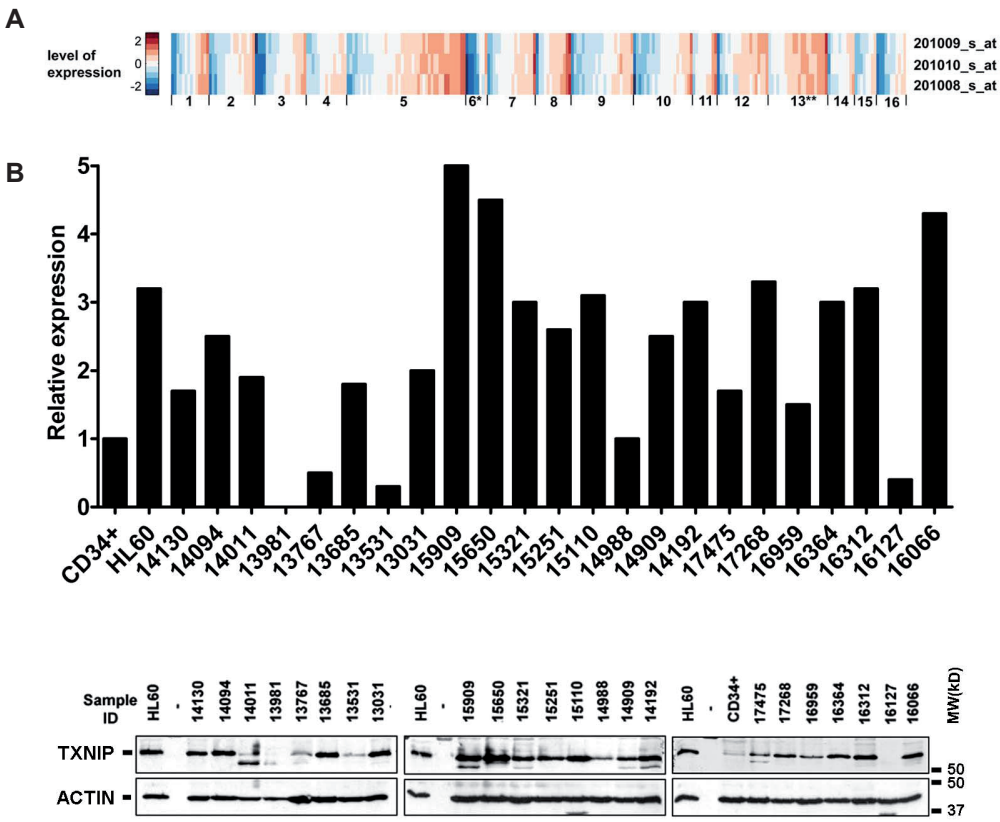

Figure 2. TXNIP levels in human AML samples

(A) Level of *TXNIP* mRNA expression in 285 human AML samples. The 16 clusters with molecular characteristics in a cohort of 285 AML patients have been published [7] and are depicted on the X-axis. Molecular characteristics associated with the clusters are: cluster 1: t(11q23), 2: FLT3-ITD, 3: FLT3ITD, 4: CEBPa mutations, 6: FLT3-ITD, 9: inv(16), 10: Evi-1, 12: t(15;17), 13: t(8;21), 15: CEBPα mutations, 16: t(11q23). *TXNIP* mRNA expression was found significantly lower (as indicated by *) or higher (**) by SAM analyses in clusters of leukemia. Probe sets of *TXNIP* were considered to be differentially expressed when Fold Change was over 1.5 or under 0.67, score was over 4 or less than 4, and a q-value less then 5%, where False Discovery Rate was less than 5%. **(B)** Western blot analysis of human AML samples, HL60 and CD34+ cells with monoclonal anti-TXNIP antibodies. Actin was stained for loading control. TXNIP expression normalized to actin levels and relative to normal CD34+ cells is plotted.

Screening for *TXNIP* mutations in AML

To investigate whether mutations in *TXNIP* frequently occur in human AML, we sequenced the entire cDNA from leukemic blasts of 270 patients. Other than some rare nucleotide changes, one of which appeared to be a relatively infrequent polymorphism at nucleotide position 741C→T, in 7 out of 270 AML cases compared to 4 out of 671 in normal controls (p=0.0169), no changes in the *TXNIP* coding region were found (Table 1). This excludes the possibility that mutant TXNIP proteins frequently interfere with normal TXNIP function in AML.

DISCUSSION

In this paper, we have identified *Txnip* as a common target of viral integrations in mouse leukemia and shown that TXNIP transcript and protein levels are elevated in a significant proportion of human AML cases. The key question that remains is how increased TXNIP levels contribute to leukemic cell growth. By interacting with thioredoxin (TRX), TXNIP causes the release of active ASK1, a stress-related serine threonine kinase that activates JNK. Although thus far mainly linked with apoptosis induction [12], a recent study indicates that this pathway also controls a G1-S cell cycle checkpoint, leading to growth arrest without detectable apoptosis [13]. This provides a plausible explanation for the observed inhibitory effects of TXNIP on normal bone marrow progenitors. Because TRX is a key anti-oxidant protein in the NADPH oxidase system, inhibition of TRX by TXNIP will result in increased levels of intracellular ROS [14]. This will predictably not only cause sustained oxidized protein and DNA damage, but also lead to increased growth and survival stimulatory activities of growth factors, through down modulation of critical phosphatases, such as the PI3K antagonist PTEN, by oxidation of catalytic sites [15]. Eventually, sustained increased cellular ROS levels will contribute to genomic instability in hematopoietic precursors that may lead to transformation and clonal outgrowth of myeloid precursor cells, resulting in development of leukemia [16]. Deregulation of redox-controlled gene expression thus may be a frequent event in the pathogenesis of myeloid leukemia. For leukemic transformation of myeloid progenitor cells via TXNIP induced ROS levels, additional hits, e.g., disrupting pro- or activating anti-apoptotic genes, would be needed. Further understanding of pathways controlled by TXNIP during myelopoiesis and a more detailed understanding of the signaling pathways influenced by TXNIP in hematopoietic cells in normal state and during cellular stresses will be required to understand whether and how increased TXNIP levels contribute to the development of human AML.

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SUPPLEMETARY DATA

Supplementary Table 1. Primers and probes used in this study

Primer name	Sequence (5'-3')
Locus specific nested PCR	
a	CTCTAGTCAGCTCCTGAGGCATCTCTCAGC
b	TTTGTTTTCGAGTTCCTGTCATTCTTTTCC
c	GCTCAGGTTTTCATAGTTTGTGTGTGTG
d	AACAGGGCAGAGAACTGGGCTCAGAGATGG
e	AAGGTTTGCCAGATAACTAAGTGAAACTAA
f	CCCTCTGTCCTCTCTCCAAATTCACTAAA
g	TGCAGGTGGGAATGTGGTGCTTAGAAC
h	GTAAAAGACGCTTTCTGCGAAGCCTGAG
L1	TGCAAGATGGCGTTACTGTAGCTAG
L1N	AGCCTTATGGTGGGGTCTTTC
L2	CCAGGTTGCCCCAAAGACCTG
L2N	AAAGACCTGAAACGACCTTGC
CL1	CGAACTTCCCTATTCTCAGTTCTGTATTT
CL1N	GCTCGCTTCTGGCTTCTGTTTG
CL2	CCCTGTGCCTTATTTGAACTAACCAAT
CL2N	TGCTAAACCTGATGGTGGGTCTTTC
P1	GCCGGTGAAATTGAAACAGGTTAGGGACAT
P2	GTCAGCTCCACTTTGTGGGACCCTGAGACC
Retroviral vector	
SmaIF	AACCCGGGATGGTGATGTTCAAGAAGATCAAGTC
SmaIR	AACCCGGGTCACTGCACGTTGTTGTTGTTGTT
Promoter constructs	
F1	AACGCGTCTCTAGTCAGCTCCTGAGGC
R2	CAGATCTGATTGAGCCGAGTGGGTTCAAG
F3	CGTCTAGAGCCTGCAGGAAATGAAGCATC
R5	CGTCTAGAAATGTAGAATGTCACCCAGG
Graffi-LTR1	GAAAGACCCCACCATAAGGCT
Graffi-LTR2	AATGAAGACCCCCGAGGTGG
TXNIP mutation analysis	
Txnip-forward-2	TCTTCCACCGTCATTTCTAA
Txnip-reverse-2	GCTGACCACCTCCTACATTA
Seq-R1	GGTATTGACATCCACCAGAT
Seq-R2	CAACTCATCTCAGAGCTGGTT
VIC	CCATCAGGAATGAAC
FAM	CCATCAGAAATGAAC

CHAPTER 5

General Discussion and Summary

Manuscript in preparation for a review

OVERVIEW OF CHAPTER 5

5.1 GENERAL DISCUSSION

5.1.1 Ptp1b and Prdx4 negatively regulate G-CSFR signaling

Endocytosis of receptors exposes the receptors to different PTPs, localized within different sub-cellular compartments, which contribute to signal termination. Although several phosphatases (Shp1, Shp2 and SHIP1) attenuate G-CSFR mediated signaling, these PTPs do not dephosphorylate the G-CSFR tyrosines. Dephosphorylation of receptor tyrosines ensures turn-off of signaling.

Studies performed using immortalized MEFs derived from *Ptp1b*-/- mice have already shown that Ptp1b plays a critical role in dephosphorylation of tyrosines of several receptors [1-3]. Our findings with respect to the role of Ptp1b in G-CSFR signal regulation are in agreement with those showing regulation of leptin receptor signaling by Ptp1b. In the context of the leptin receptor, Ptp1b was shown to physically interact with Jak2 and negatively regulate the activity of both, Jak2 and Stat3 [4-5]. Our findings clearly demonstrate the role of Ptp1b in inhibition of G-CSFR signaling and proliferation (Chapter 2). Phosphorylated tyrosines of G-CSFR, p-Jak2, p-Stat3 and p-Prdx4 were found to be targets of Ptp1b with respect to G-CSFR signaling. However, because activation of Stat3 is dependent on p-Jak2, it cannot be concluded whether p-Stat3 is a direct target of Ptp1b or whether Ptp1b regulates p-Stat3 levels indirectly by regulation of p-Jak2 levels.

Loss of either *Prdx4* or *Ptp1b* in bone marrow cells leads to increased colony formation in response to G-CSF and both *Prdx4* deficient and *Ptp1b* deficient fibroblasts show prolonged Stat3 activation upon stimulation with G-CSF. These findings suggest that Prdx4 and Ptp1b act in the same signaling pathway. We also found that the levels of phosphorylation of Prdx4 are higher in MEFs lacking *Ptp1b*. This suggests that Ptp1b positively regulates the activity of Prdx4 by dephosphorylation. Prdx4 in turn could reduce the levels of ROS produced upon G-CSFR activation and also reactivate Ptp1b by reducing the cysteine in its catalytic site (Figure 1).

The carboxyl terminus of the G-CSFR binds several proteins that are responsible for signal down modulation. Prdx4 and Ptp1b bind to the G-CSFR and inhibit G-CSFR signaling. We reported that the antioxidant, Prdx4 binds to the C-terminus of the G-CSFR where it inhibits signaling via the G-CSFR depending on the presence of cysteines in its catalytic site/s. Such a mechanism has only been reported for the PDGFR so far [6]. Knockdown of *Prdx2* resulted in increased activation of the PDGFR. Amongst the peroxiredoxin family members, only Prdx4 interacted with the G-CSFR and the most N-terminal region of Prdx4 was involved in interaction with the G-CSFR. Prdx2 and Prdx4 proteins are more than 70% identical and only differ in the additional 73 amino acids present in the N-terminus of Prdx4. Prdx2 however was not found to interact with G-CSFR in MAPPIT assays indicating that the N-terminal of Prdx4 might be critical for interaction with G-CSFR. Prdx4 could act as a tether whereby it could bring G-CSFR in the close proximity of Ptp1b, thereby allowing Ptp1b to dephosphorylate G-CSFR.

Figure 1

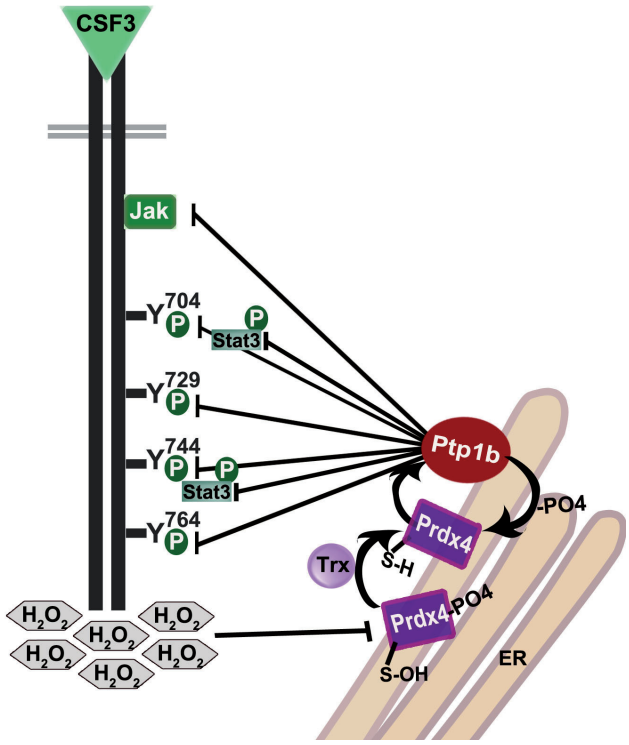

Figure 1: Positive feed back loop at the ER
Specifically at the ER, Ptp1b and Prdx4 down modulate G-CSFR signaling. The oxidized Prdx4 is reduced by Trx. Ptp1b dephosphorylates Prdx4 thereby activating it. Ptp1b also dephosphorylates tyrosines of the G-CSFR and also Jak2 and Stat3 thereby terminating signaling. Prdx4 reduces levels of ROS and also reactivates oxidized Ptp1b.

5.1.2 Dynamics and mechanisms of receptor dephosphorylation

The sub-cellular localization of Prdx4 is a much debated topic. The N-terminus of Prdx4 contains a signal peptide. Some reports show that Prdx4 is localized in the lumen of the ER while others show that Prdx4 is present on the surface of the ER [7-9]. Besides, the phosphatase Ptp1b is also localized to the surface of the ER [10-11]. Only under certain circumstances Ptp1b can be released from the ER [12].

It is believed that dephosphorylation of receptors precedes their degradation. However it is unknown whether dephosphorylation is a prerequisite for degradation [13]. Upon endocytosis, the receptors route via the late endosomes to the lysosomes where they are degraded. This pathway raises questions regarding how an ER-localized phosphatase dephosphorylates tyrosines of receptors present at the plasma membrane or in endocytotic vesicles.

The earlier concept of Ptp1b interacting only with newly synthesized receptors was proven incorrect by the findings which showed interaction of Ptp1b with activated PDGF and EGF receptors [13]. Upon stimulation with growth factors like EGF and PDGF, Ptp1b was found to cluster in regions on the face of the ER [13-14]. Using a FRET based approach

it was shown that the activated EGFR routed via the ER where it interacted with the Ptp1b [13]. As proposed by the pit stop model, after a 'pit-stop' at the ER, where the receptors are dephosphorylated, the EGFR proceeded to the lysosomes where they were degraded [15].

More recent evidence suggests that the interaction between ER-localized Ptp1b and endosomes containing EGFR occurs when the membranes of these two compartments come in contact with each other [16]. Two hours after stimulation with EGF, the cells expressing the catalytically inactive, Ptp1b substrate trapping mutant showed accumulation of EGFR in MVBs while the non-transfected cells showed presence of EGFR in the lysosomes. In cells expressing the EGFR, stimulation with EGF leads to the formation of internal vesicles within MVBs. Expression of substrate trapping mutant of Ptp1b leads to a block in formation of these internal vesicles within MVBs. Although overexpression of Ptp1b leads to accelerated degradation of EGFR, knocking down Ptp1b expression has no effect on the lysosomal routing of EGFR. The rapid degradation of EGFRs in Ptp1b overexpression conditions could be explained by the fact that other proteins of the endosomal sorting complex (ESCRT) machinery could also be targets of Ptp1b.

Indeed, STAM2, a part of the ESCRT machinery, required for targeting activated receptors for lysosomal degradation, was also found to be a target of Ptp1b [17]. Ptp1b interacts with STAM2 localized in early endosomes and dephosphorylates tyrosines of STAM2. While the phosphorylation defective mutant of STAM2 displayed prolonged co-localization in EGF positive endosomes and did not route to lysosomes, no effect was observed on the trafficking of EGFRs. Loss of Ptp1b expression may result in hyper-phosphorylation of STAM2 leading to dissociation of STAM2 from ubiquitinated EGFRs thereby preventing their trafficking into MVBs. Whether dephosphorylation of STAM2 affects its binding affinity for ubiquitinated substrates remains unknown. These findings raise the hypothesis of Ptp1b being a controller of the receptor trafficking process. Based on data from Stuible et. al. [17], it can be conceived that Ptp1b also regulates routing of G-CSFR.

Spatial segregation of Ptp1b limits its activity. Ptp1b is retained in a low activity state [14] mostly by the Nox4 mediated ROS production in the ER [18]. The role of Ptp1b in dephosphorylation of receptors might be secondary to its role in controlling receptor trafficking. Most of the targets of Ptp1b are known to recycle back to the plasma membrane upon dephosphorylation and ligand dissociation [19-20]. However, G-CSFRs do not recycle back to the plasma membrane after ligand dissociation [21]. Although the role of Ptp1b in negative regulation of G-CSFR is clear, the question still remains as to whether dephosphorylation is a prerequisite for degradation. Activity of other proteins like STAM2, involved in receptor trafficking could be regulated by phosphorylation/ dephosphorylation. These proteins could be targets of Ptp1b. Ptp1b could indirectly control G-CSFR trafficking by regulating the activity of these proteins.

Figure 2

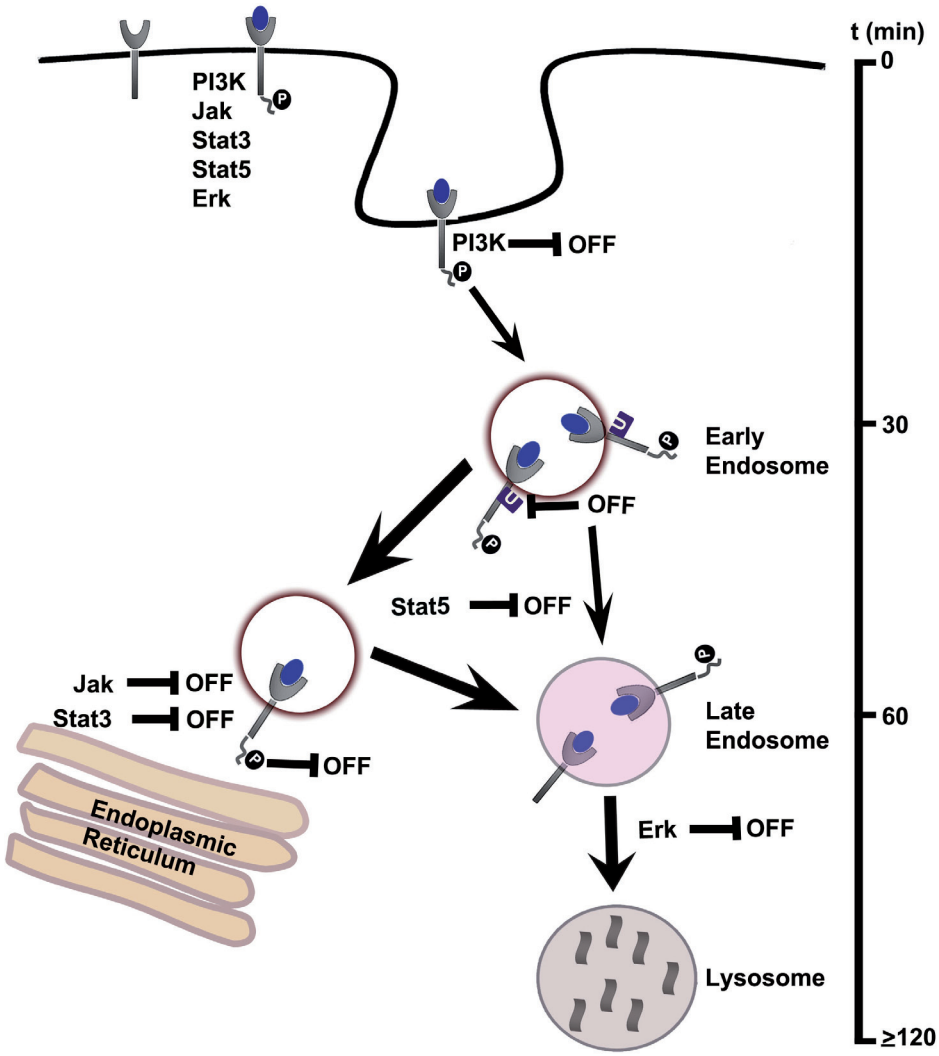

Figure 2: Spatio-temporal regulation of G-CSFR signaling
PI3K, Jaks, Stat3, Stat5 and Erk molecules are activated as a result of G-CSFR stimulation. At the plasma membrane the receptor tyrosines are phosphorylated by Jaks. The negative regulation of each of the activated pathways is restricted to certain sub-cellular compartments. PI3K signaling is regulated at the level of the plasma membrane. In early endosomes the lysines of the receptors are ubiquitinated by SOCS3. This acts as a sorting signal for routing to late endosomes. The deubiquitinating enzyme DUB2A which can deubiquitinate receptors, most probably acts before the receptor routes to the late endosomes. The receptors further route to late endosomes, alternatively via the ER, where they are dephosphorylated and the p-Jaks and p-Stat3 molecules are inactivated. In the late endosomes, Erk signaling in regulated. The receptors are finally degraded in lysosomes. Although p-Stat5 inactivation depends on the ubiquitination of G-CSFR, it is unclear whether p-Stat5 is also inactivated by degradation.

5.1.3 Spatio-temporal control of G-CSFR signal down modulation

Attenuation of different signaling pathways occurs in different cellular compartments. While Stat3, Stat5 and Erk activation is prolonged in cells expressing the lysine-less K5R receptor as compared to wild type controls, the activation of PKB is not prolonged [21]. The K5R-G-CSFR does route to early endosomes upon stimulation with G-CSF but fails to route to late endosomes/ lysosomes.

The mutant lacking the internalization motif, the Δ749-769 G-CSFR shows prolonged Stat3, Stat5, Erk and PKB activation. Δ749-769 G-CSFR mutant is defective in internalization and differs from K5R only in prolonged PKB activation. This suggests that the down regulation of PI3K pathway occurs mostly at the plasma membrane. Dephosphorylation of PIP3 occurs via the phosphatase PTEN [22]. A small fraction of PTEN is located at the plasma membrane and this fraction dephosphorylates PIP3 [23]. The control of p-Erk, p-Stat3 and p-Stat5 mediated signaling probably occurs at the level of early endosomes or further downstream in the routing cascade (Figure 2).

The basal levels of both Stat3 and Stat5 activation are higher in cells expressing the Δ715-G-CSFR as compared to wt-G-CSFR [24]. In luciferase assays, Stat3 activation was found to be sensitive to inhibition by SOCS3 [25], while Stat5 activation was found to be insensitive to inhibition by SOCS3 in Δ715-G-CSFR expressing cells as compared to those expressing the full length G-CSFR [25]. The Δ715-G-CSFR lacks the Y729 residue required for recruitment of SOCS3. SOCS3 via ubiquitination of the most membrane proximal lysine (K632) of G-CSFR brings about the routing of receptors from early endosomes to lysosomes. This suggests that SOCS3 mediated ubiquitination of K632 of G-CSFR is a key step for Stat5 down regulation, because failure to recruit SOCS3 in case of Δ715-G-CSFR renders it insensitive to inhibition by SOCS3. Stat3 activation appears to be in part regulated by other pathways, specifically those that directly interfere with JAK kinase activity.

The pErk signaling of the G-CSFR may continue even after the G-CSFR reaches late endosomes, as suggested in the case of EGFR signaling [26]. Interestingly lipid raft adaptor p18 which binds to the p14-MP1 complex provides a scaffolding surface for MAPK signaling complex on late endosomes [26-27].

Ptp1b dephosphorylates tyrosines of G-CSFR including Y729 which is critical for SOCS3 binding. Thus Ptp1b via dephosphorylation of Y729 of the G-CSFR may block the SOCS3 mediated ubiquitination of lysines of the receptor. This in turn may enhance the receptors routing via the ER where they are dephosphorylated. Termination of Jak2 and Stat3 signaling might take place primarily at the ER. This alternative routing pathway where receptors pass the ER on their way to the lysosomes may explain the differential regulation of signaling. While Stat5 signal attenuation primarily depends on the lysosomal degradation of the G-CSFR, Stat3 signal attenuation occurs by dephosphorylation of tyrosines which act as docking sites for Stat3. Hence, whereas Stat5 signal attenuation depends on SOCS3, Stat3 signal attenuation may be regulated by Ptp1b and the kinase inhibitory region of SOCS3 which

directly interacts with JAK kinases, independent of docking to the critical tyrosine (Y729) of G-CSFR (Figure 3).

5.1.4 Redox signaling in the context of G-CSFR

Ptp1b is highly sensitive to ROS-mediated inactivation. In case of the G-CSFR, Ptp1b dephosphorylates the C-terminal tyrosine of Prdx4. Loss of Ptp1b results in increased phosphorylation of Prdx4. No formal evidence yet exists for the regulation of activity of Prdx4 by post-translational modifications. The tyrosine of Prdx4 which was found to be phosphorylated is also conserved in Prdx1. Recently, a new method for post translational regulation of Prdx1 has been described, wherein; Prdx1 was shown to undergo tyrosine phosphorylation mediated inactivation upon stimulation with PDGF/ EGF [28]. Prdx2 however was shown to be inactivated by oxidation of cysteines and not by phosphorylation. The inactivation of Prdx1 led to accumulation of ROS levels in cells. By analogy to Prdx1 it can be envisaged that activity of Prdx4 could also be regulated by phosphorylation. Ptp1b could reactivate Prdx4 by dephosphorylation of the C-terminal tyrosine of Prdx4.

After binding of a ligand to its receptor, ROS may be produced in millimolar amounts. The current hypothesis is that ROS are produced by the local NADPH oxidase machinery and that they locally act on downstream signaling cascades. G-CSFR-induced ROS production occurs via the activation of the Lyn-PI3K-PKB pathways, which not only leads to activation of $p47^{phox}$ by phosphorylation but also its recruitment to the plasma membrane, thereby activating the NADPH oxidase [29]. The levels of ROS produced are higher in 32D cells and primary bone marrow cells expressing the Δ715-G-CSFR mutant as compared to those expressing the wild type receptor [29]. The inability of Δ715-G-CSFR to undergo rapid endocytosis limits its ability to interact with the negative regulatory molecules like Ptp1b and Prdx4. This leads to prolonged signaling as well as increased ROS levels in cells expressing the Δ715-G-CSFR.

Currently, one of the best documented examples for cytokine responses locally controlled by ROS comes from the IL-1 receptor. Upon stimulation with IL-1, assembly of the Nox2 complex and subsequent ROS production was shown to occur in the early endosome compartment [30]. Nox4 was also found to be involved in ROS production and further activation of downstream signaling via insulin receptor mainly by inhibition of Ptp1b activity [31-32]. In another recent study, it was shown that activation of IL-4 signaling led to generation of ROS via PI3K mediated activation of Nox1 and Nox5l [33]. Notably, the ROS produced by activation of IL-4 was shown to oxidize and thereby inactivate Ptp1b [33].

Based on the above mentioned examples, local production of ROS upon G-CSFR activation seems likely. Considering the fact that two redox sensitive proteins involved in negative regulation of G-CSFR signaling are localised at the ER, the ER appears to be the major site of redox-controlled signaling in case of G-CSFR. Whether ROS production upon G-CSFR activation occurs at the ER by the local Nox4 complex remains unknown. Based on the

Figure 3

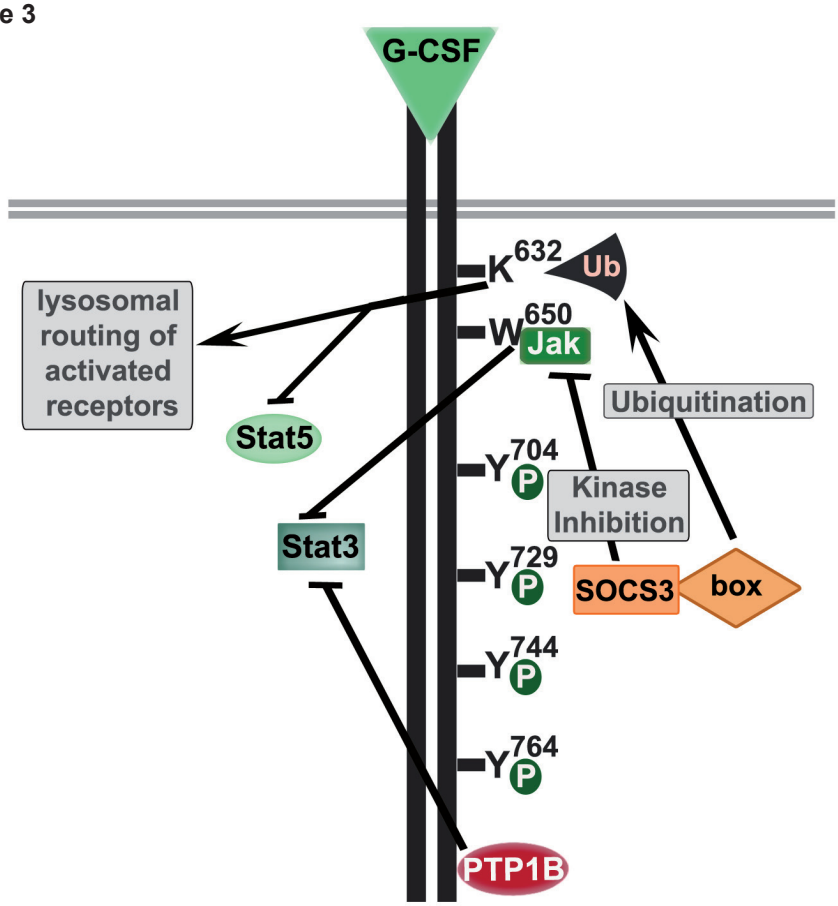

Figure 3: Down regulation of G-CSF signaling by SOCS3 and Ptp1b
Inhibition of Stat5 activity is dependent primarily on the recruitment of SOCS3 to tyrosine (Y729) whereby it brings about ubiquitination of lysine (K632). Ubiquitination of K632 leads to lysosomal routing of G-CSFRs. Inhibition of Stat3 activity is partly dependent on the phosphatase Ptp1b and partly on SOCS3 mediated inhibition of Jaks via the KIR of SOCS3. Inhibition of Jaks leads to a block in phosphorylation of tyrosines of G-CSFR and a block in recruitment of Stat3 molecules to the phosphorylated tyrosines.

findings that the Δ715-G-CSFR produces higher levels of ROS although it does not route to the ER, the possibility of another ROS producing complex activated upon stimulation with G-CSF at the plasma membrane cannot be ruled out. Whether Prdx4 indeed reduces ROS levels in the ER and reactivates Ptp1b requires further investigation. The lack of highly sensitive and reproducible techniques for measuring ROS and phosphatase activities at a local, i.e., subcellular level currently remains a major limitation for these studies.

5.1.5 Role of Prdx4 in leukemogenesis

Peroxiredoxin 4 was identified as fusion partner of AML1 in a myeloid leukemia patient carrying t(X;21)(p22;q22) [34]. In *Chapter 3* of this thesis, we described that *PRDX4* is silenced specifically in acute promyelocytic leukemia (APL), characterized by t(15;17), by H3K27-me3 of the *PRDX4* promoter. In *Chapter 3* of thesis we also show that PML-RARα binds to the transcriptional start site (TSS) of *PRDX4*. In an attempt to restore *PRDX4* expression in APLs by de-repressing the PML-RARα complex, we have treated APL cells with (combinations of) pharmacological compounds that included the "de-repressing" agents ATRA, the histone deacetylase inhibitor valproic acid and EZH2 inhibitor DZNep, which reduces EZH2 protein levels and thereby the level of H3K27me3 at transcriptional start sites of certain genes [35]. However down regulation of the methyl transferase EZH2 by DZNep, as reported in colorectal cancer samples, was not observed in APLs. The reason for this discrepancy is not known but may relate to the fact that the majority of APL cells have limited or no ability to proliferate *in vitro*.

As compared to other types of leukemias, APLs are characterized by a relative hyper proliferative response to G-CSF [36]. From the AML expression array data of Valk et al [37], it can be seen that the expression levels of *G-CSFR* are higher in APLs as compared to other sub-types of AML. Besides, based on our findings that PRDX4 down modulates G-CSFR signaling, the hyper proliferative response of APLs to G-CSF might be explained by the loss of PRDX4 binding leading to hyper-oxidation of PTP1B resulting in prolonged G-CSFR mediated signaling (*Chapter 2*).

PML-RARα is known to interact with members of the polycomb repressor complex (PRC2) complex which are responsible for H3K27-me3 mediated silencing of loci [38]. Preliminary data from PML-RARα ChIPs shows presence of PML-RARα at the TSS of *PRDX4* locus. The PML-RARα present at the TSS of *PRDX4* via its interaction with EZH2, the histone methyl transferase of PRC2, could bring about H3K27me3 mediated silencing of *PRDX4*.

Retinoic acid receptors (RARs) bind to specific sites in promoters called the retinoic acid responsive elements (RAREs). RARs suppress transcription by recruiting corepressor complexes such as SMRT/NCoR/ HDAC [39]. However, considering the fact that the promoter of *PRDX4* lacks any RAREs, the possibility of repression of *PRDX4* expression by recruitment of corepressor complexes is unlikely. PML-RARα was also shown to suppress genes indirectly by modulation of the activity of transcription factors like Sp1 and bring about suppression of the Sp1 target genes [40]. Six Sp1 sites are present within the region of -1kb to +1kb of the TSS of *PRDX4*, to which the PML-RARα fusion protein may bind in a RARE independent manner [40].

Arsenic trioxide (As_2O_3) is commonly used for treatment of APLs with PML-RARα fusion [41]. In contrast, As_2O_3 does not affect APLs with PLZF/RARα fusion [42]. Degradation of PML/RARα and eradication of leukemia initiating cells can be achieved upon treatment of APLs with As_2O_3. Treatment of APLs with a combination of ATRA and As_2O_3

results in enhanced granulocytic differentiation of APLs and regression of the leukemia [43]. Treatment of cells with As_2O_3also leads to an increase in ROS levels in cells. A result of the ROS production is the formation of intermolecular disulphide bonds between either PML and / or PML-RARα proteins which bind arsenic directly [44]. The resultant PML and PML-RARα multimers form nuclear matrix associated nuclear bodies and undergo sumoylation and are subsequently degraded [44]. Based on these findings, the treatment of APLs with a combination of G-CSF and ATRA could have similar effects. Due to low expression of *PRDX4* in APLs, the levels of ROS produced in response to G-CSF treatment could be higher leading to multimerization of PML-RARα. This could result in sumoylation mediated degradation of PML-RARα and regression of leukemia.

New technologies enable the global assessment of changes not only in the expression of genes but also the epigenetic changes associated with genes. This allows us to not only understand the underlying mechanisms involved in the transformation but also to design drugs targeting the key epigenetic modifiers involved in the process.

5.1.6 Role of Txnip in transformation of myeloid progenitors

In *Chapter 4* we describe that the thioredoxin interacting protein (*Txnip*) is a common virus integration site in murine leukemia virus (MLV)-induced leukemia. We found that *TXNIP* expression is upregulated in a subset of human AMLs characterized by t(8;21). The viral integrations in the 5' UTR and 3' UTR result in increased expression of *Txnip*, as shown by the luciferase assays. Treatment of BaF3/ 32D cells with hydrogen peroxide leads to increased expression of *Txnip*.

Txnip / Vdup1 is mostly known as a tumor suppressor gene [45-46] whose expression is reduced in human breast, colon and lung cancer tissues and also different cell lines derived from tumours [53-55]. Our findings however suggest that *TXNIP* expression is higher as compared to control samples in myeloid leukemia. Whether Txnip is directly involved in the process of transformation still remains a question. Txnip has been linked to perturbation of signaling pathways, which might provide indirect evidence for the role of Txnip in transformation. Txnip has been shown to be a stress response gene which is activated in response to ROS, ultraviolet (UV) light, γ-ray exposure and under conditions of high density culture and heat shock treatment [47-48]. Interaction of Txnip with Trx might block the transcription factor activating ability of Trx [49-50]. Trx interacts with apoptosis signaling kinase 1 (Ask1) and promotes ubiquitination and degradation of Ask1 [51-52]. Over-expression of Txnip leads to inhibition of Trx activity and thereby increased Ask1 activation [9]. Ask1 is a MAPKKK and plays a key role in cytokine induced or oxidative stress induced activation of JNK and p38 pathways [53].

Thioredoxin is essential for reactivation of peroxiredoxins [47]. Inhibition of thioredoxin thereby also leads to increased intracellular ROS levels [54]. Txnip might execute its function via increased ROS levels leading to inhibition of PTPs. Inhibition of PTPs will result in pro-

longed/ increased signaling in cells. High amounts of ROS induce damage to DNA, proteins and lipids. The damaged DNA that escapes the DNA replication machinery can contribute to the development of cancer. High levels of ROS are known to induce apoptosis in cells by targeting the anti-apoptotic Bcl family members for proteasomal degradation. Overexpression of anti-apoptotic Bcl-2 family members like Bcl-2 and Bcl-XL protects cells from ROS induced apoptosis [55-56]. However whether the apoptotic stimuli induced by ROS can be counteracted by the anti-apoptotic Bcl proteins remains unknown. More studies have to be performed in order to shed light on the exact role of this protein in transformation.

5.2 PERSPECTIVE

Scavenging peroxide in cancer cells can lead to a block in proliferation due to inhibition of growth factor signaling. However this process might prove to be complicated due to existent redundancy in pathways resulting in tumor growth. Many inhibitors of antioxidants are currently being used as chemotherapeutic agents with the rationale that accumulation of ROS will result in apoptosis in cells. However these approaches do not target specific molecules. It is clear that there exists a complex correlation between cellular signaling pathways and cellular redox status. Although the role of redox regulation in cellular signaling is evident, its importance for many cellular functions remains unknown. It is important to study the role of redox regulation and the underlying molecular mechanisms in cells in order to be able to target the key players involved in these pathways for therapeutic purposes.

5.3 SUMMARY

During the last few years more evidence has gathered for the role of ROS as second messengers in signaling. Although it has been shown that activation of receptors leads to production of reactive oxygen species, the exact targets of ROS have not been identified.

Chapter 1 of this thesis focuses on G-CSFR signaling. Activation of downstream signaling pathways which either activate signaling or switch off signaling have been described in detail. Also, the endocytotic pathway taken by the G-CSFR has been described. Special attention has been paid to the role of ROS in controlling signaling. ROS are produced locally. However they are highly unstable and reactive molecules which can diffuse easily throughout the cell. How do these molecules which are produced locally execute their function on different signaling molecules? Several antioxidant proteins with distinct sub-cellular localizations play an important role not only in limiting the amount of ROS but also in recycling of phosphatases which are inactivated due to ROS.

Chapter 2 describes the role of an antioxidant protein, Prdx4 which was found to negatively regulate signaling via the G-CSFR. Although, Prdx4 is predominantly localized in the

endoplasmic reticulum (ER) it was found to interact with internalised G-CSFR vesicles indicating that G-CSFR vesicles pass the ER on their way to the lysosomes. The C-terminal domain of the G-CSFR was found to be critical for interaction with Prdx4. Negative regulation of G-CSF mediated proliferation and signaling by Prdx4 in myeloid progenitor cells was strictly dependent on the presence of catalytic cysteines in the active site of Prdx4. Loss of *Prdx4* expression led to increased colony formation in myeloid progenitor cells as compared to wild type controls. Loss of *Prdx4* also led to increased Stat3 levels as compared to wild type control MEFs.

Chapter 2 also reports that another ER resident protein tyrosine phosphatase, Ptp1b negatively regulates proliferation via G-CSF in myeloid progenitor cells. Loss of *Ptp1b* led to prolonged phosphorylation of tyrosines of the G-CSFR and also increased activation of Stat3 and Jak2 as compared to *Ptp1b* reconstituted MEFs. Ptp1b is also responsible for dephosphorylation-mediated reactivation of Prdx4. The activated Prdx4 then could reduce ROS and reactivate Ptp1b completing a positive feed back loop for down regulation of G-CSF signaling.

In *Chapter 3* it is shown that *PRDX4* expression is very low or absent in acute promyelocytic leukemia (APL) patients characterised by the chromosomal translocation t(15;17). Also the proteins levels of PRDX4 are very low in APLs as compared to other leukemias. The low expression of PRDX4 is only associated with APL and not promyelocytes, the cells from which this leukemia is derived, indicating that this is a leukemia-related phenomenon. Although a bivalent chromatin methylation mark is present at the TSS of *PRDX4*, H3K27me3 is the dominant repressive mark. No aberrant methylation of CpG islands in the *PRDX4* promoter region of was detected in APL. The fusion product, PML-RARα resulting from the t(15;17) was found to be associated with the promoter of *PRDX4*. PML-RARα is known to recruit the PRC2 complex, responsible for H3K27-me3. This could explain the correlation between PML-RARα and H3K27-me3 presence at the TSS of *PRDX4* and silencing of *PRDX4* in APLs.

In *Chapter 4* it was shown that *Txnip*, the gene encoding a protein that is also involved in oxidative stress mediated signaling, is a common virus integration site in murine myeloid leukemia induced by murine leukemia virus. Ectopic expression of *Txnip* in myeloid progenitor cells, leads to decreased proliferation of these cells. Furthermore, the expression (both mRNA and protein) of TXNIP was found to be higher in certain AML samples, particularly those carrying the t(8;21). No mutations in *TXNIP* were reported in AMLs. Combining the murine and human AML data, may suggest that abnormal expression of Txnip is associated with AML.

Finally, in *Chapter 5* the results reported in this thesis have been summarized and discussed in a broader perspective.

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NEDERLANDSE SAMENVATTING

In de voorbije jaren is bekend geworden dat zuurstof radicalen (reactive oxygen species, afgekort als ROS) een rol spelen bij de intracellulaire signaalafgifte (signaaltransductie) door groeifactorreceptoren. Hoewel het nu vast staat dat activering van groeifactorreceptoren leidt tot verhoogde aanmaak van ROS en veranderingen in de signaalfunctie, zijn de onderliggende werkingsmechanismen nog grotendeels onbekend.

Hoofdstuk 1 van dit proefschrift richt zich op de belangrijkste principes van G-CSF receptor (G-CSFR) signalering. Mechanismen die verantwoordelijk worden geacht voor de activering en uitschakeling van de aan de G-CSFR gekoppelde signaalwegen worden hier ingeleid. Ook wordt het principe van G-CSFR endocytose beschreven. Speciale aandacht is gericht op de regulerende rol van ROS in signaaltransductie. Het wordt steeds duidelijker dat ROS zeer lokaal in de cel worden geproduceerd. ROS moleculen zijn echter zeer instabiel en kunnen zich gemakkelijk door de hele cel verplaatsen. Een belangrijke vraag is hoe ROS moleculen die lokaal worden geproduceerd hun functie op verschillende signaalmoleculen uitvoeren. Meerdere antioxidant eiwitten en oxidatiegevoelige fosfatasen, gelokaliseerd in verschillende subcellulaire compartimenten zoals het endoplasmatisch reticulum (ER), blijken hierbij een belangrijke rol te spelen.

Hoofdstuk 2 handelt over de remmende werking van het antioxidant eiwit peroxiredoxin-4 (Prdx4) op de signaalfunctie van G-CSFR. Hoewel Prdx4 voornamelijk in het ER is gelokaliseerd werd een interactie met de geïnternaliseerde, in endocytotische blaasjes aanwezige G-CSFR eiwitten waargenomen, wat duidt op een passage van deze G-CSFR bevattende blaasjes langs het ER. Het carboxyl-terminale gedeelte van de G-CSFR bleek essentieel te zijn voor de binding met Prdx4. De remmende werking van Prdx4 op de door G-CSF geïnduceerde proliferatie van myeloide voorlopercellen bleek strikt afhankelijk van de aanwezigheid van de katalytisch actieve cysteine residuen in Prdx4. Verlies van Prdx4 expressie resulteerde in een toename van G-CSF geïnduceerde kolonievorming door myeloide voorlopercellen. Verlies van Prdx4 expressie leidde ook tot verhoogde activering van het STAT3 eiwit in vergelijking tot normale controle cellen.

In *Hoofdstuk* 2 wordt verder uiteengezet hoe het eiwit tyrosinefosfatase Ptp1b, ook een enzym dat zich in het ER bevindt, de door G-CSF geïnduceerde proliferatie van myeloide voorlopercellen remt. Verlies van Ptp1b leidde tot een verlengde tyrosinefosforylering van de G-CSFR en ook tot verhoogde activiteit van STAT3 en Jak2, in vergelijking tot controle cellen. Bovendien werd aangetoond dat Ptp1b verantwoordelijk is voor de defosforylering van Prdx4, wat mogelijk een verhogend effect heeft op de activiteit van Prdx4. De resultaten passen in een model waarin het actieve Prdx4 lokaal, d.w.z. nabij het ER, de hoeveelheid ROS verlaagt waardoor het oxidatie gevoelige Ptp1b actief blijft.

In Hoofdstuk 3 wordt beschreven dat de expressie van *PRDX4* transcripten zeer laag of zelfs afwezig is in acute promyelocyten leukemie (APL) cellen, gekenmerkt door de chromo-

soom translocatie t(15;17). Ook op eiwitniveau is de expressie van PRDX4 in APL cellen zeer laag vergeleken met andere typen leukemiecellen. De lage expressie van PRDX4 werd niet in normale, d.w.z. niet leukemische promyelocyten gezien, wat aangeeft dat verlaagde PRDX4 expressie een leukemie gerelateerd fenomeen is. Hoewel er een zogenaamde bivalente histon methylering markering aanwezig is op de transcriptie start site (TSS) van *PRDX4*, overheerst histon H3K27 tri-methylering (me3), leidend tot onderdrukking van *PRDX4* genexpressie. Er werd geen afwijkend DNA methyleringspatroon van CpG eilanden in de *PRDX4* promoter regio waargenomen. Het PML-RARα fusie-eiwit, ontstaan door de t(15;17) chromosoomfusie, bleek te binden aan de *PRDX4* promoter. Van PML-RARα is bekend dat het aan het PRC2 complex kan binden dat verantwoordelijk is voor de trimethylering van H3K27. Dit vormt de mogelijke verklaring voor de betrokkenheid van PML-RARα bij de vorming van H3K27-me3 op de TSS van PRDX4, leidend tot de onderdrukte expressie van het *PRDX4* eiwit in APL.

In *Hoofdstuk 4* wordt getoond dat *Txnip*, het gen dat codeert voor thioredoxin-interacting protein (Txnip), een eiwit betrokken bij ROS gereguleerde signaalprocessen een zogenaamde common virus integration site (CIS) is in door retrovirus veroorzaakte leukemie bij de muis. Ectopische expressie van Txnip in myeloide voorlopercellen leidde tot verlaagde proliferatie van deze cellen. Bovendien was de expressie (zowel op mRNA als op eiwitniveau) van Txnip verhoogd in humane AML cellen, in het bijzonder in AML met de chromosoom translocatie t(8;21). Er werden geen mutaties in het *TXNIP* gen gevonden bij AML patiënten. De gecombineerde resultaten van de studies bij de muis en in humane AML suggereren dat afwijkende expressie van TXNIP een rol kan spelen in AML, maar welke redox-gevoelige mechanismen hierbij betrokken zijn is nog onduidelijk.

In *Hoofdstuk 5*, tenslotte, worden de resultaten beschreven in dit proefschrift samengevat en in een breder perspectief bediscussieerd.

ABBREVIATIONS

AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
Ask1	Apoptosis signaling Kinase 1
CAT	Catalase
CFP	Cyan Flourescent protein
ChIP	Chromatin Immunoprecipitations
CIS	Common Integration Site
CLSM	Confocal Laser Scanning Microscopy
CML	Chronic Myeloid Leukemia
CSF3R	Granulocyte colony stimulating factor receptor
DSPs/ DUSPs	Dual Specificity Phosphatases
DUBs	de-ubiquitinating enzymes
DZNep	3-deazaneplanocin A
EEA1	Early Endosome Antigen 1
EED	Embryonic ectoderm development
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPO	Erythropoietin
EPO	Erythropoietin Receptor
ER	Endoplasmic Reticulum
ERAD	ER-associated degradation
ERGIC	Endoplasmic Reticulum Golgi Intermediate Compartment
ESCRT	Endosomal sorting complex required for transport
EZH2	Enhancer of zeste homolog 2
FCS	Fetal Calf Serum
FISH	Flourescent in-situ hybridization
G-CSF	Granulocyte Colony Stimulating Factor
G-CSFR	Granulocyte Colony Stimulating Factor Receptor
GFP	Green Flourescent protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GM-CSFR	Granulocyte-Macrophage Colony-Stimulating Factor Receptor
GPX	Glutathione peroxidise
Gr 1.4	Graffi 1.4
H_2O_2	Hydrogen Peroxide
H3K27-me3	Trimethylation of lysine 27 of histone 3
H3K4-me3	Trimethylation of lysine 4 of histone 3
H3K36-me2	Bimethylation of lysine 36 of histone 3

HAC	Histone acetylases
HDAC	Histone deacetylases
HEK cells	Human embryonic kidney cells
HGFs	Hematopoietic growth factors
HSCs	Hematopoietic Stem Cells
IL-R	Interleukin Receptor
Jak	Janus Tyrosine Kinase
K	Lysine
LTR	Long Terminal Repeat
MAPPIT	Mammalian Protein Protein Interaction Trap
MBD	Methyl-CpG binding domain protein
MDS	Myelodysplastic Syndrome
MEFs	Mouse Embryonic Fibroblasts
MuLV	Murine Leukemia Virus
MVBs	Multi Vesicular Bodies
NAC	N-acetyl Cysteine
NOX	NADPH Oxidase
O_2^-	Superoxide Radical
OH^-	Hydroxyl Radical
PDGFR	Platelet-Derived Growth Factor Receptor
PH2	Pleckstrin Homology 2
PI3K	Phosphoionositol 3 kinase
PKB	Protein kinase B
PLZF	Promyelocytic leukemia zinc finger
PML	Promyelocytic Leukemia
PRC	Polycomb Repressor Complex
Prdx	Peroxiredoxin
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
Ptp1b	Protein Tyrosine Phosphatase 1b
RAR	Retinoic Acid Receptor
RAREs	Retinoic Acid Responsive Elements
ROS	Reactive Oxygen Species
RPTPs	Receptor Protein Tyrosine Phosphatases
S	Serine
SCF	Stem Cell Factor
SCN	Severe Congenital Neutropenia
SH2	Src Homology 2
SHIP1	SH2 Domain-containing Ionositol Phosphatase

SHP1	Src Homology 2 domain phosphatase 1
SHP2	Src Homology 2 domain phosphatase 2
SOCS	Suppressor of Cytokine Signaling
SOD	Superoxide dismutase
STAM2	Signal transducing adapter molecule 2
STAT	Signal Transducer and Activator of Transcription
SUZ12	Enhancer of zeste homolog 12
T	Threonine
t(15;17)	Translocation between chromosomes 15 and 17
t(8;21)	Translocation between chromosomes 8 and 21
TPO	Thrombopoietin Receptor
TPβ	Thromboxane A2 receptor
TRAIL	TNF-related apoptosis inducing ligand
TRX/ TXN	Thioredoxin
TSS	Transcriptional Start Site
Txnip	Thioredoxin interacting protein
UTR	Untranslated Region
Vdup1	Vitamin D3 upregulated protein 1
VIS	Viral Integration Site
Wsb	WD-repeat domain and SOCS box-containing protein
Y	Tyrosine

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Karishma

CURRICULUM VITAE

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PUBLICATIONS

1. *The gene encoding thioredoxin-interacting protein (TXNIP) is a frequent virus integration site in virus-induced mouse leukemia and is over-expressed in a subset of AML patients*
   Stefan J. Erkeland, Karishma K. Palande, Marijke Valkhof, Judith Gits, Astrid Danen-van Oorschot and Ivo P. Touw
   Leukemia Research, March 2009

2. *The antioxidant protein peroxiredoxin 4 is epigenetically down regulated in acute promyelocytic Leukemia*
   Karishma K. Palande, Renee Beekman, Lotte van der Meeren, Berna Beverloo, Peter J. M. Valk, Ivo P. Touw
   PLoS one- 2011 Jan 20;6(1):e16340.

3, *G-CSF receptor signaling at the endoplasmic reticulum-early endosome interface*
   Karishma Palande, Onno Roovers, Judith Gits, Carola Verwijmeren, Yoshihito Iuchi, Junichi Fujii, Benjamin G. Neel, Robert Karisch, Jan Tavernier and Ivo P. Touw
   Submitted

4 *G-CSFR signaling: An update*
   Karishma K. Palande, Annemarie Meenhuis, Ivo P. Touw
   Manuscript in preparation

COLOR SECTION
CHAPTER 2

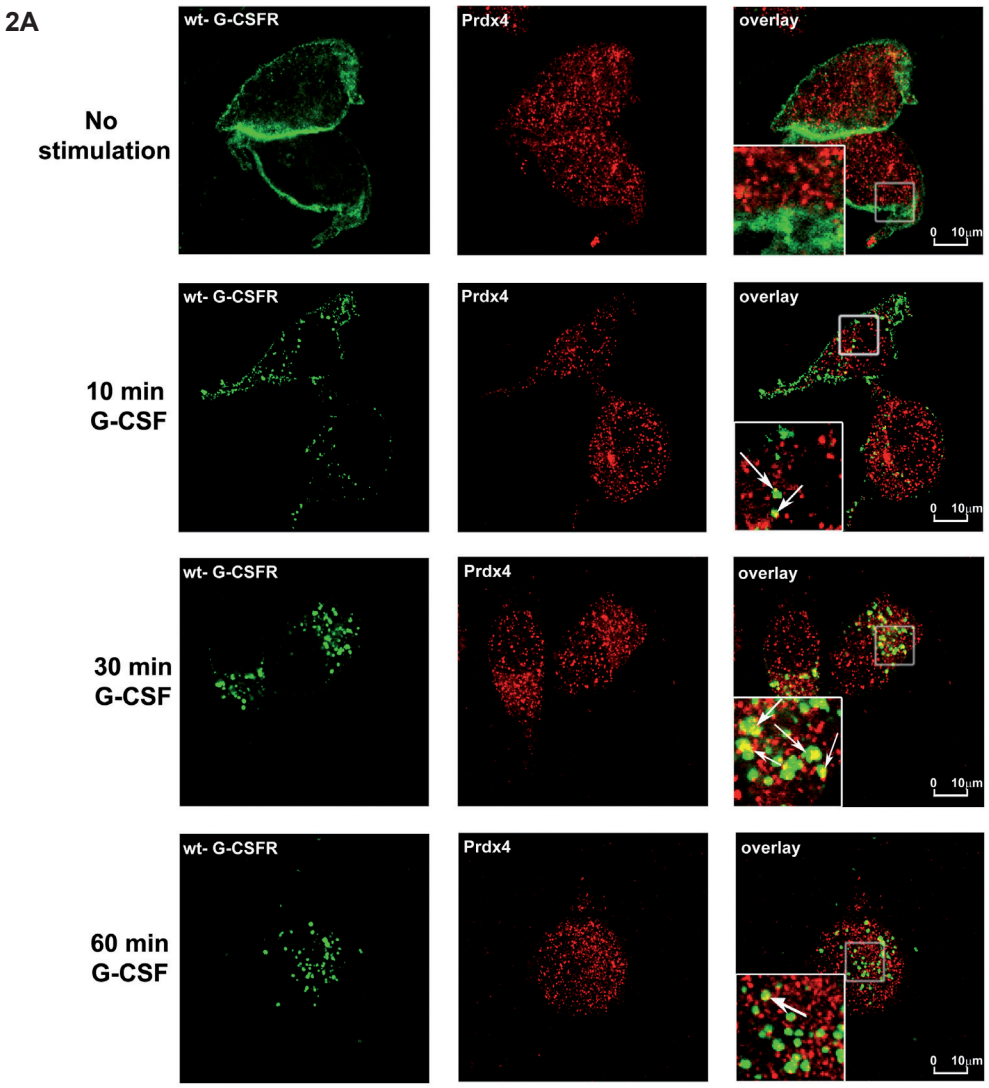

Figure 2. G-CSFR and Prdx4 interaction and co-localization
(A) HeLa cells ectopically expressing WT G-CSFR were growth factor-deprived for 4 hrs. Surface membrane G-CSFRs were labelled with anti-G-CSFR antibody prior to stimulation with G-CSF for different time points. Cells were permeabilised, fixed and stained with anti-Prdx4 antibody, followed by secondary and anti-rabbit Cy3 and anti-mouse Cy5 antibodies, and analyzed by CLSM.

2B

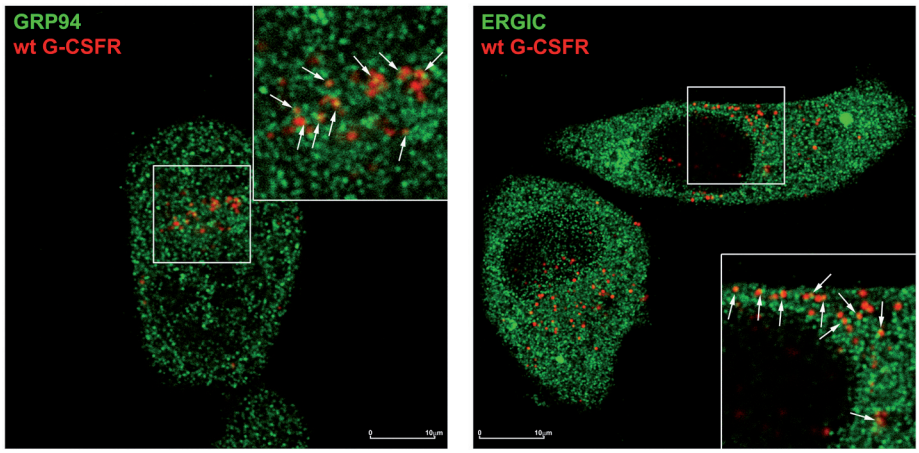

Figure 2. G-CSFR and Prdx4 interaction and co-localization
(B) Co-localization of endocytosed G-CSFR with Grp94-stained ER and with ERGIC 30 min. after ligand stimulation. Experimental conditions were the same as under B.

S3

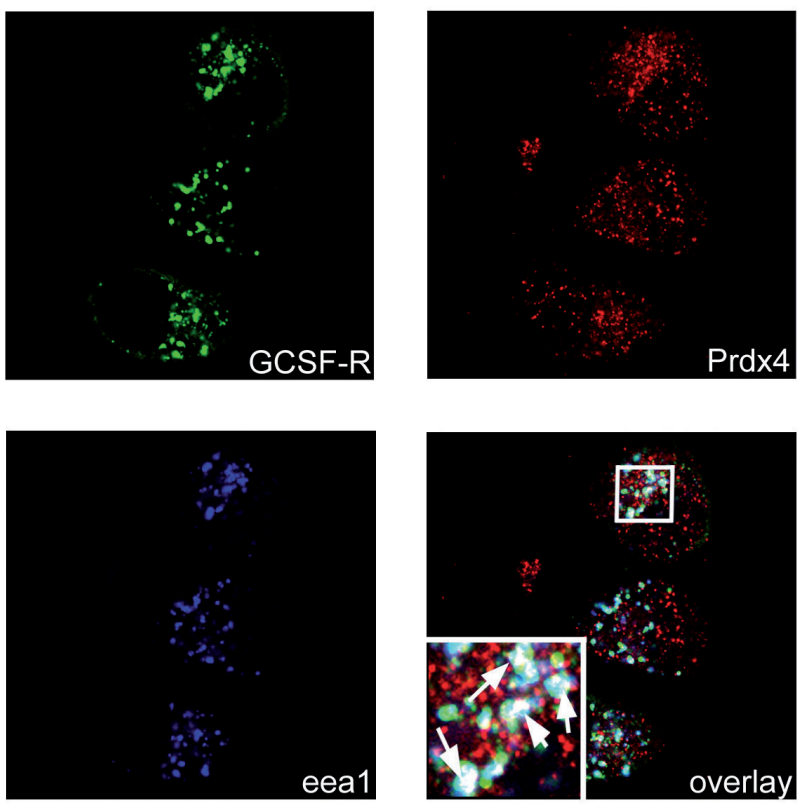

Figure S3: HeLa cells ectopically expressing wt-G-CSFR were growth factor deprived for 4 hrs. Surface membrane G-CSFRs were labeled with anti-G-CSFR antibody prior to stimulation with G-CSF for different time points. Cells were permeabilised, fixed and stained with anti-Prdx4 and eea1 antibodies followed by secondary anti-goat 488, anti-rabbit Cy3 and anti-mouse Cy5 antibodies antibodies and analyzed by CLSM.

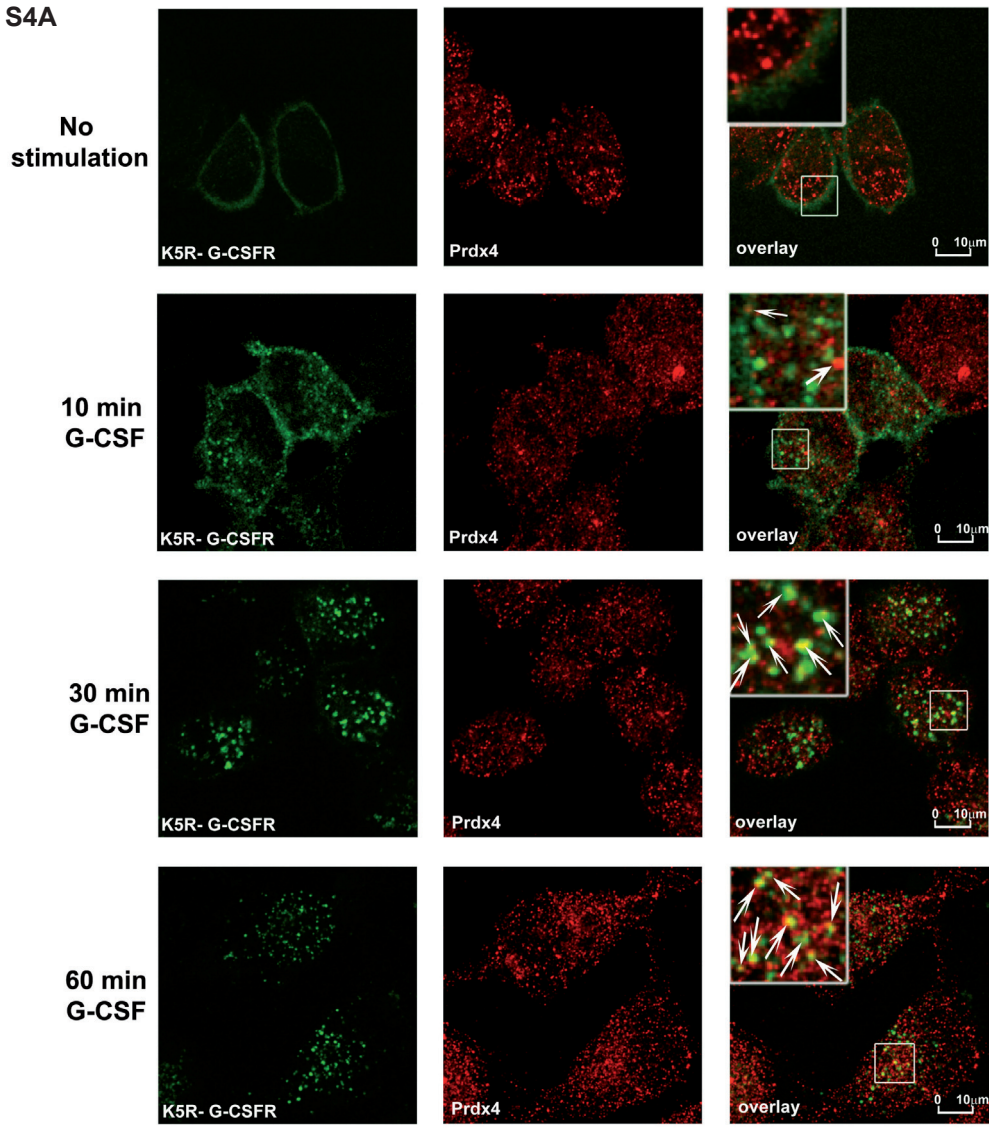

Figure S4a: HeLa cells ectopically expressing K5R-G-CSFR were growth factor deprived for 4 hrs. Surface membrane G-CSFRs were labeled with anti-G-CSFR antibody prior to stimulation with G-CSF for different time points. Cells were permeabilised, fixed and stained with anti-Prdx4 antibody followed by secondary and anti-rabbit Cy3 and anti-mouse Cy5 antibodies antibodies and analyzed by CLSM.

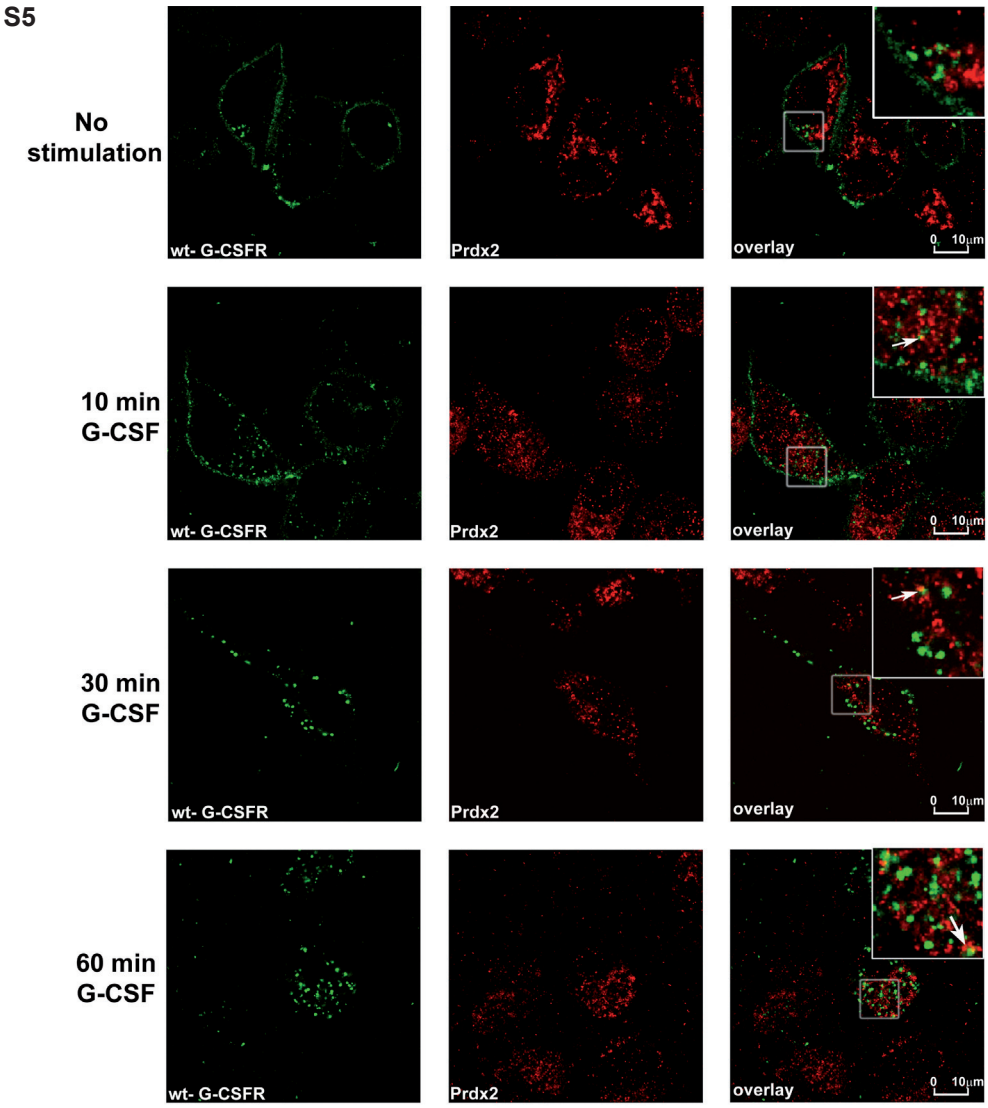

Figure S5: HeLa cells ectopically expressing wt-G-CSFR were growth factor deprived for 4 hrs. Surface membrane G-CSFRs were labeled with anti-G-CSFR antibody prior to stimulation with G-CSF for different time points. Cells were permeabilised, fixed and stained with anti-Prdx2 antibody followed by secondary and anti-rabbit Cy3 and anti-mouse Cy5 antibodies antibodies and analyzed by CLSM.

S6

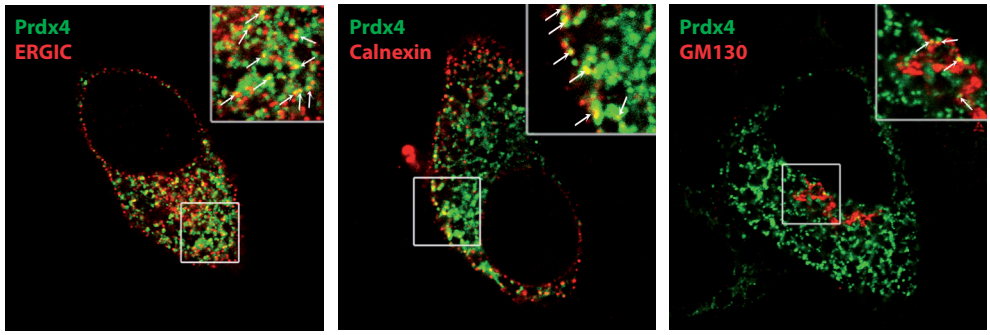

Figure S6: CLSM showing the co-localization of Prdx4 with markers for various sub-cellular compartments in HekT cells. The ER-Golgi-Intermediate Compartment was stained using an antibody against ERGIC, the ER with anti-calnexin and the Golgi with anti-GM130.

S7

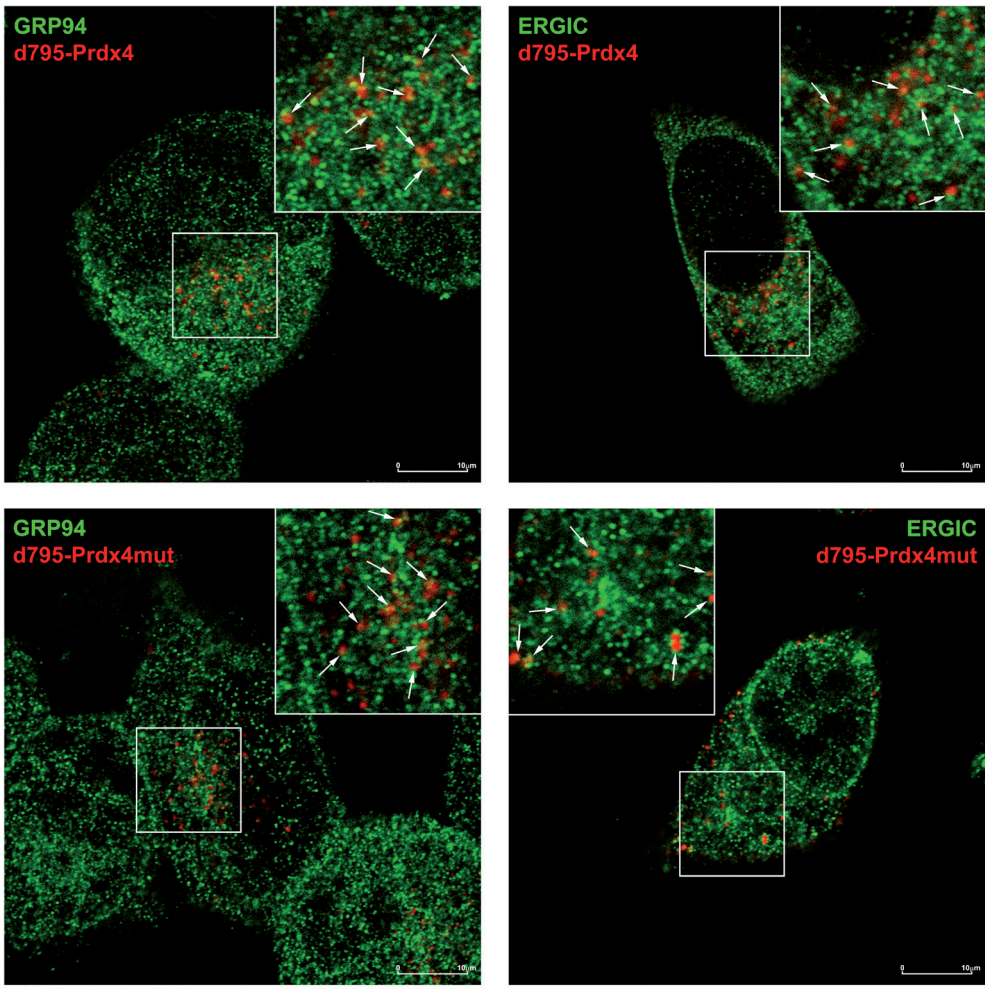

Figure S7: (A) CLSM showing routing of G-CSFR-Prdx4 fusion protein towards ER and ERGIC comparable to WT G-CSFR (Figure 2B); (B) Flow cytometric analysis of cell surface expression of G-CSFR constructs in 32D cells

S8

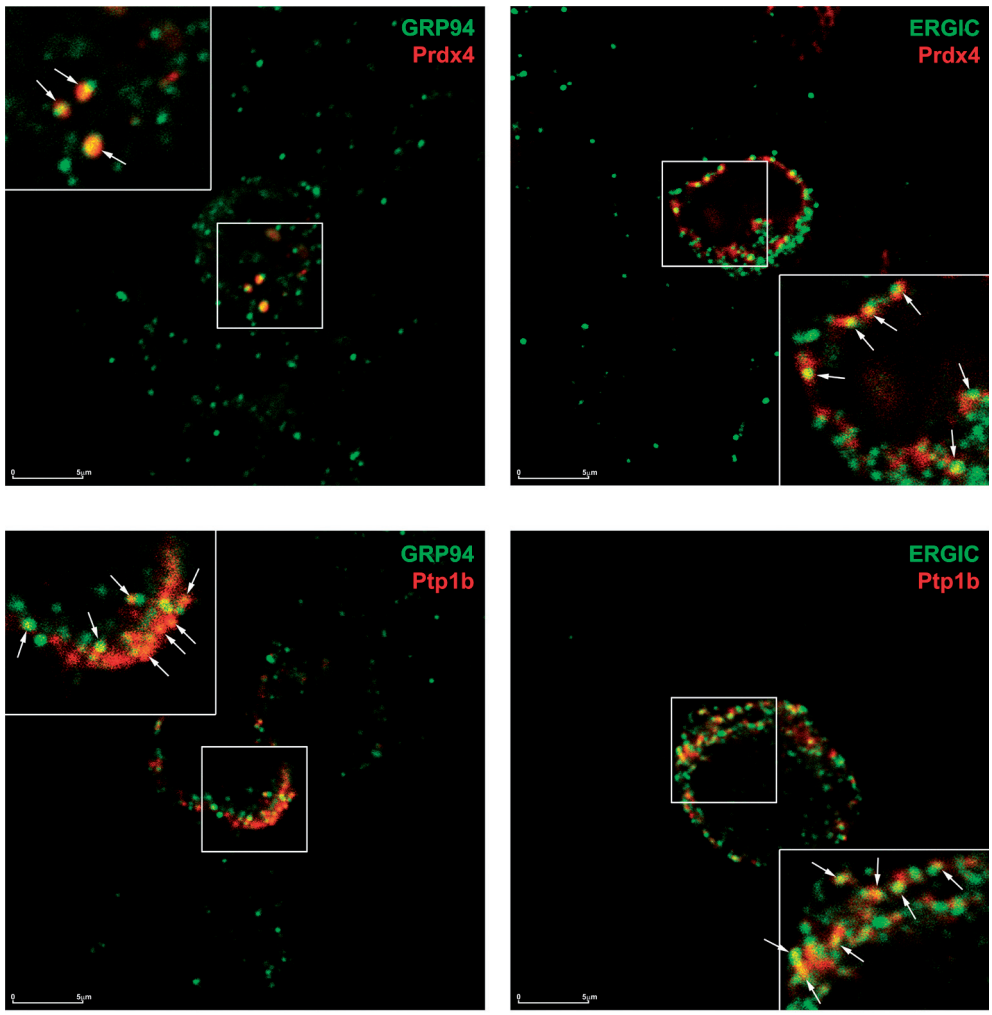

Figure S8: CLSM showing co-localization of Prdx4 or Ptp1b with ER (Grp94) or ERGIC markers in mouse bone marrow cells.

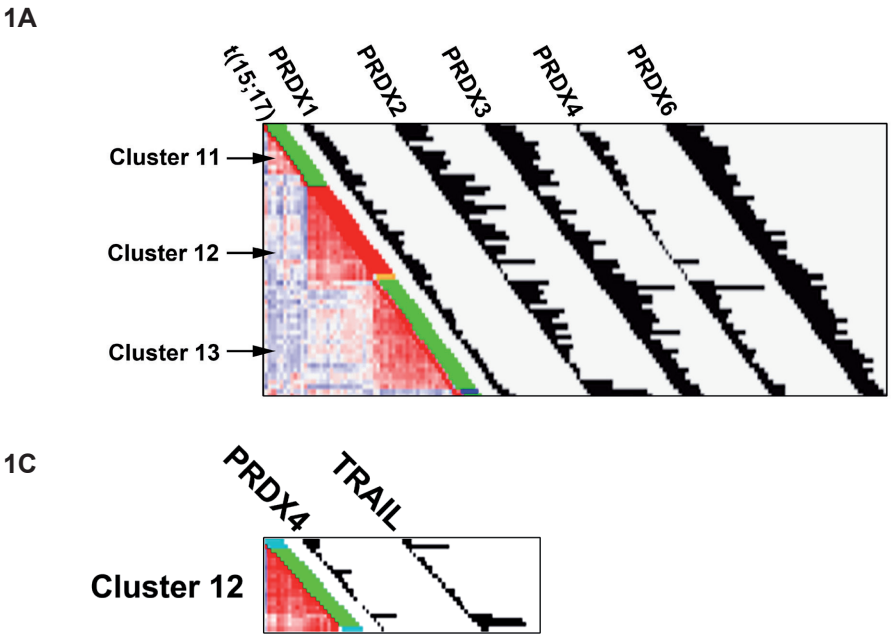

Figure 1: Expression of *PRDX* transcripts in AML and APL
(A) Graphical representation of expression of *PRDX* family members in APL patients clustered based on expression of ~2000 genes as described [14]. Cluster 12 is exclusively formed by APL patients, as indicated by the red bars indicating the presence of t(15;17). Cluster 11 comprises AML patients with normal karyotype and an underlying NPM1 mutation. Cluster 13 is formed by AML patients with t(8;21). Histograms represent MAS5-normalized expression values.
(C) APLs are not associated with high expression of *TRAIL* ligand excluding the possibility that low expression of *PRDX4* could be attributed to high levels of *TRAIL*

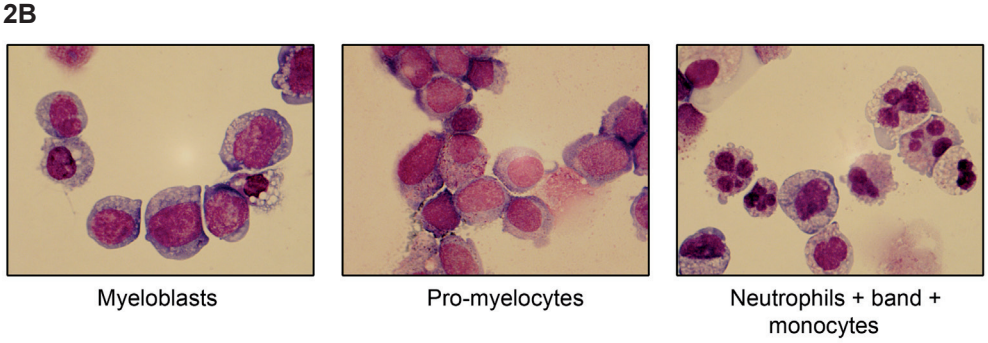

Figure 2: Reduced PRDX4 protein levels in APL
(B) Lower panel: Micrographs of May Grunwald Giemsa stained fractions of cells used for Western blotting.

2A

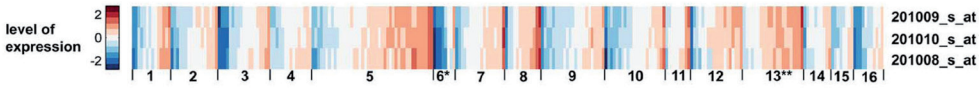

Figure 2. *TXNIP* levels in human AML samples

(A) Level of *TXNIP* mRNA expression in 285 human AML samples. The 16 clusters with molecular characteristics in a cohort of 285 AML patients have been published [247] and are depicted on the X-axis. Molecular characteristics associated with the clusters are: cluster 1: t(11q23), 2: FLT3-ITD, 3: FLT3ITD, 4: CEBPα mutations, 6: FLT3-ITD, 9: inv(16), 10: Evi-1, 12: t(15;17), 13: t(8;21), 15: CEBPα mutations, 16: t(11q23). *TXNIP* mRNA expression was found significantly lower (as indicated by *) or higher (**) by SAM analyses in clusters of leukemia. Probe sets of *TXNIP* were considered to be differentially expressed when Fold Change was over 1.5 or under 0.67, score was over 4 or less than 4, and a q-value less then 5%, where False Discovery Rate was less than 5%.