REGULATION OF THROMBOPOIETIN RECEPTOR EXPRESSION AND FUNCTION

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SUMMARY

Of the many cells in the body, the hematopoietic cells are among those with the highest rate of self-renewal and turnover. The production and destruction of these cells are tightly controlled by a number of hematopoietic growth factors, in particular by members of the family of helical cytokines. Studying the thrombopoietin receptor, I focused on two aspects of cytokine receptor signaling: attenuation of signaling by receptor isoforms and the biological function of cytokine receptor target genes.

Cytokine receptor signaling has profound effects on cell survival, proliferation and differentiation. It is therefore not surprising that components of the signaling cascade are tightly regulated at the level of expression. An important mechanism for controlling gene expression is alternative splicing. Alternate isoforms have been identified for many cytokine receptors and a regulatory function and/or altered expression in disease have been described for some of these isoforms

The cytokine thrombopoietin (TPO) and its cognate receptor c-mpl are the primary regulators of platelet production and also play an important role in hematopoietic stem cell biology. Several isoforms of unknown function exist for both mouse and human mpl and it is possible that they play an important role in modulating mpl signaling. In my thesis work, I have analyzed the function of a truncated receptor isoform (mpl-tr) which is the only alternate mpl isoform conserved between mouse and humans. Although mpl-tr lacks a transmembrane domain, classifying it as a ‘secreted’ or ‘soluble receptor’, it is retained intracellularly. My results provide evidence that mpl-tr acts as a dominant-negative variant of mpl for both proliferation and survival. I also demonstrate that mpl-tr mediates protein degradation of the full-length receptor by a cathepsin-like cysteine protease activity. Due to a shift of the reading frame at a splice acceptor site, the C-terminus of mpl-tr consists of a peptide of unique sequence, 30 amino acids in length. I show that this peptide sequence is essential for the inhibition of TPO-dependent proliferation and for mpl protein degradation mediated by mpl-tr. Together, these data suggest a

new paradigm for the regulation of cytokine receptor expression and function through a proteolytic process directed by a truncated isoform of the same receptor.

To test for the *in vivo* function of alternative mpl isoforms, a c-mpl cDNA was expressed as a transgene in mpl knockout mice. These mice express mpl full-length as the only mpl isoform and develop severe thrombocytosis with platelet numbers, elevated about five times higher than normal. The reintroduction of the endogenous mpl allele restores normal platelet counts and I attribute this to the *in vivo* effect of dominant-negative mpl isoforms. A mpl knock-in allele, which does not express mpl-tr but still expresses the second known alternate variant of murine mpl, mpl-II, normalizes platelet numbers, similar to the endogenous mpl allele. This result demonstrates that the absence of mpl-tr is not sufficient to cause thrombocytosis. I propose that mpl-II is an additional dominant-negative mpl isoform and attenuates the expansion of the megakarocytic lineage *in vivo.* In summary, these results impressively demonstrate the importance of alternate cytokine receptor isoforms *in vivo* and emphasize the need to study the function of the many uncharacterized cytokine receptor isoforms.

In a second project, I studied the role of mpl signaling in regulating the expression of a gene with a potential role in cell differentiation and proliferation. The diversification of cell types is controlled through the use of both lineage-restricted and more widely expressed transcriptional regulators and the combinatorial actions of these regulators specify gene expression. The differentiation of megakaryocyte precursors is dependent on the proper function of the GATA-1 transcription factor. Mice lacking GATA-1 selectively in megakaryocytes have dramatically fewer platelets but more megakaryocytes, altered platelet size and shape and prolonged bleeding times. Further, GATA-1-null megakaryocytes hyperproliferate *in vitro*, suggesting that GATA-1 is both a differentiation factor and negative regulator of megakaryocyte cell proliferation. However, GATA-1 regulated genes which are responsible for this growth inhibition are presently unknown. In this thesis work, I describe a novel gene, GASIP (GATA-1 regulated SIAH Interacting Protein), which is

dramatically downregulated in mpl-transfected hematopoietic cell lines, identifying mpl as a negative regulator of GASIP expression. The presence of juxtaposed GATA and Ets-binding *cis*-elements in the GASIP promoter are typical for a megakaryocytic gene. I found that GASIP expression in platelets is indeed robust and correlates with mRNA levels of GATA-1, but not GATA-2 or –3, identifying GATA-1 as a positive regulator of GASIP expression. The finding that mpl and GATA have opposite effects on both proliferation and on GASIP expression, make GASIP a candidate GATA-1 target gene involved in growth inhibition. To investigate the potential role of GASIP in growth regulation, I screened for potential protein binding partners. Interestingly, I identified the p53-inducible tumor suppressor seven in absentia homologe (SIAH) as a GASIP interacting protein. I speculate that GASIP may contribute to the anti-proliferative effect mediated by SIAH.

GENERAL INTRODUCTION

Hematopoiesis

Vertebrate blood consists of multiple cell types that perform varied and specific functions. Though distinct, all blood cell types are derived from a common, pluripotent precursor or hematopoietic stem cell (HSC) [3]. The onset of vertebrate hematopoietic development, generally termed primitive hematopoiesis, occurs during embryogenesis when the extraembryonic yolk sac (or an equivalent site) gives rise to blood precursors, which are primarily erythroid in nature [4]. A second round of de novo hematopoietic development, termed definitive hematopoiesis, occurs in the mesodermal aorta/gonad/mesonephros (AGM) region of the embryo proper and gives rise to cells that will seed subsequent hematopoietic sites, such as the fetal liver and the bone marrow in mammals, as well as to all blood cell types found in the mature organism [5]. The myeloid lineage gives rise to multiple cell types including erythrocytes, megakaryocytes (from which platelets are derived), and macrophages, which primarily function as professional phagocytes in the context of both development and innate immunity [6]. The lymphoid lineage primarily mediates adaptive immunity through the production of B and T cells and natural killer cells [7]. Proliferation and differentiation of the HSC and lineage restricted precursor cells are largely regulated by the interaction of these cells with soluble signaling molecules, especially cytokines.

Cytokines and their receptors

Many aspects of cell behavior, such as growth, motility, differentiation, and apoptosis, are regulated by signals which cells receive from their environment. Upon ligand binding, receptors become activated and thereby initiate a cascade of intracellular events. The initial ligand/receptor interaction seems to be the most specific step in this cascade. The specificity of

ligand/receptor interaction has been well studied in the field of cytokines and their receptors [8].

The helical cytokines constitute a family of secreted proteins of a molecular weight of usually 20 to 30 kDa. The amino acid sequences of different cytokines display no apparent homology but share a common three-dimensional structure of an anti-parallel “four-helix bundle” with a characteristic up-up-down-down topology [9]. This topology demands that the three loops connecting the four helices are long-short-long (Fig.1).

All these cytokines transmit their signal into the cell by multi-subunit receptor complexes. Subfamilies of cytokines have been classified based shared signal-transducing receptor subunits. One such receptor-subunit is gp130, which is shared by the Interleukin-6 (IL-6) type cytokines as part of the signal-transducing complex. Similarly, the β-subunit of the IL-3 receptor is shared by IL-3, IL-5, and the granulocyte macrophage colony stimulating factor (GM-CSF), while the IL-2β receptor is the common subunit for IL-2, IL-4, IL-7 and IL-15 [10].

The growth hormone (GH) receptor complex was the first four-helical cytokine receptor to be crystallized together with its ligand [11] and has therefore become pivotal for the understanding of four-helical cytokine receptor complexes. One GH molecule binds to two receptor molecules via two contact epitopes designated as site I and II. Remarkably, the two receptors use identical amino acid residues to bind to the two different epitopes of the cytokine [11,12]. One conclusion derived from these studies is that cytokines are recognized by their cognate receptors at sites equivalent to site I and II of GH [8,11]. Biochemical studies demonstrated the existence of an additional site III on IL-6 and a requirement for two distinct binding epitopes on the receptor for activation [13-16].

The cytokine receptor superfamily is defined by the presence of the cytokine-receptor homology region (CRH) consisting of two immunoglobulin domains, whereby the loops connecting the β-sheets establish the specific contact to the ligand [17]. Within the cytokine receptor superfamily, two subclasses can be distinguished. The CRH domain of class I receptors contains two conserved disulfide bridges and a typical sequence motif tryptophan-serine-X-

tryptophan-serine (WSXWS) in the membrane proximal immunoglobin domain. In contrast, the CRH domain of class II receptors lacks the WSXWS motif and the first conserved disulfide bridge. Instead, a distinct disulfide bridge is formed at the C-terminus of the membrane proximal immunoglobulin domain [8]. In addition to CRH domains, the extracellular domain of cytokine receptors can contain several membrane proximal fibronectin type III (FN III) domains. The intracellular portion of cytokine receptors is very diverse in length and shows little homology. No enzymatic activity has been identified for the intracellular domain. However, many members of the cytokine receptor family are associated with kinases of the *janus* activated kinase (JAK) family through two membrane-proximal proline rich sequence motifs called box 1 and box 2. In contrast, TNF receptor (TNFR)-1 and –2 show neither intrinsic enzymatic activity nor are they directly associated with enzymes. Although TNFR-1 and –2 are activated by the same ligand, they initiate different signaling cascades due to their different intracellular modules. TNFR-1 contains an intracellular death domain, which interacts with proteins named TRADD leading to the activation of a number of proteases of the caspase family. The TNFR-2 does not contain a death domain but interacts with the FADD protein [18].

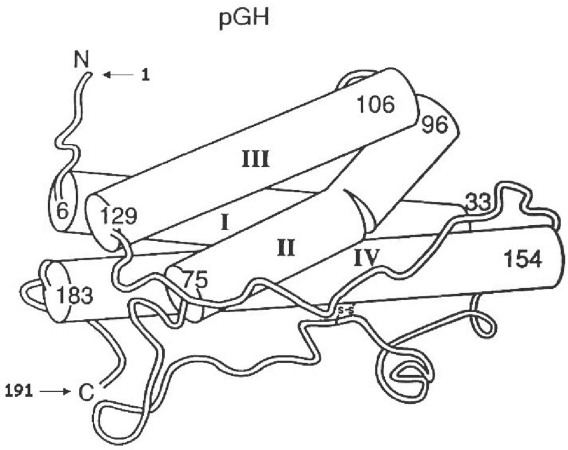

Fig.1. Computer representation of porcine GH crystal structure. The four antiparallel -helical bundles are represented as *cylindrical rods* and labeled with *Roman numerals I–IV*. The amino and carboxyl termini are indicated with an N and C, respectively. The *numbers* indicate amino acid positions at the N and C termini of each independent -helix. (Adapted from S. S. Abdel-Meguid et al. [2])

Most cytokines cause receptor dimerization or oligomerization upon binding. In these cases, dimerization or oligomerization is required and sufficient for the onset of the signal transduction. Interestingly, it has been shown that dimerization is required but not sufficient for some of the homodimeric

receptors to signal and that these receptors exist as preformed dimers on the cell surface. Crystallographic and biochemical studies have demonstrated that the erythropoietin receptor (EpoR) as well as TNFR-1 can form dimers in the absence of their ligands [19,20]. It has been demonstrated that distinct dimeric configurations exist for EpoR dependent on whether it is liganded or unliganded, or bound to agonistic or antagonistic peptides [19,21,22]. The presence of a ligand and its agonist or antagonist activity resulted in different distances and orientations of the receptor parts close to the membrane [19]. Beyond the evidence that preformed dimers exist, the structure of the unliganded receptors revealed that the dimerization occurs via epitopes that are also involved in ligand binding [19]. Therefore, ligand binding to EpoR can be regarded as a competition event in which the ligand binds the dimerized receptor and replaces the direct physical interaction between the two receptor chains. As a consequence, the intracellular domains of the two receptor molecules change their relative orientation to one another.

JAK/ STAT signaling

Janus kinases (JAK)s include four family members (JAK1-3 and TYK2) with molecular masses of 120-130 kDA. Whereas, JAK1, JAK2, and TYK 2 are expressed ubiquitously, expression of JAK3 is confined to hematopoietic and lymphoid cells [23,24]. These nonreceptor tyrosine kinases associate with members of the cytokine receptor family through conserved membrane proximal motifs, termed box 1 and 2, in the receptor [25]. JAKs lack true *src* homology 2 or 3 domains (SH2 and SH3), and the precise nature of the physical association between receptor and kinase is unknown. Box 1, defined by two appropriately spaced prolines (PXXP), is located within the first 30 cytoplasmic residues of most cytokine receptors and is similar to the core binding sequence of SH3 proteins. Box 2 is defined by the frequent occurrence of acidic and serine residues and is located between cytoplasmic amino acids 45 and 69 in mpl. The exact boundaries of box 2 are unknown. However, mutations or deletions of either box 1 or 2 completely disrupt all measurable JAK activity. Characteristic of the structure of JAKs is the

presence of two JAK homology (JH) domains, of which the C-terminal (JH1) domain has tyrosine activity [25]. Upon receptor oligomerization, JAKs are activated, presumbly by trans-'auto' phosphorylation on tyrosines. Subsequently, JAKs phosphorylate signal transducer and activators of transcription (STAT) proteins, which form homodimeric or heterodimeric complexes via their SH2 domains. These complexes translocate to the nucleus, where they bind to specific enhancer sequences and influence gene transcription [26]. Thus far, six mammalian STAT proteins (plus several isoforms) have been identified [27]. Two homologS exist of STAT 5 (STAT5A and STAT5B) that are encoded by different genes. Expression of STAT proteins is ubiquitous, except for STAT4, which is expressed in several tissues including spleen, heart, brain, peripheral blood and testis [28]. While STAT1, STAT3 and STAT5 are activated by multiple receptors, activation of STAT4 and STAT6 is restricted to the receptors for IL-12 and IL-4/IL-13, respectively [29-31]. Phosphorylated tyrosines and flanking amino acid residues in the activated cytokine receptor determine this specificity by providing docking sites for the SH2 domains of STATs [32,33]. The tyrosine phosphorylation of the receptor proteins is also likely to be directly mediated by JAKs.

Negative regulatory mechanisms of cytokine signaling

Of equal importance to the positive mechanisms initiated by cytokine signals are the negative regulatory mechanisms that serve to dampen or terminate cytokine signals [34,35]. Three classes of negative regulatory proteins have been discovered: phosphatases, PIAS and SOCS proteins – their importance being vividly demonstrated by mice lacking these inhibitors. Each of these protein families appears to act at a distinct point and at a particular time in the cytokine signaling cascade (Fig. 2). Additionally, alternatively spliced cytokine receptor variants have emerged as important negative regulators of cytokine signaling [36-38].

The SOCS family of cytokine signal suppressors

One recently identified protein family of inhibitors of cytokine signaling is the SOCS (suppressor of cytokine signaling) family. The first member of this family, CIS (cytokine-inducible SH2-domain-containing protein), was identified as an immediate-early gene, induced by IL-3, that inhibited signaling and proliferation [35]. Using different strategies, three other family members, termed SOCS1, JAB (JAK binding protein) and SSI1 (STAT-induced STAT inhibitor 1), were subsequently separately cloned [39,40]. The SOCS family now includes eight members, CIS and SOCS1-SOCS7 [41].

The mRNA and protein expression for SOCS members is rapidly induced by a variety of stimuli, including cytokines and growth factors. Importantly, SOCS expression is reduced in mice lacking various STAT genes but, in addition, there are examples of SOCS induction in response to stimuli that do not activate STATs, such as lipopolysaccharide or IL-1. Functioning as a classic negative feedback loop, SOCS proteins are structurally conserved but inhibit signaling by different mechanisms. SOCS-box-containing proteins can be divided into five subfamilies of proteins with SH2 domains, WD-40 repeats, ankyrin repeats, SPRY domains or GTPase domains [41]. The function of the SOCS-box has not been established but several lines of evidence point to a role in ubiquitin-mediated protein degradation, potentially through the binding of the elongin BC complex [42]. The elongins bind to E3 ligase complex of CUL 2 and Skp1 and this may regulate protein turnover. The function of SOCS proteins other than CIS and SOCS1-SOCS3 are currently not well understood.

CIS

CIS, the first member of the SOCS family to be identified, is induced by Epo, IL-3, GH, IL-2 and by Prl, and it competes with STATs for the receptor phosphotyrosine residues that serve as a docking site. CIS transgenic mice have low birth weight, stunted growth and defective mammary gland development, possibly due to a complete block of Prl signaling, a phenotype similar to that observed with Stat5-/- mice [43]. However, the lack of a clear

phenotype in the CIS-/- mice suggests that, unlike other SOCS family members (see below), it may be redundant [44].

SOCS1

SOCS1 mRNA is induced by many cytokines, including IL-6, IL-2, IL-4, LIF, IFN-α/β, IFN-γ, GH and G-CSF. SOCS1 inhibits JAK1, JAK2 and TYK2 but affects JAK3 kinase activity minimally. Mutational analysis of SOCS1 and SOCS3 has established that both the SH2 domain and an amino-terminal kinase-inhibitory region (KIR), but not the SOCS-box, are essential for blocking JAK activity and cytokine signaling. Interessingly, the KIR is not found in other SOCS [45-47] although SOCS4 and SOCS5 have amino-terminal regions with sequences similar to KIRs. SOCS1-/- mice, though born healthy, rapidly succumbed to a complex, multiorgan disease characterized by leukocytic infiltration and necrosis with severe B cell lymphopenia. SOCS1-/- mice have activated STAT1 in the liver, have elevated expression of IFN-γ-inducible genes, are hypersensitive to IFN-γ and have elevated circulating levels of this cytokine [48,49]. Accordingly, mice lacking both SOCS1 and IFN-γ are healthy, as are doubly deficient SOCS1-/- Rag2-/- mice.

SOCS2

SOCS2-/- mice are born at the expected Mendelian ratio, survive weaning and are healthy and fertile as adults [50]. Male SOCS2-/- mice grow more rapidly than their wild-type counterparts; SOCS2-/- females are also heavier, but less dramatically so. With the notable exception of the brain, most organs in SOCS2-/- animals are proportionately large and the long bones are 7-15% longer. The increased weight of SOCS2-/- animals is not due to increased fat; rather the animals are if anything leaner than normal, a phenotype similar to that of GH transgenic mice. The mechanism of SOCS2 action in regulating body size is not entirely clear but an important mechanism of GH action is the induction IGF1 (insulin-like growth factor 1). Notably, SOCS2-/- animals exhibit elevated expression of IGF1 mRNA in a number of tissues, including the heart, lung and spleen.

SOCS3

Like SOCS1, SOCS3 can inhibit STAT activation in response to many cytokines, including GH, leptin, IL-2, IL-4 and IL-10; SOCS3 is also induced in response to most four--helical cytokines tested. SOCS3 only partially inhibits JAK activation but its effect is enhanced in the presence of receptors, suggesting that SOCS3 inhibits cytokine responses by binding to phosphorylated components of the receptor complex [51,52]. For gp130, an SH2-domain-containing protein tyrosine phosphatase 2 (SHP-2)-interaction site (YEPY757STV) is also a SOCS3 contact site; SOCS3 may compete for the SHP2-gp130 interaction site [53,54]. SOCS3-/- mice die during mid-gestation, (between day 11.5 and day 16.5) though the basis of this embryonic lethality is unclear. Some mice display marked erythrocytosis, consistent with the finding that a SOCS3 transgene results in severe anemia [44]. However, reconstitution of irradiated mice with SOCS3-/- fetal liver results in normal hematopoiesis, suggesting that the critical role of SOCS3 may be limited to embryogenesis. Clearly, many questions remain regarding the physiological role of SOCS3.

PIAS: negative regulators of STATs

In mammals, the PIAS (protein inhibitors of activated STATs) proteins were first discovered as transcriptional coregulators of the JAK/STAT pathway [55]. Four members have been identified: PIAS-1, -3, -X and -Y. They share homology amongst themselves but have no previously characterized motifs [56]. PIAS proteins may not be specific for STAT interaction, as PIAS haven been shown to interact with a number of different pathways [57]; the *in vivo* relevance of this family of proteins will need to be assessed by the production of the relevant knockout mice.

Phosphatases in attenuating cytokine mediated signals

SHP-1

Given the importance of tyrosine kinases in initiating signaling, it comes as no surprise that tyrosine phosphatases are also important inhibitors. For

instance, SHP-1 is an important negative regulator, as illustrated by the `motheaten mice' phenotype. The lack of SHP-1 affects almost all hematopoietic lineages and results in the characteristic motheaten appearance of the coat and fatal pneumonitis resulting from unchecked neutrophil and macrophage proliferation [58]. Several hematopoietic receptors, including the IL-4R, EpoR, GHR and IL-2R have been shown to recruit SHP-1. The mechanism by which SHP-1 inhibits signaling has not been entirely elucidated but it presumably occurs via SH2-phosphotyrosine interaction. SHP-1 may directly dephosphorylate and inactivate JAKs or dephosphorylate other key tyrosines on the cytokine receptors that are involved in signaling. Loss of SHP-1 expression has been implicated in human lymphoproliferative disease and may be involved in malignant transformation, as reported with HTLV-1 associated leukemia and other tumors.

SHP-2

The action of SHP-2 is more complicated. Though it has been reported to inhibit signaling by some cytokines, it also acts as an adaptor protein, enhancing PI-3'K and Ras activation. SHP-2 binds the receptor and recruits Grb-2 and SOS, thus initiating Ras activation. SHP-2 does not bind all receptors directly; it also binds docking proteins such as Gab-2 [59,60].

SHIP

Two phospholipid phosphatases, SHIP (SH2-containing inositol phosphatase) and PTEN, target PIP-3 but at different positions on the inositol ring [61]. Normally PTEN antagonizes the activation of PI3'-K and PKB/Akt by growth factors. Many tumors and cell lines have PTEN mutations, resulting in activation of the Akt/PKB and phosphatidylinositol PI3'-K pathway. This can antagonize the apoptosis induced by cytokine withdrawal.

CD45 and putative STAT phosphatases

Recently, it has been shown that the absence of CD45 leads to augmented JAK and STAT phosphorylation in hematopoietic cells. Whether the major role of CD45 is to statically influence JAKs or whether there is a dynamic

component of regulation is not known. Whether other receptor phosphatases affect JAKs in nonhematopoietic cells will also need to be determined [62]. Finally, STATs are dephosphorylated over time and this presumably reflects the activity of a STAT phosphatase. The identity of the major STAT phosphatase and its intracellular location remains enigmatic.

Dominant-negative receptor isoforms

Most members of the family of hematopoietic growth factor receptors have been shown to be capable of giving rise to soluble forms [63]. Because most of these soluble receptors, when expressed as recombinant proteins, act to neutralize their cognate ligands, they are considered to function as receptor antagonists [64-66]. A different class of dominant-negative isoforms has been described for the erythropoietin receptor (EpoR) and the common β-receptor subunit of the granulocyte-macrophage colony-stimulating factor receptor (β-GMR). EpoR-T is an intracytoplasmic truncated splice variant and believed to function by forming a non-functional heterodimer with EpoR [67]. Recently, Wagner et al. have
characterized the intracytoplasmic truncated variant of β-GMR (β-GMR_{IT}) as a dominant-negative isoform [68]. They demonstrated that β-GMR_{IT} is trafficking to the plasma membrane and that surface expression of the α/β-GMR complex remains unchanged in the presence of β-GMR_{IT}. In analogy to EpoR-T, they proposed that the inhibitory effect of β-GMR_{IT} was due to the formation of non-functional complexes between β-GMR and the truncated β-GMR form. The vast number of cytokine receptor isoforms suggests that the play an important role in modulating cytokine receptor signaling.

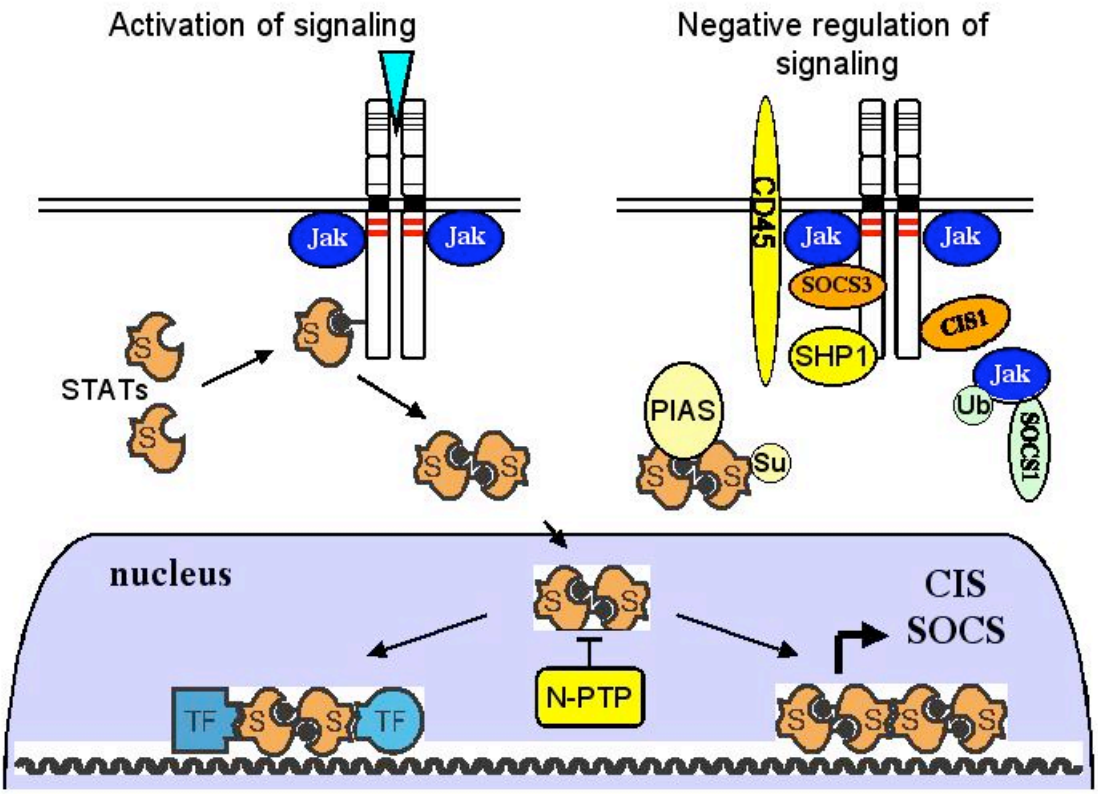

Fig. 2. Overview of Cytokine Signaling: Positive and Negative Regulation. Cytokines bind to homodimeric or heterodimeric receptors, which bind Janus kinases (JAKs). JAKs are activated by transphorylation and they in turn phosphorylate cytokine receptors, allowing STATs to bind via SH2-phosphotyrosine interactions. STATs themselves are phosphorylated, permitting STAT dimerization and translocation to the nucleus where STATs bind DNA and regulate gene expression. This process is regulated at multiple steps, some of which are summarized here: tyrosine phosphatases such as SHP-1, CD45, and PTP1b may regulate phosphorylation of receptors and JAKs. Dimerized STATs can be bound by PIAS members, which have been found to be SUMO E3 ligases; the depicted sumoylation of STATs, however, is a speculative, albeit reasonable, possibility. Additionally, cytokine stimulation induces the transcription of a family of SH2 containing proteins known as SOCS proteins. SOCS proteins inhibit signaling by multiple means: (1) binding and inhibiting JAKs, (2) binding cytokine receptors and blocking STATs recruitment, and (3) promoting ubiquitination and degradation of the JAK/receptor complex. STATs are dephosphorylated in the nucleus, but the identity of the predominant nuclear STAT phosphatase (N-PTP) remains to be determined.

Thrombopoietin and Thrombopoietin receptor c-mpl

Thrombopoietin (TPO) and its receptor c-mpl support all of the developmental stages necessary for megakaryocytopoiesis, i.e. the generation of

thrombocytes or platelets [69]. The first evidence for the existence of a humoral thrombopoietic growth factor came from studies of the murine myeloproliferative leukemia virus (MPLV) [70]. The viral oncogene, v-*mpl*, was recognized to encode a truncated but novel member of the cytokine receptor superfamily [71]. Cellular homologs were cloned from human and mouse tissues [72-74]. The mpl ligand, TPO, was cloned soon thereafter [75]. The N-terminal portion of TPO has marked homology to erythropoietin and is predicted to display the structure typical for helical cytokines [76]. The gene encoding mpl is located on human chromosome 1p34 and is composed of 12 exons [77] (Fig. 3). The corresponding murine gene is on chromosome 4 and has a similar organization [78]. Four distinct mRNA species exist in humans. The predominant form encodes the full-length receptor. The Mpl-K variant is due to a read through beyond the exon 10 splice donor site and encodes a protein that diverges form the native Mpl sequence after the ninth cytoplasmic amino acid and terminates within intron 10 with 66 predicted cytoplasmic residues [73]. Mpl-del, a third isoform, arises as a consequence of alternative splicing between exons 8 and 9 and encodes a protein with an in-frame deletion of 24 amino acids [79]. The fourth mRNA species is the only one found both in human and murine cells. It results from splicing of exon 8 directly to exon 11, eliminating the juxtamembrane WSXWS motif and the transmembrane domain. The resulting isoform mpl-tr lacks the cytoplasmic domain and terminates instead in a short stretch of residues of novel sequence due to an altered reading frame at the splice acceptor site of exon 11 [72,74]. Additionally, an alternative splice acceptor site in exon 4 of murine mpl (but not human) generates an isoform with an in-frame deletion of 60 residues in the extracytoplasmic domain [80] (Fig. 3).

Both TPO [81] and mpl [82,83] knockout mice are viable but have a 90% reduction in platelet counts. A reduction in progenitor cell numbers and a decrease in megakaryocyte ploidy cause the thrombocythemia in these mice. However, the megakaryocytes and platelets produced in the absence of TPO or mpl appear to be morphologically and functionally normal, indicating that *in vivo* the main role of TPO is to control their numbers, rather than their maturation. Analysis of these gene-targeted mice provides substantial evidence to a model where the circulating TPO level is directly regulated by platelet mass

through binding to mpl receptors at the platelet surface. This elegant feedback mechanism allows a tight regulation of the amount of TPO available to stimulate megakaryopoiesis [84]. In addition to its effects on megakaryopoiesis, TPO also affects hematopoietic stem cells [85] as measured by the reduction of repopulating capacity of bone marrow cells from mpl-deficient mice [86].

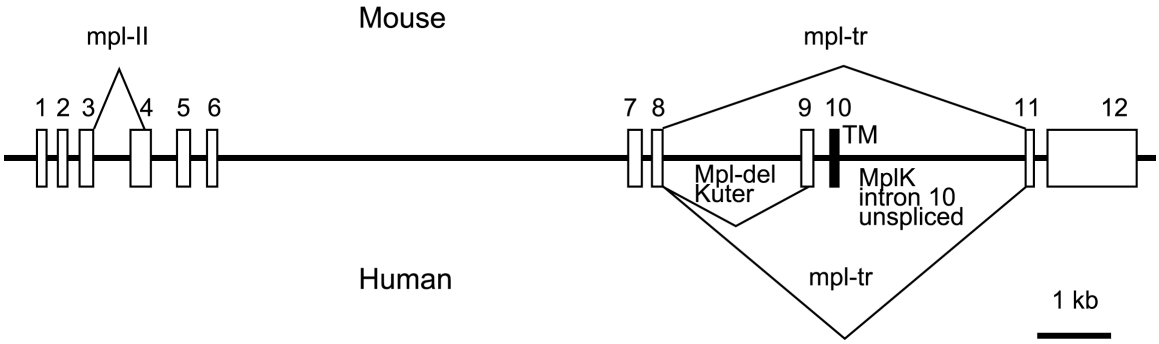

Fig. 3 Alternative mpl splice variants. The gene encoding c-*mpl* is composed of 12 exons [77]. In humans four distinct mRNA species are known. The predominant form encodes the full-length protein. The human Mpl K-form is due to a readthrough beyond the exon 10 splice donor site [73]. Mpl-del, a third isoform, arises as a consequence of alternative splicing between exons 8 and 9 [79]. The fourth mRNA species is the only one found both in human and murine cells. It results from splicing of exon 8 directly to exon 11, eliminating the transmembrane domain (TM), which is encoded by exon 10 [72,74]. Additionally, an alternative splice acceptor site in exon 4 of murine *mpl* (but not human) generates an isoform (mpl-II) [80].

Thrombopoietin signaling

Like other class I cytokine receptors, the TPO receptor mpl makes a single pass through the cell membrane. The extracellular domain consists of two CRH domains, which are most similar to the CRH of EpoR. The cytoplasmic portion of mpl is highly conserved across species and lacks any intrinsic enzymatic function. Instead, JAK family members associate with mpl through interaction with box 1 and box 2. The family member JAK2 is the predominantly phosphorylated JAK after TPO activation of purified murine megakaryoctes [87]. TPO signaling leads to the activation of STAT3 and 5 . Recently, it has been demonstrated that members of the Src family of tyrosine kinases are also activated by mpl signaling [88].

Many if not most of the secondary signaling pathways activated by TPO are also activated in response to binding of other hematopoietic growth factors to their cognate receptors, making mpl signaling a good representative of cytokine signaling in general [89].

Specificity of cytokine signaling

In principle, each cytokine receptor could activate a unique signaling pathway, but this does not appear to happen. One theme that has emerged from studies of cytokine receptors and receptor tyrosine kinases is that different receptors stimulate similar collections of intracellular signaling pathways. For instance, 34 known or predicted type I cytokine receptors exist [90], but only four *Janus* kinases [25]. Given this similarity of response, an important question has arisen about the role of cytokine receptor signaling in cell differentiation. There are two basic models for how unique responses might be generated by different cytokine receptors with apparently similar intracellular signaling events. The first model postulates that there are intrinsic differences in the intracellular signaling pathways activated by cytokine receptors. These differences could either be quantitative (strength or duration of the signal) or qualitative (a different combination of intracellular pathways being activated). In this model, the outcome of a specific cytokine signaling event is unique. The instructive model of differentiation proposes that such unique signals bring about the induction of lineage specific genes resulting in a distinct developmental outcome. The second model postulates that cytokine receptors generally signal in similar ways, but cells interpret these signals based on their distinct developmental histories. In this model, the primary reason for the existence of so many cytokines and their cognate receptors is to allow the temporally and spatially appropriate activation of general signaling pathways. It suggests that cytokine receptors employ similar generic signals that permit the expression of predetermined, tissue-specific differentiation programs. Examples supporting both the instructive [91,92] and the permissive [93-95] model of cytokine function exist. In several of the experiments addressing the validity of the instructive versus the permissive model, intracellular domains of

cytokine receptors were swapped and the function of chimeric receptors was tested. It can be argued that the outcome of the experiments was dependent on the arbitrary choice of the novel intracellular domain in the chimeric receptor. Therefore, expression profiling of different cytokine receptors remains an important task in studying cytokine receptor biology. Furthermore, our understanding of the regulatory mechanisms of cytokine receptor signaling is far from complete. The importance of studying the regulation of cytokine signaling is emphasized by the existence of a number of pathological conditions with mutations in cytokine receptors or associated signaling molecules [96].

RESULTS I:

ALTERNATE C-MPL ISOFORMS IN SIGNAL ATTENUATION

A truncated isoform of c-mpl with an essential C-terminal peptide targets the full-length receptor for degradation

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Running title: An isoform of c-mpl targets the full-length receptor for degradation

Key words: Mpl, thrombopoietin, splicing, receptor, degradation,

Abstract

Thrombopoietin and its cognate receptor c-mpl are the primary regulators of megakaryopoiesis and platelet production. They also play an important role in the maintenance of hematopoietic stem cells. Here, we have analyzed the function of a truncated mpl receptor isoform (mpl-tr), which results from alternative splicing. The mpl-tr variant is the only alternate mpl isoform conserved between mouse and humans, suggesting a relevant function in regulating mpl signaling. Despite the presence of a signal peptide and the lack of a transmembrane domain, mpl-tr is retained intracellularly. Our results provide evidence that mpl-tr exerts a dominant-negative effect on thrombopoietin-dependent cell proliferation and survival. We demonstrate that this inhibitory effect is due to downregulation of the full-length mpl protein. The C terminus of mpl-tr, consisting of 30 amino acids of unique sequence, is essential for the suppression of TPO-dependent proliferation and mpl protein downregulation. Cathepsin inhibitor-1 (CATI-1), an inhibitor of cathepsin-like cysteine proteases, counteracts the effect of mpl-tr on mpl protein expression, suggesting that mpl-tr targets mpl for lysosomal degradation. Together, these data suggest a new paradigm for the regulation of cytokine receptor expression and function through a proteolytic process directed by a truncated isoform of the same receptor.

Introduction

Cytokine receptor signaling has profound effects on cell survival, proliferation and differentiation of the receiving cell [97]. It is therefore not surprising that components of the signaling cascade are tightly regulated at several levels. An important mechanism for controlling gene expression is alternative splicing allowing the synthesis of structurally and functionally distinct protein isoforms [98]. Many alternative splice variants of different cytokine receptors have been described, but the function of most of the resulting protein isoforms remains unknown.

Cytokine receptor isoforms may be classified according to the presence or absence of a transmembrane domain. Isoforms lacking a transmembrane domain are often termed ‘soluble cytokine receptors’ and can fulfill different physiological functions [63,99]. In general, soluble receptors may function as agonists by stabilizing their ligands, e.g. growth hormone and tumor necrosis factor [100,101], or contrarily act as antagonists by competing with the membrane bound receptor for ligand binding, e.g. epidermal growth factor and interleukin (IL)-1 [102,103]. Soluble receptors can arise from alternative splicing or from proteolytic receptor shedding on the cell surface. Isoforms generated by alternative splicing often contain additional protein sequence due to unspliced intron sequence and/ or a shift of the reading frame. Generally, no biological function has been attributed to these additional stretches of amino acids.

Thrombopoietin (TPO) and its receptor “cellular homolog of myeloproliferative leukemia” (c-mpl) are the primary regulators of megakaryopoiesis [69]. The c-mpl gene is composed of 12 exons (Fig. 1A) [77]. In the mouse, two distinct alternate mRNA isoforms are known. The transmembrane variant mpl-II is due to usage of a cryptic splice acceptor in exon 4 resulting in a in-frame deletion of 60 amino acids [80]. No function has yet been assigned to this isoform. The second mRNA variant encodes a truncated soluble receptor, mpl-tr, and is the only one found both in human and mouse. This variant results from splicing of exon 8 directly to exon 11, eliminating the juxtamembrane extracellular part and the transmembrane domain [72,74] . Due to an altered reading frame at the splice acceptor site of exon 11, mpl-tr protein terminates in a short stretch of novel amino acid sequence (Fig. 1).

Mpl-tr mRNA accounts for approximately 30% of mpl mRNA in mouse spleen [74]. In spite of the presence of a signal sequence and the lack of a transmembrane domain, mpl-tr is not secreted into the cell supernatant when ectopically expressed in cell line [74]. In human, two alternate mRNA mpl species are known in addition to mpl-tr. The mpl-K variant is due to a readthrough beyond the exon 10 splice donor site [73]. The resulting K-form of the receptor diverges from the native sequence after the ninth cytoplasmic amino acid and terminates within intron 10. Mpl-del, a second isoform, arises as a consequence of alternative splicing between exons 8 and 9 and encodes a protein with an in-frame deletion of 24 amino acids and unknown function [79]. Because of the lack of secretion of mpl-tr, we analyzed whether mpl-tr plays a physiological role intracellularly. Here we demonstrate that mpl-tr specifically inhibits TPO-dependent proliferation and survival. We show that mpl-tr is responsible for initiating protein downregulation of the full-length mpl receptor by a cathepsin-like cysteine protease activity. As a consequence, the amount of total mpl protein in the cell is drastically reduced. Further, our data show that for this effect a short peptide sequence at the C terminus of mpl-tr is essential. The ability of mpl-tr to antagonize mpl function represents a novel mechanism by which cytokine signaling is regulated.

Materials and Methods

DNA constructs

The plasmid pCD4 (pMICD4) is a gift from Dr. Harvey A. Lodish. It contains an intraribosomal entry site (IRES) followed by a truncated cDNA of the human CD4 gene and is derived from the retroviral expression vector pMX [104]. To generate pCD4-mpl-tr, mpl-tr was cloned into the restriction sites *Xho*I and blunted *Bam*H1 of the multiple cloning site of pCD4. For the generation of the mpl-tr mutants, site-directed mutagenesis was performed using the QuikChange XL mutagenesis kit (Stratagene, Cedar Creek, TX) according to the manufacturer's protocol. The following primers were used: GAAGGCCGTGAGGACTGGAAGTAGACTGAGGCAAGCTTTGTGG (sense), CCACAAAGCTTGCCTCAGTCTACTTCCAGTCCTCACGGCCTTC (anti-sense) for the stop codon in Δpep_{30}; GAAGGCCGTGAGGACTGGAAGAGACTGAGGCAAGCTTTGTGG (sense), CCACAAAGCTTGCCTCAGTCTCTTCCAGTCCTCACGGCCTTC (anti-sense) for the frame shift in tr-pep_{mpl}; GCCCTAAGTCCTTCTTAAGGCCACGGTTACCGATAGCTGTG (sense), CACAGCTATCGGTAACCGTGGCCTTAAGAAGGACTTAGGGC (antisense) for the stop codon in tr-pep_{mpl}. Mpl, mpl-tr and pep_{30} cDNAs were cloned into the 5myc-pcDNA1 vector (gift from Dr. Eva Reinhard, Biozentrum, University of Basel), which contains at its 5' end a sequence encoding a HA-signal sequence followed by 5 myc epitopes [105]. For stable transfections, myc-tagged mpl cDNA was cloned into the pGD expression vector [106] as a *Xho*I-*Not*I fragment. For transient transfections into human kidney 293T cells, mouse mpl, myc-mpl and mpl-tr cDNAs were subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) as *Xho*I-*Not*I fragments.

Cell transfection and culture

BaF3 cells were cultured as described [84]. UT-7 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2ng/ ml recombinant human GM-CSF (PromoCell, Heidelberg, Germany). For transfections of BaF3 and UT7 cells, 0.5 – 1 $\times 10^7$ cells were electroporated at 270 V and 975 μF at ambient temperature in the presence of 20 μg plasmid. UT-7/myc-mpl cells were cultured in the presence of 450 μg/ml G418. A pool of stably transfected UT-7/myc-mpl cells was used for the transfection with different pCD4 constructs. The BaF3/mpl cell clone used in this study (TM17) has been described [84]. The BaF3/mpl clone TM17 was used for transfections with different pCD4 constructs. Cells expressing human CD4 were selected by the usage of CD4 microbeads according to the manufacture's protocol (Miltenyi, Auburn, CA). For transient transfections of 293T cells, the transfection reagent FuGENE was used according to the manufacturer's protocol (Roche, Switzerland).

Proliferation assay

An XTT proliferation kit (Roche, Switzerland) was used according to the manufacture's protocol to determine cytokine-dependent cell proliferation. In brief, cells were plated in 96-well plates at 10^4 cells per well in 100 µL of medium containing the indicated concentrations of cytokine. After 3.5 days of stimulation for BaF3 cells and 5 days of stimulation of UT7 cells, 50 µL of a 1mg/mL stock solution of XTT with 5 mmol/L phenazine methosulfate, an electron coupling agent, was added to each well. The product of XTT reduction by viable cells, reflecting the number of cells per well, was measured at 4 hours at 450 nm.

Protein expression analyses

Surface expression of myc-mpl was analyzed by fluorescence-activated cell sorting (FACS) analysis. For this analysis, 5×10^5 cells were incubated with the mouse monoclonal antibody 9e10 directed against the N-terminal myc-tag of myc-mpl for 60 minutes on ice followed by incubation with the fluorescein isothiocyanate (FITC)-labeled goat antimouse antibody (BD Biosciences, San Diego, CA). Untransfected UT7 cells served as a control. Total amounts of c-mpl and mpl-tr were determined by immunoblot analysis using a rabbit polyclonal antibody directed against mpl, as described [74]. The same polyclonal antibody and 9e10 were used for the detection of myc-mpl protein by immunoblot analysis. To normalize for protein loading, the membranes were reprobed using a mouse monoclonal antibody AC-40 directed against actin (Sigma, St. Louis, MO).

Puls-chase analysis

293T cells cultured in 60-mm dishes were transiently transfected with 3 µg myc-mpl and 3 µg mpl-tr expressed off the pcDNA3 expression vector. Pulse–chase analysis was begun 40 h post-transfection. To perform the pulse–chase, cell monolayers were washed twice with warm phosphate-buffered saline (PBS), and starved of methionine and cysteine by incubation for 40 min at 37°C in 1 ml of methionine/cysteine-free DMEM (Gibco-BRL), supplemented with 5% dialyzed FCS (Gibco-BRL). Following amino acid starvation, cellular proteins were pulse-labeled by incubating each plate of cells with 400 µCi of methionine/ cysteine (Tran35S-label; NEN) for 30 min at 37°C. The radioactive medium was then removed, the cells washed twice in warm PBS, re-fed with DMEM supplemented with 2 mM methionine and 2 mM cysteine, and incubated for the indicated times. Cells were collected, and labeled proteins recovered by denaturing immunprecipitation using the 9E10 antibody and the method of Hofmann *et al*. [107]. Immune complexes were analyzed on 10% SDS–PAGE gels. The dried gels were quantitated on a Molecular Imager FX (BIO-RAD, Hercules, CA) using Quantity One software (BIO-RAD, Hercules, CA).

Apoptosis

For quantification of apoptosis, UT-7/myc-mpl cells were grown at 2×10^5 cells/mL in the presence of either 2 ng/mL of human GM-CSF or 2 ng/mL or 20 ng/mL of human TPO (gift

from Dr. Frederic J. de Sauvage). After 48 hours, cells were washed twice and stained with annexin V with the use of the annexin V-FITC kit (Roche, Switzerland). FITC positive cells were quantitated by flow cytometry.

Generation of cDNA and Quantitative-Polymerase chain reaction (Q-PCR)

RNA was extracted from cell lines by means of Trizol reagent (Life technologies, Rockville, MD). RNA was treated with RNase-free Dnase (Promega, Madison, WI) for 90 minutes at 37°C, heat-inactivated and then purified with RNAeasy (Qiagen, Germany) according to manufactures protocol. For reverse-transcriptase-polymerase chain reaction (RT-PCR), 1□g of RNA was reverse-transcribed after random hexamer priming in a 30-□L reaction mix containing 100 U of omniscript RT (Qiagen, Germany). The reaction was performed for 90 minutes at 37°C followed by 30 minutes at 40°C and denaturing at 95 °C for 10 minutes. The cDNA was diluted 1:10 and used for Q-PCR. The differential quantification of mpl and mpl-tr was performed on an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA), as described [1]. The sequences for the isoform specific primers and for MGB probes were as follows: for the exon 10/11 boundary of murine c-mpl: GCAATTTCCTGCGCACTACA, GGAAGCGAGGGCCACAA, GAGACTGAGGCATGC (probe); for mpl-tr: AGCGAGGGCCACAAAGC, CAGCTCAAGAGACCTGCTACCA, CCTCAGTCTCCTTCCAGT (probe). The □C_T values were derived by subtracting the threshold cycle (C_T) values for mpl and mpl-tr from the C_T value for mouse ribosomal protein L19 (RPL19), which serves as an internal control [1,108]. All reactions were run in duplicates.

Results

Co-expression of mpl-tr with c-mpl inhibits TPO-dependent mitogenic and survival signaling. To study the effect of mpl-tr on TPO-dependent proliferation, we used two cytokine-dependent cell lines for cell growth assays: the murine cell line BaF3 and the human megakaryoblastic cell line UT-7. Both cell lines do not express mpl protein endogenously. To assay potential effects of mpl-tr on the function of the full-length mpl protein, we used BaF3 cells stably transfected with murine mpl (BaF3/mpl) [84] (Fig.1). These cells were subjected to a second round of transfection with an expression vector containing the cDNA for murine mpl-tr. The presence of an IRES followed by a truncated form of human CD4 in the vector allowed selection of mpl-tr expressing cells using magnetic anti-CD4 microbeads.

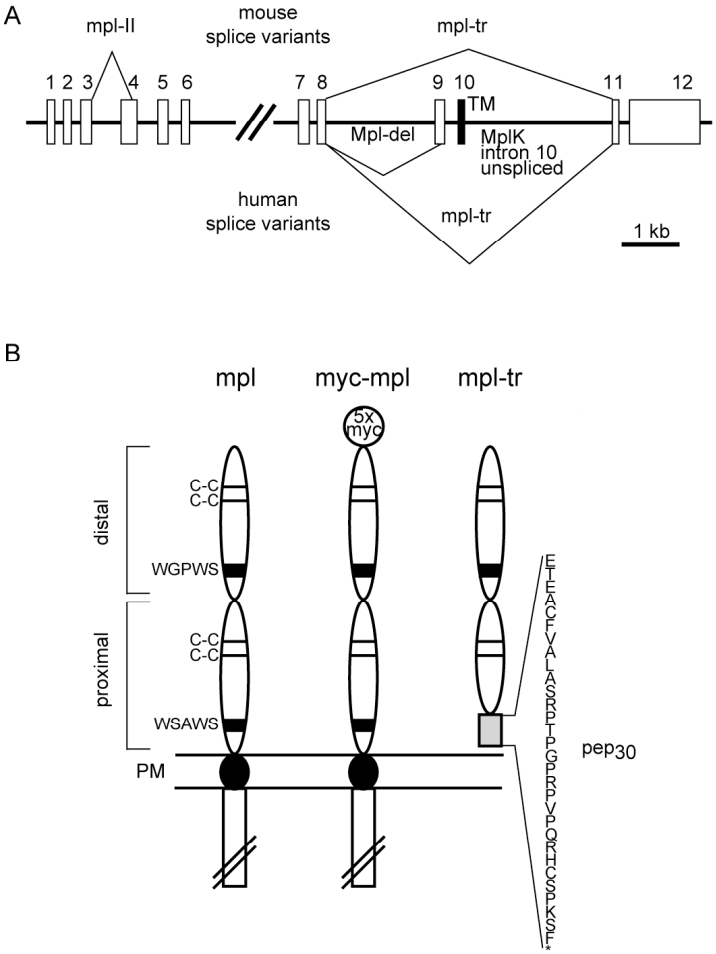


Fig. 1. Schematic representation of the c-mpl gene and of mpl and mpl-tr protein. (A) The known alternate splice variants of human and mouse c-mpl are depicted in a schematic drawing of the exon/ intron structure of c-mpl. **(B)** The extracellular domain of mpl protein consists of two cytokine receptor modules, one proximal and one distal to the plasma membrane (PM). A black oval represents the transmembrane domain and white boxes the cytoplasmic portion. Horizontal lines indicate the position of the conserved cysteines. The black bars indicate the positions of the conserved WGXWS and WSXWS motifs. The myc-mpl variant has five consecutive myc epitopes added in frame to the N-terminus of the mature mpl protein. The mpl isoform mpl-tr lacks the juxtamembrane extracellular portion and the transmembrane domain. Due to an altered reading frame, the C-terminus comprises a stretch of 30 residues of novel sequence termed pep_{30}.

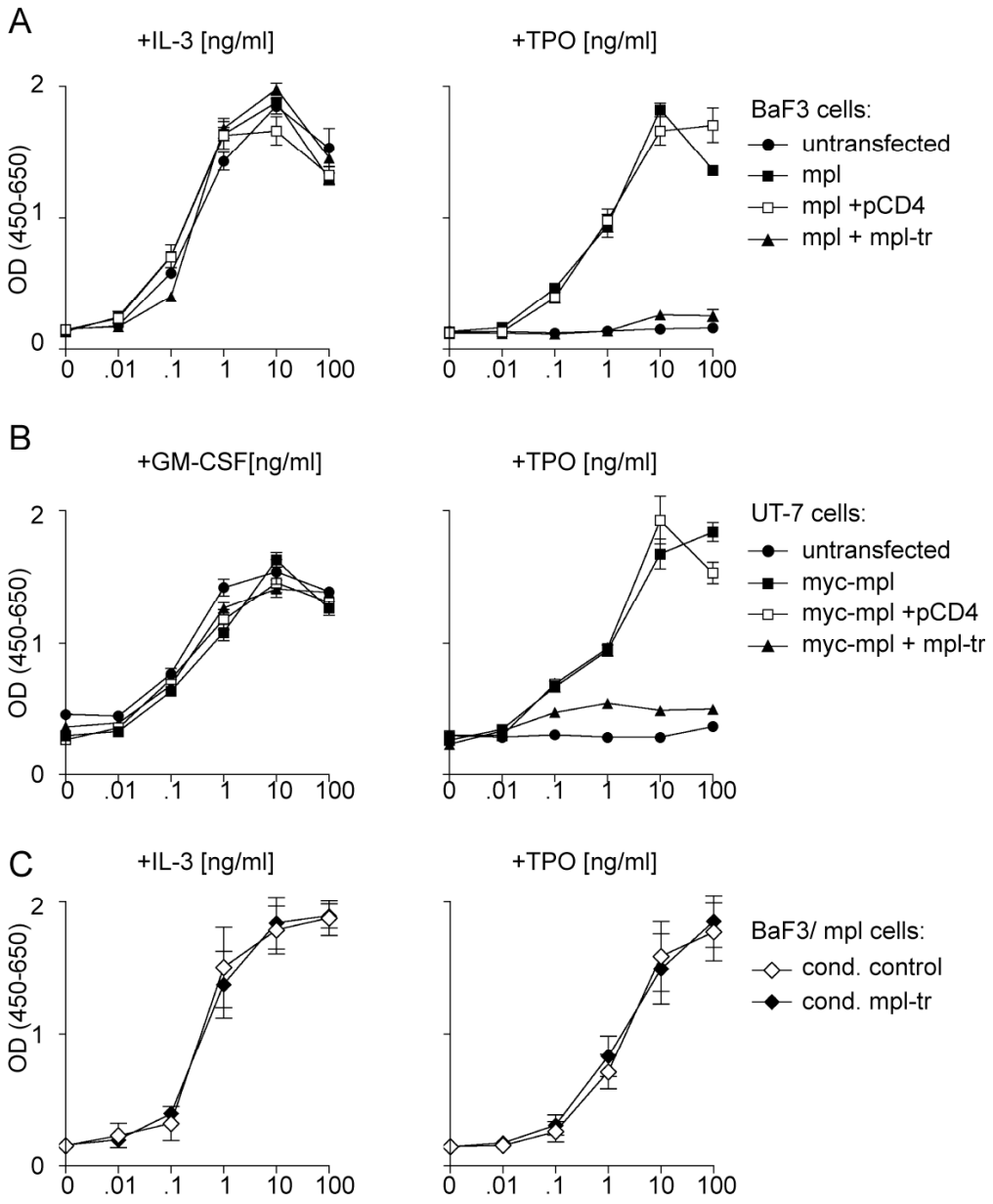


Fig. 2. Effect of mpl-tr on proliferation of cytokine-dependent cell lines. We used two cytokine-dependent cell lines for cell growth assays: the murine IL-3 dependent cell line BaF3 and the human GM-CSF-dependent cell line UT-7. Both cell lines do not express mpl protein endogenously. Upon expression of mpl BaF3 and UT-7 cells proliferate in response to TPO. The isoform mpl-tr was expressed off a retroviral vector containing an IRES followed by a truncated form of human CD4, used as a quantitative selectable marker. **(A)** BaF3 cells stably transfected with mpl (BaF3/mpl) were additionally transfected with pCD4 or pCD4-mpl-tr and sorted with anti-CD4 microbeads. Proliferation of transfected and parental BaF3 cells was determined by a XTT assay with increasing concentrations (ng/ml) of IL-3 or TPO, as indicated. Error bars indicate the standard deviation on triplicate samples. **(B)** UT-7 cells stably transfected with myc-mpl (UT-7/myc-mpl) were additionally transfected with pCD4 or pCD4-mpl-tr and sorted with anti-CD4 microbeads. Proliferation of transfected and parental UT-7 cells in the presence of GM-CSF and TPO is shown. Annotation as above. **(C)** BaF3/mpl cells were were cultured with increasing concentrations (ng/ml) of IL-3 or TPO in the presence of mpl-tr conditioned media (filled diamonds) or control media (open diamonds).

This procedure was repeated 3-4 times to enrich for CD4 positive cells and expression of CD4 was confirmed by flow cytometry (data not shown). The sorted cells were cultured with different concentrations of either IL-3 or TPO. As expected, parental BaF3 cells failed to respond to TPO (Fig. 2A). On the other hand, BaF3/mpl cells and BaF3/mpl cells transfected with the parental pCD4 plasmid proliferated in dependence on TPO concentration. In contrast, BaF3/mpl cells expressing mpl-tr failed to show a proliferative response to TPO, behaving similar to parental BaF3 cells. Importantly, mpl-tr did not have a general inhibitory effect on proliferation, because in the presence of IL-3, BaF3/mpl cells expressing mpl-tr grew as efficiently as control cells. The same result was obtained when mpl-tr was expressed in human UT-7 cells that were stably transfected with mpl. In this experiment we used a mpl construct with 5 myc-tags at the N terminus (UT-7/myc-mpl) (Fig. 1). The myc-mpl protein conveyed TPO-responsiveness to UT-7 cells, demonstrating that the myc-tag did not interfere with mpl function (Fig. 2B). When mpl-tr was expressed in UT-7/myc-mpl cells, TPO-mediated proliferation was abolished, but GM-CSF-dependent proliferation remained unchanged. To test whether the observed inhibitory effect could be mediated by secreted mpl-tr protein, medium conditioned by mpl-tr-expressing cells was transferred onto BaF3/mpl cells. TPO-dependent proliferation of BaF3/mpl cells was not inhibited by the presence of this conditioned media (Fig. 2C) demonstrating that no secreted inhibitory activity exists. Since mpl signaling exerts an anti-apoptotic effect, we analyzed the effect of mpl-tr on TPO-dependent cell survival. UT-7/myc-mpl cells transfected with either mpl-tr or control vector were cultured with GM-CSF or with TPO. After 48 hours, cells were incubated with annexin V and analyzed by flow cytometry. In the presence of GM-CSF, mpl-tr had no effect on the number of annexin V-positive cells. However, with TPO, most cells co-expressing mpl and mpl-tr stained positive for annexin V (Fig. 3). This indicates that mpl-tr expression inhibits the anti-apoptotic signal delivered by TPO.

Mpl-tr mediates downmodulation of mpl protein expression in a post-transcriptional manner. To investigate the inhibitory mechanism exerted by

mpl-tr, we analyzed the effects of mpl-tr on mpl protein expression. First, we asked whether mpl protein surface expression was affected by the presence of mpl-tr. Cells were stained with anti-myc antibodies (Fig. 4A). As expected,

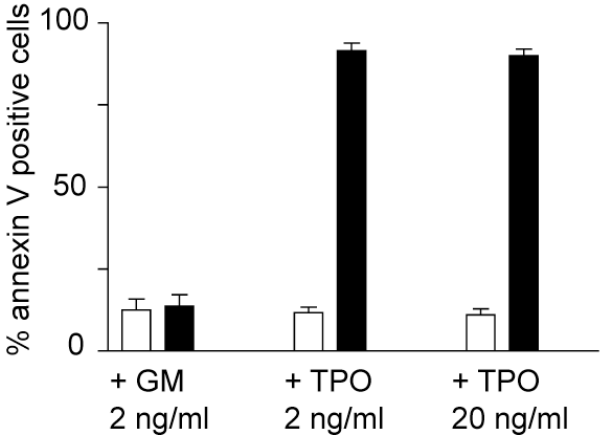
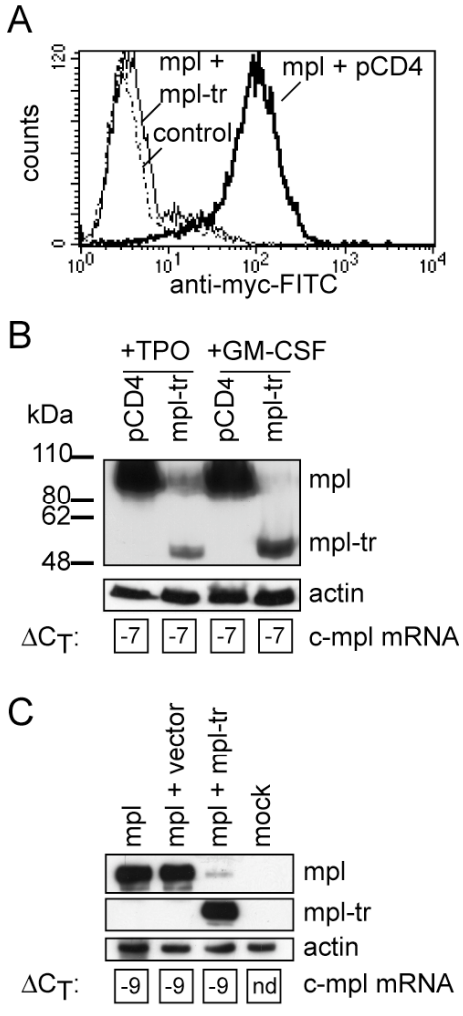


Fig. 3. Effect of mpl-tr on cell survival. UT-7/myc-mpl cells were transfected with pCD4 (open bars) or pCD4-mpl-tr (black bars) and sorted with anti-CD4 microbeads. Cells were cultured in the presence of GM-CSF or TPO at the indicated concentrations for 48 hours, stained with FITC-labeled annexin V and analyzed by flow cytometry. The results of 3 independent experiments are shown. Error bars indicate the standard deviation.

Fig. 4. Analysis of mpl protein expression in UT-7 cell line. (A) UT-7/myc-mpl cells stably transfected with pCD4 (thick line) or pCD4-mpl-tr (thin line) or the parental UT-7 cells (dotted line) were incubated with the mouse monoclonal anti-myc antibody 9E10 and a secondary FITC-labeled goat anti-mouse antibody to detect myc-mpl on the plasma membrane. Cells were analyzed by flow cytometry and one representative result is shown. **(B)** The same UT-7/myc-mpl cells transfected with pCD4 or pCD4-mpl-tr as in (A) were cultured either on 2ng/ml GM-CSF or 2ng/ml TPO for 24 hours and total mpl protein expression in total cell lysate was determined by immunoblot analysis. Proteins of 93 kDa corresponding to myc-mpl and of 55 kDa for mpl-tr were detected. The same membrane was probed for actin to control for equal loading. To determine an effect of mpl-tr on mpl mRNA expression, the amount of myc-c-mpl mRNA was determined by Q-PCR. The ΔC_T values were derived by subtracting the threshold cycle (C_T) values for c-mpl from the C_T value for RPL19, which serves as an internal control. All reactions were run in duplicates. The ΔC_T values are shown underneath the corresponding lanes. **(C)** 293T cells were transiently transfected with myc-mpl or transiently co-transfected with myc-mpl and mpl-tr or control plasmid, respectively.



UT-7/myc-mpl cells showed marked myc-mpl surface expression. In contrast, the staining of UT-7/myc-mpl cells expressing mpl-tr did not significantly differ from the control staining of the parental UT-7 cells, indicating that mpl-tr interferes with myc-mpl cell surface expression. We then asked whether the lack of detectable myc-mpl surface expression correlated with a decrease in total myc-mpl protein. Immunoblot analysis of total cell lysates demonstrated a massive reduction of myc-mpl protein in cells that expressed mpl-tr (Fig. 4B). The decrease in myc-mpl protein was observed irrespective of whether the cells were grown with TPO or GM-CSF. Importantly, mpl-tr did not alter mpl mRNA levels, as indicated by the unchanged ΔC_T values for mpl (Fig. 4B). Expression of mpl-tr also led to a dramatic decrease in expression of the untagged mpl protein in BaF3/mpl cells without affecting mRNA levels showing that mpl-tr targets mpl protein regardless of the presence of an N-terminal myc-tag (Fig. 6C). To determine whether this phenomenon was limited to hematopoietic cells, we performed transient co-transfections of mpl and mpl-tr cDNAs into human 293T cells. As shown in Fig. 4C, expression of mpl-tr in 293T cells lowered the amount of mpl protein without altering mpl mRNA expression.

Mpl-tr mediates dose-dependent reduction of mpl protein expression and a decrease of mpl protein half-life while steady-state levels of mpl-tr remain unchanged. To quantify the effect of mpl-tr on mpl expression, we transiently co-transfected 293T cells with a constant amount of plasmid encoding mpl and varying amounts of plasmid for the expression of mpl-tr. In this experiment mpl protein amount was affected by mpl-tr in a dose-dependent manner (Fig.5 A). Measuring mRNA levels by Q-PCR confirmed that mRNA expression correlated with the amount of plasmid DNA transfected. For example, the mpl-tr (ΔC_T) value for cells transfected with 0.1 μg of mpl-tr was –4. Cells transfected with 30 times the amount of mpl-tr (3 μg) had a mpl-tr (ΔC_T) value of –9. This corresponds to a decrease of 5 CTs which equals 2^5 = 32 times higher expression of mpl-tr (Fig.5 A).

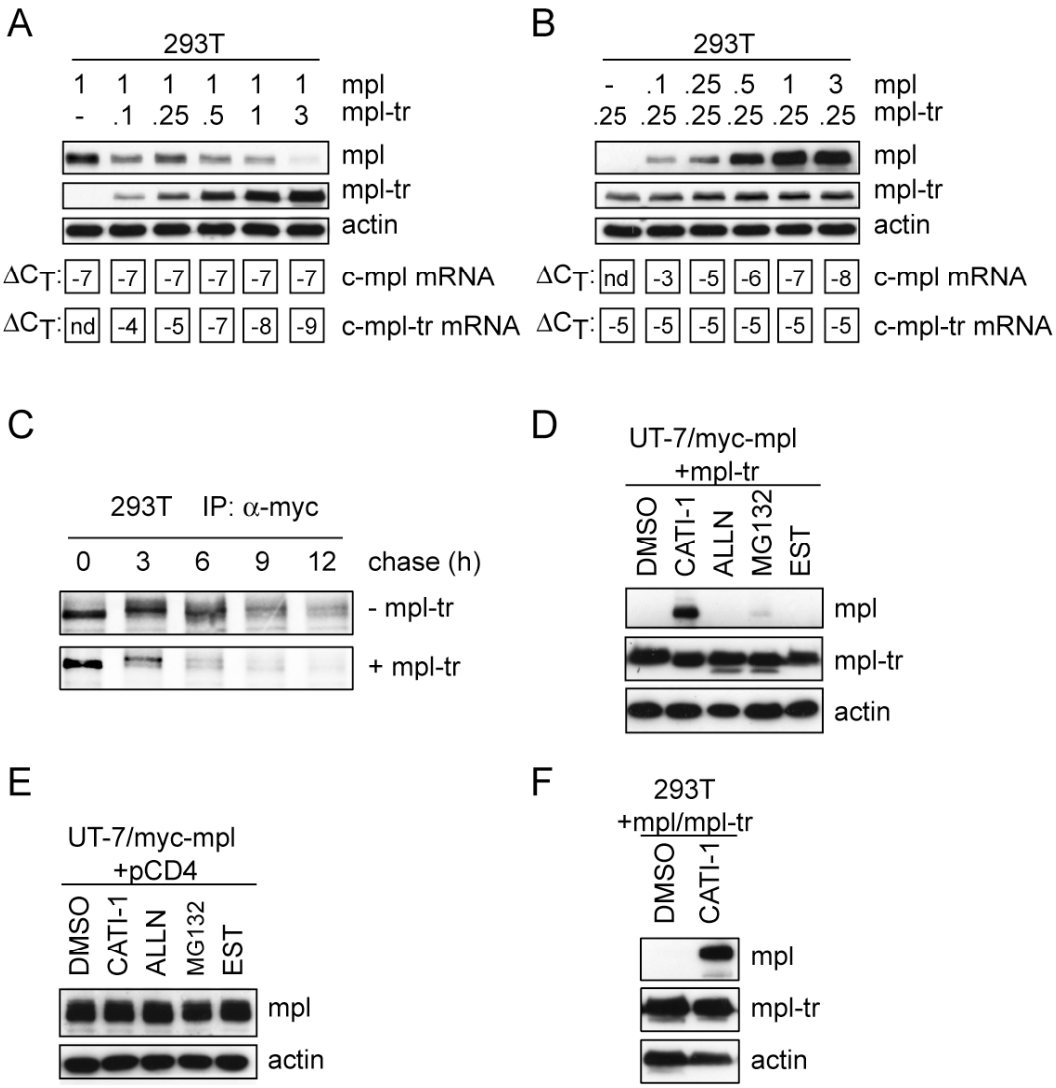


Fig. 5. Dose response, protein turnover, and sensitivity to protease inhibitors of mpl downregulation. (A) 293T cells were transiently co-transfected with varying amounts of plasmid DNA encoding mpl and mpl-tr (numbers above the lanes indicate DNA amount in mg). Mpl, mpl-tr and actin proteins were detected by immunoblot analysis. The mRNA expression of c-mpl and c-mpl-tr was determined by Q-PCR: ΔC_T values for mpl and mpl-tr are shown below the corresponding lanes of the immunoblots. The amount of mpl DNA was kept constant while the amount of mpl-tr DNA varied. **(B)** In this experiment, mpl DNA was varied and the amount of mpl-tr DNA was kept constant. **(C)** Pulse-chase analysis of the turnover of myc-mpl protein in the presence and absence of mpl-tr. 293T cells transiently transfected with pcDNA3-myc-mpl alone (-) or in combination with pcDNA3-mpl-tr (+ mpl-tr) were metabolically labeled with of (48) methionine/ cysteine and then chased in the presence of excess cold methionine and cysteine for 3, 6, 9, and 12 h. The amount of labeled myc-mpl was determined by immuno-precipitation with 9E10 and SDS-gel analysis. **(D)** UT-7/myc-mpl cells stably transfected with pCD4-mpl-tr were treated with protease inhibitors at a final concentration of 25 mM each or DMSO as indicated. After 6-8 hours of incubation, total cell lysates were subjected to immunoblot analysis. **(E)** UT-7/myc-mpl cells stably transfected with pCD4 were treated as described for the pCD4-mpl-tr transfected cells. **(F)** 293T cells were transiently co-transfected with mpl and mpl-tr and then treated with CATI-1 or DMSO for 6-8 hours.

In the converse experiment, we transfected 293T cells with a constant amount of mpl-tr and varied the concentration of mpl. We found that steady-state expression of mpl-tr was not altered by the presence of increasing amounts of mpl (Fig. 5B). Since mpl-tr affects the steady state levels of mpl protein, we determined the half-life of mpl by a pulse-chase experiment (Fig. 5C). In the presence of mpl-tr, the half-life of mpl was decreased from 5-6 hours to 2-3 hours (Fig. 5C).

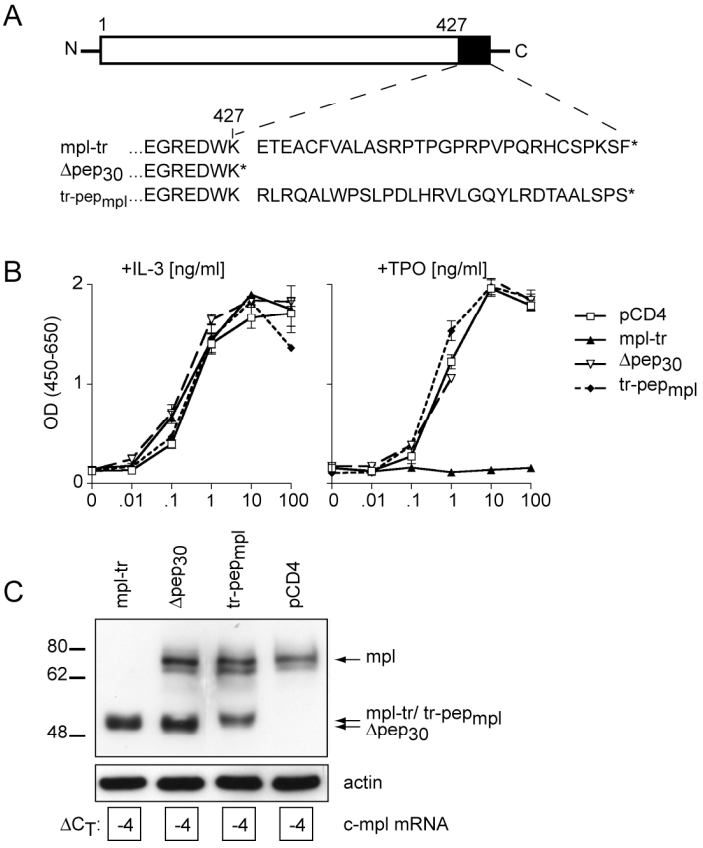


Fig. 6. Functional analysis of the C-terminal peptide of mpl-tr. (A) Schematic draw of mpl-tr protein and two mpl-tr mutants used in this study. The amino acid sequence of the junction and the C-terminal 30 amino acids are shown. In the tr-Δpep_{30} mutant, all C-terminal 30 amino acids of mpl-tr are removed. In the tr-pep_{mpl} mutant, the pep_{30} peptide is replaced by 30 amino acids derived from the mpl reading frame of exon 11. **(B)** Proliferation of BaF3/mpl cells stably transfected with pCD4 (open square), pCD4-mpl-tr (filled triangle), pCD4-Δpep_{30} (open triangle), and pCD4-tr-pep_{mpl} (filled diamond) in the presence of increasing concentrations of IL-3 or TPO (numbers indicate concentration in ng/ml). Error bars indicate the standard deviation. **(C)** Immunoblot analysis of total lysates from the same cells as in (B). Expression of mpl mRNA is indicated by the ΔC_T values below the corresponding lanes.

The cysteine cathepsin inhibitor I (CATI-1) restores mpl protein expression in the presence of mpl-tr. To identify the mechanism that underlies diminished mpl protein expression in the presence of mpl-tr, we treated mpl-tr transfected UT-7/myc-mpl cells with the cathepsin inhibitor CATI-1, the proteasome inhibitor MG132 or the calpain inhibitors ALLN or EST. Inhibitors were added at a final concentration of 25 μM each and cells were cultured for 6-8 hours in the presence of GM-CSF. Only CAPI-1 restored

mpl protein expression (Fig. 5D), a weak mpl band was detectable with MG132 treatment, whereas the other inhibitors had no effect. None of the inhibitors changed the steady-state mpl protein levels in the UT-7/myc-mpl cells lacking mpl-tr (Fig. 5E). To confirm these results in a different cell system, we also treated 293T cells with CATI-1 and determined mpl protein expression. Similar to UT-7/myc-mpl, 293T cells co-transfected with mpl and mpl-tr showed a rescue of mpl protein expression in the presence of CATI-1 (Fig. 5F). These results argue that mpl-tr mediates mpl protein degradation by a cathepsin-like protease activity.

The C-terminal peptide sequence of mpl-tr is necessary but not sufficient for the inhibition of cell proliferation and for mpl protein degradation. The amino acid sequence of mpl-tr is identical to the N terminus of mpl except for a stretch at the C terminus of mpl-tr, 30 amino acids in length (Fig. 1). We therefore speculated that this unique C-terminal peptide in mpl-tr could be of functional importance. To test this hypothesis, we made two mutants of mpl-tr: in the first mutant, Δpep_{30}, we introduced a stop codon at position 427, removing the entire C-terminal peptide (Fig. 6A). In the second mutant, tr-pep_{mpl}, the sequence of the C-terminal peptide was changed by adding two base pairs, which restored the reading frame of full-length mpl and by introducing a stop codon terminating the reading frame after 30 amino acids (Fig. 6A). With these mpl-tr mutants we stably transfected BaF3/mpl cells and assayed TPO-dependent proliferation. As shown in Fig. 6B, only mpl-tr abrogated mpl-mediated cell growth, whereas tr-Δpep_{30} and tr-pep_{mpl} did not interfere with TPO-dependent proliferation. As expected, the mpl-tr mutants did not interfere with the proliferative responses to IL-3 (Fig. 6B). Immunoblot analysis showed that Δpep_{30} and tr-pep_{mpl} were expressed at levels similar to mpl-tr (Fig. 6C). This indicates that the degree of expression does not explain the failure of the mutants to inhibit TPO-dependent growth. Importantly, the levels of mpl protein in cells expressing the mpl-tr mutants were similar to BaF3/mpl control cells, but mpl was undetectable in cells expressing mpl-tr.

These results demonstrate that the C-terminal peptide sequence from mpl-tr is required for the ability of mpl-tr to promote a decrease in mpl protein and to inhibit TPO-dependent cell proliferation. Furthermore, these results illustrate that the function of mpl-tr in growth inhibition directly correlates with its role in mpl protein reduction. To determine whether pep_{30} is sufficient to interfere

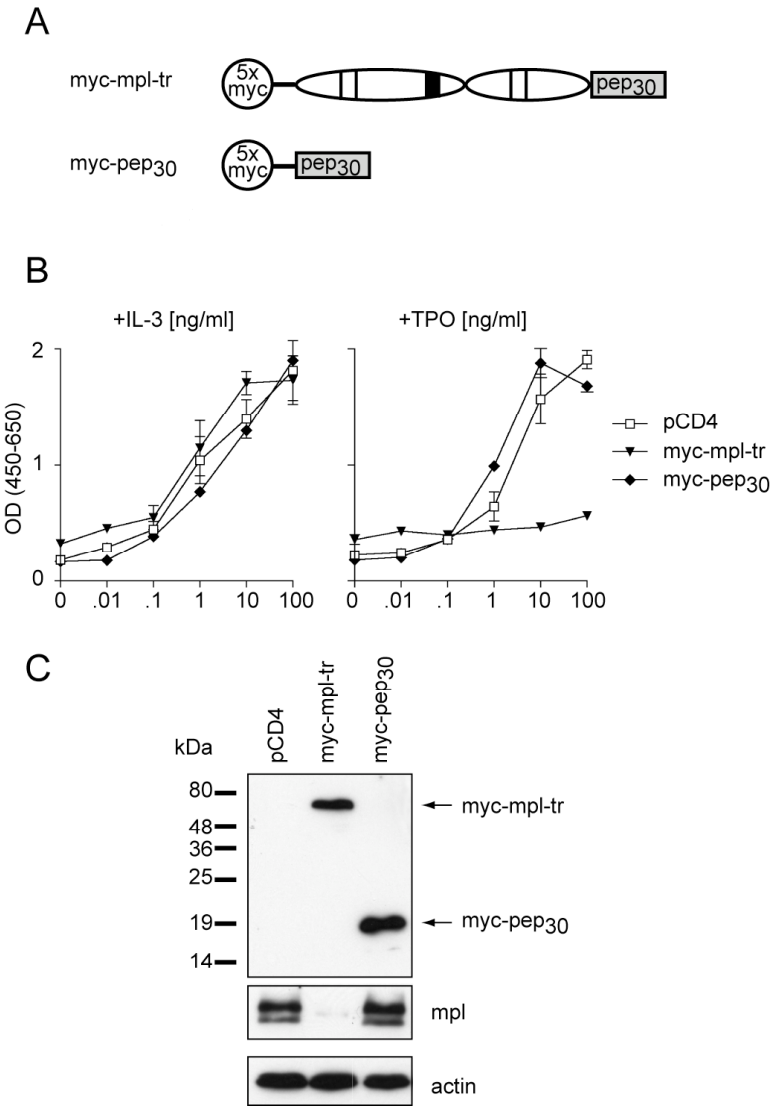


Fig. 7. Pep_{30} sequence alone is not sufficient to inhibit mpl function. **(A)** Schematic drawing of myc-tagged pep_{30} and mpl-tr (not to scale). **(B)** Proliferation of BaF3/mpl cells expressing vector alone (pCD4), myc-pep_{30} and myc-mpl-tr. **(C)** Immunoblot of total cell lysates probed with anti-myc (9E10), anti-mpl and anti-actin antibodies.

with TPO-induced proliferation and to mediate mpl degradation, we generated a myc-tagged versions of pep_{30} and mpl-tr (Fig. 7A). Myc-pep_{30} did not affect TPO-dependent proliferation (Fig. 7B) nor alter the expression levels of mpl protein (Fig. 7C), whereas myc-mpl-tr behaved comparable to untagged mpl-tr. Thus, pep_{30} sequence alone is not sufficient to inhibit mpl function.

Discussion

Alternatively spliced cytokine receptor variants are emerging as regulators of cytokine signaling [36-38,68,109,110]. The c-mpl locus gives rise to the full-length mpl protein and the mpl-tr isoform, which is present in both human and mouse [72,74,77]. Here, we characterized mpl-tr as a specific inhibitor for TPO-dependent proliferation and described a novel mechanism of receptor protein downmodulation mediated by an alternate isoform of the same receptor through a cathepsin-like protease activity.

Alternatively spliced isoforms are frequently found among members of the cytokine receptor superfamily and most family members have been shown to be capable of giving rise to soluble forms [63]. So far, two functions have been assigned to soluble cytokine receptor isoforms: soluble receptors can prolong the half-life of the ligand and activate signal transduction by associating with a transmembrane receptor complex, as exemplified by the soluble IL-6 receptor [111,112]. Alternatively, soluble receptors can act as antagonists of their membrane-bound counterparts by binding and neutralizing the ligand, e.g. IL-5 or GM-CSF [65,66,113,114]. In either model, the function of the soluble receptor is dependent on its secretion. However, the serum levels of some soluble receptor are low or undetectable [63,115], suggesting additional functions of soluble receptors as non-secreted forms.

Because the deletion in mpl-tr removes the transmembrane domain, mpl-tr is expected to give rise to a secreted form, which might antagonize mpl signaling by sequestering TPO. However, this model cannot explain the observed dominant-negative effect of mpl-tr for three reasons: 1) mpl-tr could not be detected in the cell supernatant [74]; 2) high concentrations of TPO did not titrate out the effect of mpl-tr (Fig. 2); and 3) media conditioned by mpl-tr expressing cells did not affect TPO-dependent growth of BaF3/mpl cells (Fig. 2C).

We demonstrated that over-expression of mpl-tr leads to a dramatic decrease in mpl protein amounts, which can explain the dominant negative effect of mpl-tr on TPO-dependent cell proliferation and survival. The decrease in mpl protein was not due to differences in c-mpl mRNA expression (Fig. 4 and 5). A

specific inhibitor for cysteine protease cathepsins, CATI-1, can counteract the effects of mpl-tr and restore full-length mpl protein expression. In contrast, inhibitors of Ca^{2+}-dependent cysteine proteases, ALLN and EST, had no detectable effect on mpl protein amounts. It is therefore tempting to speculate that in the presence of mpl-tr, mpl traffics to the lysosome where it is degraded. The small increase in mpl expression in the presence of the proteasomal inhibitor MG132 may be explained by the finding that some receptor trafficking events from endosomes to lysosomes require a functional proteasome [116,117].

The observed effects of mpl-tr appear to specifically target the mpl protein, since IL-3 and GM-CSF dependent cell proliferation and survival were not affected. This specificity could be based on an inherent capability of the extracellular domains of cytokine receptors to form dimers. It has been shown by crystallography that Epo-R form dimers in the absence of ligand [19]. In analogy to the structure-function studies performed for Epo-R, we expect that the extracellular portion of mpl-tr is sufficient to allow hetero-dimerization with the full-length form of mpl. So far, we have been unable to detect this physical interaction through co-immunoprecipitation (data not shown), possibly because the interaction is only transient.

We demonstrated that the unique sequence at the C terminus of mpl-tr is essential for mediating mpl degradation. In one possible scenario, the C terminus of mpl-tr prevents the secretion of mpl-tr and directs the putative mpl/mpl-tr dimer to traffic along the endosomal-lysosomal route. Increasing acidification of the endosomal vesicle could cause the hetero-dimer to disassemble allowing mpl-tr to be recycled. This model would explain why mpl-tr protein expression is apparently not affected by the presence of mpl protein (Fig. 5B). Further structure function analysis the C-terminal sequence of mpl-tr should allow us to define a minimal functional peptide motif.

Alternatively, mpl-tr may interfere with proper folding of mpl. In both mammalian cells and in yeast it has been shown that a post-ER quality control mechanism appears to be responsible for targeting and lysosomal/ vacuolar degradation of misfolded membrane proteins [118-121]. The presence of a cytosolic portion may be a prerequisite for the detection of partly unfolded mpl by the post-ER quality surveillance system. Since mpl-tr only contains the

luminal portion of mpl, it may evade detection by the quality control system. This would explain why mpl-tr steady state levels are not altered in the presence of full-length mpl.

Cytokine receptor variants may be important in the regulation of receptor function. Interestingly, Nakamura et al. demonstrated that transgenic mice overexpressing the intracytoplasmic truncated erythropoietin-receptor isoform EpoR-T reveal mild anemia [109]. Because overexpression of EpoR-T diminishes the number of erythrocytes in the mouse, these results suggest a role of cytokine receptor variants as regulators of proliferation and differentiation *in vivo*.

In this study, we have demonstrated that an alternate mpl isoform (mpl-tr) exerts a dominant negative effect on proliferation and survival. Because mpl-tr is an abundant c-mpl splice variant accounting for about 30% of total mpl mRNA in mouse spleen [74], mpl-tr is likely to play a physiological role in megakaryopoiesis. Differential expression has been shown for alternatively spliced isoforms of cytokine receptors like IL-5 receptor and GM-CSF receptor [110,122]. The relative expression of different isoforms of the same cytokine receptor can determine cell differentiation and cell lineage expansion [110,123]. In analogy to isoforms of other receptors, we speculate that the relative expression of c-mpl-tr varies with different cell types and developmental stages and that this serves to modulate mpl function.

Substitution of the endogenous *mpl* locus by an mpl transgene causes thrombocytosis in the mouse

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Running title: Absence of c-mpl isoforms causes thrombocythosis in the mouse
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Abstract

Multiple mechanisms for the downmodulation of cytokine receptor signaling are known. These include post-translational modifications of JAK and STAT molecules, dephosphorylation of receptor tyrosines and the effects of dominant-negative cytokine receptor isoforms. Thrombopoietin (TPO) and its receptor c-mpl are essential for normal megagkaryopoiesis and play an additional role in regulating hematopoietic stem cell numbers *in vivo*. We have recently characterized the alternate mpl variant mpl-tr as a dominant-negative isoform, which specifically inhibits TPO-dependent cell proliferation and survival signaling. To test for the *in vivo* function of alternative mpl isoforms, we generated mice expressing full-length mpl as a transgene in a mpl knockout background. These mice express mpl full-length as the only mpl isoform and develop severe thrombocytosis with platelet numbers, elevated about five times higher than normal. The reintroduction of the endogenous mpl allele restores normal platelet counts and we attribute this to the *in vivo* effect of dominant-negative mpl isoforms. A mpl knock-in allele, which does not express mpl-tr but still expresses the second known alternate variant of murine mpl, mpl-II, normalizes platelet numbers, similar to the endogenous mpl allele. This result demonstrates that the absence of mpl-tr is not sufficient to cause thrombocytosis. We propose that mpl-II is an additional dominant-negative mpl isoform and attenuates the expansion of the megakarocytic lineage *in vivo.*

Introduction

Of the many cells in the body, the hematopoietic cells are among those with the highest rate of self-renewal and turnover. The production and destruction of these cells are tightly controlled by a number of hematopoietic growth factors. Interestingly, many of these hematopoietic growth factors have primarily lineage-restricted effects as demonstrated in knockout analyses. For example, gene deletion of erythropoietin (EPO) or of its receptor Epo-R lead to a specific reduction in erythrocytes [124-126]. Similarly, the disruption of G-CSF and G-CSF-R expression solely affects granulopoiesis [127-129]. Finally, thrombopoietin (TPO) and TPO-R deficiencies result in a profound reduction of platelet and bone marrow megakaryocyte numbers, but do not significantly alter megakaryocyte differentiation [82,130,131]. Although it is clear that the primary function of TPO is to regulate platelet numbers, several lines of evidence indicate that this cytokine modulates multiple aspects of hematopoiesis. Importantly, all hematopoietic stem cells (HSC) are positive for mpl expression and genetic elimination of mpl reduces the numbers of murine HSCs by 7-8 fold [86]. In humans, null mutations in mpl lead to congenital amegakaryocytic thrombocytpenia, a disorder that almost invariably leads to aplastic anemia [132-134]. Together, these findings suggest that mpl activity supports mostly the expansion of HSC and megakaryocytic precursors, but has little importance in megakaryocytic differentiation. In this context, it is worthwhile remembering that the c-mpl gene was first discovered as the cellular homolog of the retroviral oncogene v-mpl [71,72,74], emphasising the primary role of mpl as a transducer of a proliferation signal. Because cytokines like mpl are potent growth factors, stringent mechanisms of signal attenuation are essential for ensuring an appropriate, controlled cellular response. Several protein tyrosine phosphatases including SHIP-1, CD45, and PTP1b have been shown to be negative regulators of cytokine signaling [62,135]. Members of the suppressor of cytokine signaling (SOCS) family of proteins act in a negative feedback loop, inhibiting the cytokine activated Janus kinase/ signal transducers and activators of transcription (JAK/ STAT) signaling pathway to modulate cellular responses [136]. Thirdly, protein

inhibitors of activated STAT (PIAS) bind activated STAT dimers and block transcription in a process that may involve sumoylation of STATs [57].
An additional mechanism of modulating the cytokine response is the expression of alternative cytokine receptor variants, which act as dominant-negative isoforms [68,109,137]. Mechanistically, dominant-negative receptor variants can form non-functional heterodimers with the full-length receptor or, when expressed as secreted 'soluble' isoforms, compete with the receptor for ligand binding [63]. We have recently discovered a third mechanism, in which the mpl isoform mpl-tr mediates protein degradation of the full-length receptor by a cathepsin-like cysteine protease activity (Coers et al., *in press*).
Altered expression of alternative spliced mRNA variants has been linked to a number of diseases [138]. We were therefore interested in determining whether mpl isoforms are important for normal megakaryopoiesis. To address this question we generated mice which express c-mpl under the control of the mpl promoter in a mpl knockout background, thereby deleting all alternate isoforms while retaining full-length mpl receptor expression. These mice have platelet numbers elevated five fold compared to wild type and show additional characteristics which are reminiscent of human essential thrombocythemia (ET), a myeloid disorder characterized by a persistent increase in circulating platelets. Expression of the endogenous mpl allele in these thrombocthemic mice restores the wild type phenotype, showing that the mpl allele contains a dominant-negative property. A mpl knock-in allele, which does not express mpl-tr but retains expression of the alternate isoform mpl-II [80], acts similar to the endogenous mpl allele in reducing the number of platelets. This demonstrates that an additional dominant-negative mpl isoform other than mpl-tr must exist and we propose that this isoform is the recently described mpl variant mpl-II.

Materials and Methods

Generation of transgenic mice

To generate the transgenic construct, a 2kb *Hind* III-*Apa* I promoter fragment was isolated from a lambda FIX II genomic DNA library and subcloned into pKS II. The mpl cDNA was added as a blunt ended fragment into an EcoRV site. At the 3' end an SV40 polyadenylation signal was added. The transgenic construct was excised as a *Xho I-Not I* fragment. The resulting 5kb fragment, which contains no vector sequences, was used for oocyte microinjection. We have generated 5 transgenic founder mice in the $B6D_2F_1$ strain (BRL, Switzerland) by standard oocyte injection.

Blood and tissue analysis

Blood was obtained by cardiac puncture without anticoagulants. Approximately 500 □l of blood was immediately mixed with EDTA and blood counts were performed with an automated blood counter (Technicon H-3, Bayer Diagnostics, Tarrytown, NY). For mouse TPO ELISA (mouse TPO Quantikine kit, R&D Systems), the remaining blood was either also mixed with EDTA to obtain plasma or allowed to coagulate and serum was collected. For histology, freshly dissected tissues were fixed in Optimal*Fix (American Histology Reagent Co., Stockton, CA). Fixed specimens were embedded in paraffin, sectioned, and stained by the Transgenic Pathology laboratory at the University of California at Davis. Megakaryocyte frequency was determined by two-color flow cytometry as described in (Arnold et al., 1997). In brief, bone marrow cells from one femur and one tibia were isolated in CATCH (0.84 mM adenosine, 2 mM theophilline, 0.38% sodium citrate, 3.5 % BSA and mM Hepes in Hanks BSS (GIBCO) at pH 7.0). Cells were filtered through a 100 □m nylon mesh. Megakaryocytic cells were stained using the monoclonal 4A5 rat anti-mouse platelet antibody and a Tago FITC–goat anti rat IgG F(ab')2 antibody. Staining was performed for each antibody at 4°C for 30 minutes. Samples for electron microscopy were processed essential as described [139]. In brief, spleen tissue was diced into pieces of roughly one square millimeter fixed for 1 hour in 3% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, washed, and embedded in Epon. Ultrathin sections were collected on grids and imaged using a Philips Morgani 268D electron microscope. Representative fields of sections performed on the entire samples were chosen for illustration.

Clonal culture of hematopoietic progenitors

Clonal cultures of hematopoietic cells were performed as described [140]. Briefly, to assay for myeloid and granulocytic progenitors, $5x10^4$ bone marrow cells for wild type and knockout animals or $2.5x10^4$ bone marrow cells for transgenic animals were placed in 0.3% agar in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% horse serum and 10mg/ml BSA (Boehringer Mannheim) and stimulated with Hemostim M2100 at a final

concentration of 2% (StemCell Technologies, Inc.) and 100ng/ml human G-CSF (Roche). These conditions resulted in optimal stimulation of neutrophil (G), neutrophil-macrophage (GM), and macrophage (M) colony forming cells. To assess the number of megakaryocytic (Meg) progenitors, $2x10^5$ and $1x10^5$ bone marrow cells, respectively were grown in 0.3% agar under serum free conditions [140] and were stimulated with 2% Hemostim M2100 and 10% of serum free conditioned media of a TPO expressing NIH/3T3 cell line [84]. Finally, to assess erythroid colony growth $5x10^4$ and $2.5x10^4$ bone marrow cells were cultured in 2.7% methylcellulose in IMDM supplemented with 20% fetal bovine serum (FBS) and 1mg/ml BSA and stimulated with 2% Hemostim M2100 and 2.7 U/ml EPO (Eprex). The cultures were incubated for 7 days at 37°C in a humidified atmosphere with 5% CO_2. Myeloid, granulocytic and erythroid colonies were scored for morphology, whereas CFU-Meg were identified using a histochemical staining for acetylcholinesterase. Colonies containing at least five acetylcholinesterase positive cells were scored as CFU-meg [141].

RNA isolation and ribonuclease protection assay

Spleen tissue was homogenized in 4 M guanidium isothiocyanate with a Polytron homogenizer and RNA samples were prepared by the acid phenol method [142]. For ribonuclease (RNase) protection analysis [143], we constructed a riboprobe for the detection of the transgenic and the endogenous mpl mRNA by subcloning a 368 bp PCR fragment of mouse mpl cDNA into pBluescript. As primers we used 5'-CTCGCTTCCAGACCTACACC-3' and 5'-GGATAAGGTAGT GTGTAGGA-3'. The resulting vector was digested with Hind III, transcribed with T7 RNA polymerase and hybridized to 30□g of total RNA at 50°C as described [143]. This riboprobe protects a 368 nucleotides (nt) fragment for the endogenous mpl cDNA and a shorter 318 nt fragment for transgenic mpl transcripts. RNA loading was normalized with a riboprobe for mouse hypoxanthine-guanine phosphoribosyl transferase (HPRT), a house keeping gene. A. Sca I – Hind III fragment representing nucleotides 679 to 840 of mouse HPRT cDNA [144] was subcloned into pBluescript. This probe protects a 161 nt fragment. HPRT riboprobe was mixed with the mpl riboprobe and added as an internal standard to each sample. Protected fragments were separated on 6% polyacrylamide / 8M urea sequencing gels. Dried gels were exposed to phosphorimager screens and quantitations of radioactive bands were performed on a phosphorimager 425 using the ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

Platelet and megakaryocyte isolation

For platelet isolation, blood was drawn by cardiac puncture into a syringe containing 3,8% Citrate. Platelet rich plasma (PRP) and afterwards pure platelets were isolated by centrifugation at 350g and 1300g, respectively, for 10 minutes. Platelets were then washed with PBS and purity was determined by automated blood counter (Technicon H-3, Bayer Diagnostics, Tarrytown, NY). For megakaryocyte isolation, two femurs per mouse were flushed with CATCH medium (129 mM NaCl, 8.614 mM Na_2HPO_4, 1.6 mM KH_2PO_4, 0,4% Na-

citrate, 11.1 mM glucose, 1mM adenosine, 2 mM theophilline, 2.3 μM PGE1 and 1% BSA). 4ml of a single bone marrow cell suspension was then mixed with 3 ml of Percoll/ PBS (1.02g/ ml, Pharmacia), gently layered on top of 4 ml Percoll/ PBS (1.05 g/ml, Pharmacia) and spun for 20 minutes at 400g. The interface was then collected and washed with CATCH buffer. Finally the purity of the megakaryocyte preparation was assessed by acetylcholine esterase stain of a cytospin.

Protein expression in platelets and megakaryocytes

Isolated platelets and megakaryocytes were lysed by boiling 5 minutes at 94° C in 2xSDS Page loading buffer at a concentration of $2x10^6$ platelets/μl and 50'000 megakaryocytes/ μl. Protein lysates were then separated by SDS Page and transferred to nitrocellulose by Western Blot. The membranes were blocked with 4% non-fat dry milk in PBS/ 0.05% Tween 20 and incubated overnight at 4°C with 1:5'000 diluted biotinylated monoclonal mouse anti mpl antibody (kindly provided by F. de Sauvage). After three washes with PBS/ 0.05% Tween 20 the membranes were incubated with 1:1000 diluted horseradish peroxidase-coupled streptavidine and washed. Enhanced chemiluminescence (ECL) was detected following the instructions of the manufacturer (Amersham, Buckinghamshire, England). Before reprobing the membranes were stripped for 30 minutes at 50°C in 62.5 mM Tris HCl (pH 6.8), 2% SDS, 100mM β-mercaptoethanol, blocked in 4% non-fat dry milk and incubated with biotinylated 4A5 rat anti-mouse platelet monoclonal antibody.

Results

Generation of transgenic mice expressing full-length mpl. To assess the contribution of alternate mpl isoforms to megakaryopoiesis and platelet production *in vivo*, we have generated transgenic mice expressing full-length mpl in a mpl knockout background. The transgenic construct consists of a 2kb mpl promoter, which directs expression specifically to the megakaryocytic lineage [145], murine mpl cDNA and an SV40 polyadenylation signal at the 3' end of the constructs for stability of the construct (Fig. 1A). The construct was then injected into oocytes. We obtained five founders and detected mpl transgene (mpl-TG) expression in three (Table 1). These mice are viable, fertile and show no overt abnormalities. They were then backcrossed into the mpl knockout background.

Analysis of transgene expression in mouse tissues. To verify that the mpl-TG was expressed properly, we isolated RNA from mouse spleens. To distinguish between the mpl-TG and endogenous mpl, an RNase protection assay was performed. The riboprobe protects a 368 nt fragment for the endogenous mpl transcrpt and a shorter 318 nt fragment for the mpl-TG transcript (Fig. 1B). RNA loading was normalized with a riboprobe for mouse hypoxanthine-guanine phosphoribosyl transferase (HPRT), a house-keeping gene. Fig 1C shows that the mpl-TG is expressed at levels comparable to endogenous mpl in non-transgenic wild type controls. To our surprise we could also detect endogenous mpl mRNA in mpl knockout mice. This is explained by the gene-targeting strategy used for the generation of these mice, which consisted of an insertion of a *neo*-casette in exon 3 of mpl [82]. Further analysis showed that the mpl mRNA expressed in these mpl knockout mice lacks exons 1 and 2 of mpl (data not shown), which are upstream of the *neo*-cassette insertion. It is therefore likely that the mpl mRNA found in these mice is expressed off the promoter of the *neo*-cassette. When tested for proper targeting, protein expression and responsiveness to TPO, these mice proved to be true knockouts[81] (and data not shown).

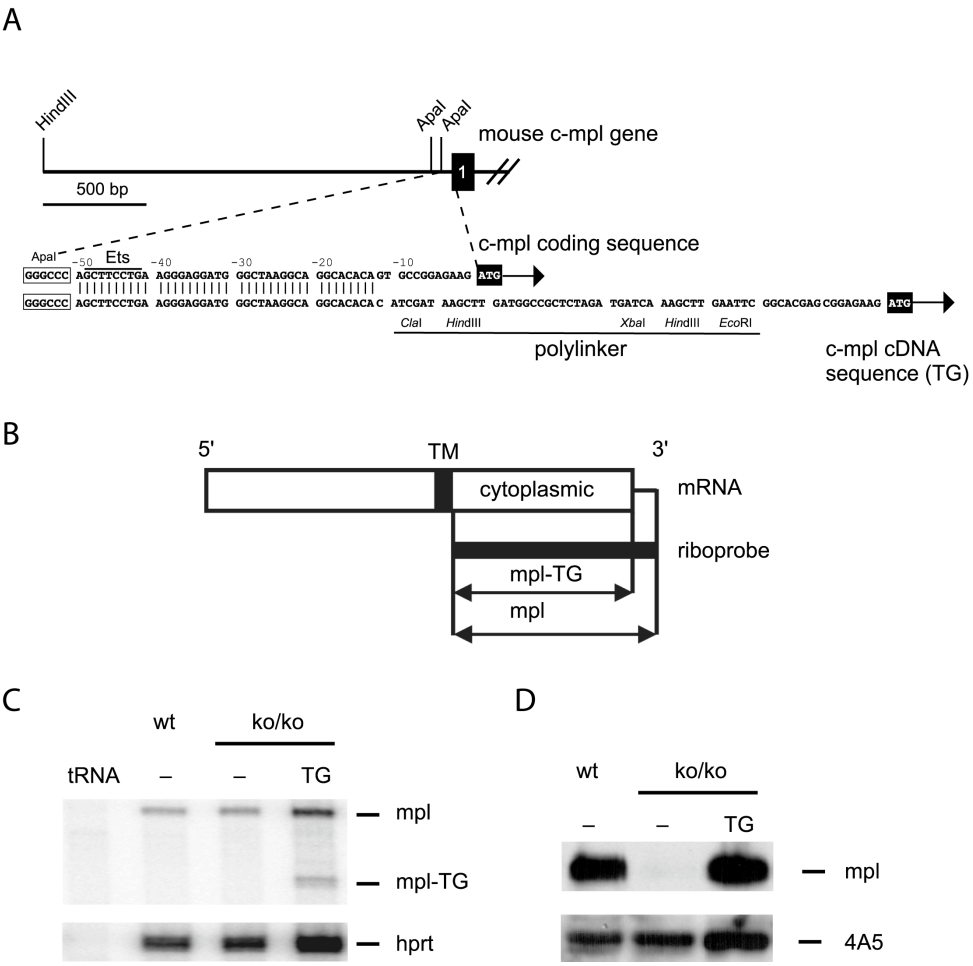

Fig. 1. Transgene expression (A) A 2kb *Hind* III-*Apa* I mpl promoter fragment drives the expression of the mpl TG. **(B)** To distinguish between the TG and endogenous mpl, an RNase protection assay was performed. The riboprobe protects a 368 nt fragment for the endogenous mpl cDNA and a shorter 318 nt fragment for transgenic mpl transcripts. **(C)** RNA from mouse spleen tissue of the indicated genotpye was used for an RNase protection assay. Protected fragments corresponding to endogenous mpl and mpl TG mRNAs are depicted. Loading was normalized with a riboprobe for mouse HPRT. **(D)** Immunoblotting blotting for mpl protein in isolated megakaryocytes.

number of TG strains	*number of strains expressing the TG*	*platelet count on mpl ko/ko background (x 10^6 /ml)*		
		a	b	b
5	3	5034	6165	3367

Table 1. Five transgenic founder mice were obtained after oocyte injection. Three of the five resulting mouse strains tested positive for TG expression and were backcrossed into the mpl ko/ko background. All three lines had clearly elevated platelet numbers.

Next, we isolated megakaryocytes from the bone marrow of mpl-TG mice and wild type controls. In Western blot analysis a strong signal of approximately 80 kDa was detected in wild type megakaryocytes, as expected. This band was absent in the knockout (Fig. 1D). As observed in Fig.1D, the transgenic version of mpl protein is expressed at levels similar to the non-transgenic wild type molecule in megakaryocytes.

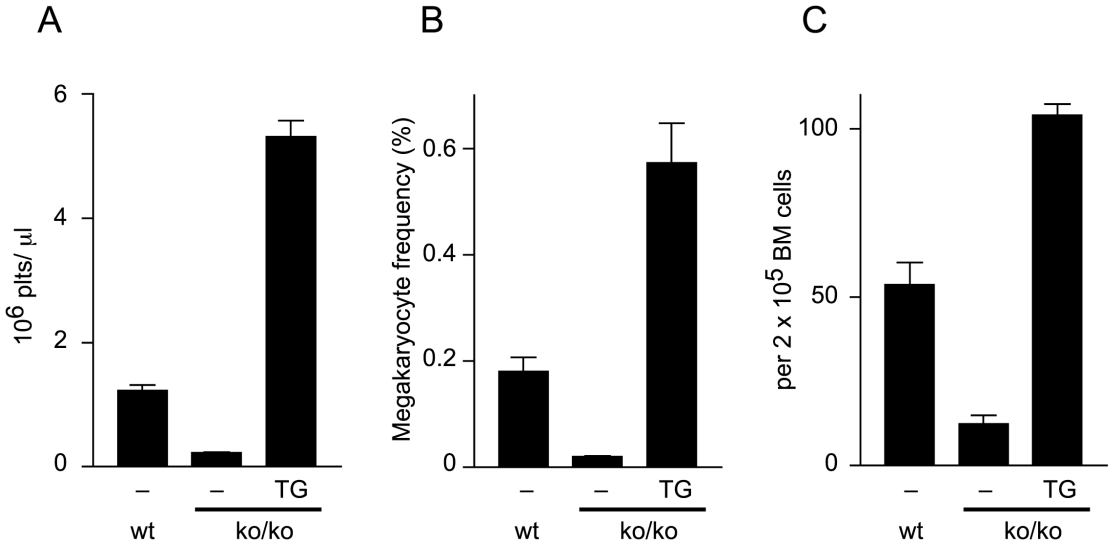

Fig. 2 Platelet counts and megakaryocytic precursors in transgenic mice. (A) Platelet counts in circulating blood from wild type (wt), and transgenic (TG) and non-transgenic mpl (-) knockout mice (ko/ko). **(B)** Frequency of megakaryocytes in bone marrow from the same mice was determined by flow cytometry. **(C)** CFU-Meg assays were performed to determine the number of megakaryocytic precursors.

Blood cell counts and progenitor analysis. To investigate the effect of the removal of alternate mpl isoforms on the production of platelets, complete blood cell counts were performed. While the absence of mpl in knockout mice leads to a 90% reduction in platelet count [82,83], mice expressing the full-length receptor as a mpl-TG in the mpl knockout background have elevated platelet levels compared to wild type mice (Fig. 2A and Table 1). The presence of the mpl-TG leads to a five fold increase in platelet numbers compared to normal. Similarly, the megakaryocyte percentage (Fig. 2B) and the frequency of megakaryocytic precursors (Fig. 2C) in the bone marrow is increased in transgenic animals. The aberration in cell numbers is restricted to the megakaryocytic lineage and circulating blood cell numbers are otherwise normal (Table 2). Further, we found that all the hematopoietic progenitor

numbers are elevated in TG-mpl expressing mice compared to the wild type control (Table 3). This increase in progenitor frequency in the transgenic mice is probably due to mpl expression on hematopoietic stem cells, as previously described [86,146]. Consequently, enhancement of the HSC pool through mpl-TG signals can explain the increase in progenitor numbers. The expression off the mpl promoter is, however, downregulated in lineage restricted progenitor cells other than the megakaryocytic progenitor cells [86] and therefore no further amplification of blood cells different from the megakaryocytic lineage is likely to be supported by the mpl-TG. This would explain why the numbers of circulating blood cells in mpl knockout mice expressing the mpl-TG are in the normal range (Table 2).

TG	mpl locus											
	+ / +				+ / ko				ko / ko			
	n	*HGB g / dl*	*WBC x(10^6/ml)*	*platelets x(10^6/ml)*	*n*	*HGB g / dl*	*WBC x(10^6/ml)*	*platelets x(10^6/ml*	*n*	*HGB g / dl*	*WBC x(10^6/ml)*	*platelets x(10^6/ml)*
none	12	14.1 ± 0.1	14.3 ± 0.7	1‘180 ± 50	5	12.3 ± 0.5	7.4 ± 0.8	1‘665 ± 40	5	14.2 ± 0.9	6.7 ± 1.7	208 ± 30
mpl	10	14.5 ± 0.2	13.1 ± 2.1	1‘390 ± 70	5	14.5 ± 0.7	10.9 ± 0.5	1944 ± 100	5	12.9 ± 0.4	9.5 ± 0.8	5‘248 ± 320

Table 2. Blood was obtained from non-transgenic and transgenic mice by cardiac puncture and analyzed as described in Materials and Methods. Shown are the concentrations of hemoglobin (HGB), and the numbers of white blood cells (WBC) and platelets. The mice were either homozygous for the endogenous mpl allele (+/+), or heterozygous (ko/+) or homozgous (ko/ko) for the mpl knockout allele.

Histopathology of mpl-TG mice. Morphologically and functionally, platelets and megakaryocytes in transgenic mice are normal. We could not observe changes in platelet activation or in megakaryocyte ploidy, a measure for the maturation stage of megakaryocytes (data not shown). Histopathological analysis of transgenic mice, however, revealed not only an increase of megakaryocytes in bone marrow but in spleen (Fig. 3). Furthermore, the spleen sections contained aggregates of small particles (Fig. 3). To identify

the nature of these particles, electron microscopic analysis of spleen sections was performed. In mice carrying the mpl-TG but not in wild type controls, we detected areas containing densely packed platelets, as identified by size, morphology, and the presence of usually one identifiable dense granule per platelet [139] (Fig. 4). Moreover, the spleens in mpl-TG animals are enlarged (data not shown), which can be explained by the congestion of the spleen with platelets and the increase in megakaryopoiesis.

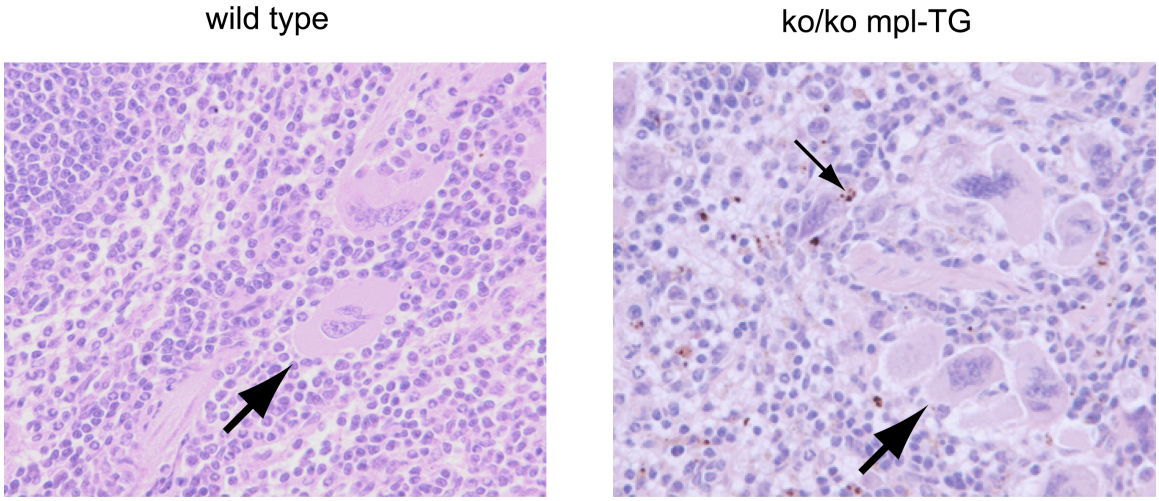

Fig. 3 Platelet-like particles are found in the spleen of transgenic mice. Representative sections show an increase in the number of megakaryocytes (big arrow) in spleen of transgenic (ko/ko TG-mpl) compared to wild type mice (wt). The small arrow points at aggregates of small platelet-like particles, which are only found in transgenic mice.

TPO levels in circulation and c-mpl expression on platelets. In experimental murine models, it has been shown that TPO over-expression causes thrombocythosis *in vivo* [147,148]. Further, mutations in the TPO gene, which result in elevated TPO plasma levels, are the underlying cause of one form of hereditary thrombocythemia in humans [149,150]. We therefore tested whether TPO levels in circulation were altered in mice expressing the mpl-TG. We found that this was not the case (Fig. 5A). Therefore, higher levels of circulating TPO do not cause the observed thrombocytosis. Contrarily, it would have been expected that thrombocythemic mice have reduced TPO plasma levels due to increased platelet numbers, because platelets have been shown to play a key role in plasma clearance of TPO [84]. It has been demonstrated that platelets actively bind TPO via mpl, and internalize and

degrade the protein [151,152]. This situation, where thrombocytosis is accompanied by normal TPO levels, resembles human ET. In patients with ET, for reasons not yet understood, expression of c-mpl protein is dramatically reduced in platelets, which may account for the normal or slightly elevated TPO levels in circulation [153]. We therefore tested mpl-TG protein expression on platelets (Fig 5B). Despite the equal expression levels on megakaryocytes, mpl-TG protein expression is reduced on platelets. The expression of mpl-TG mRNA levels is, however, similar to endogenous mpl mRNA in non-transgenic wild type controls (data not shown). This indicates that the transgenic protein is less stable or less efficiently translated. Factor independent growth of megakaryocytic progenitors as a second possible explanations for the observed thrombocytosis could also be ruled, because CFU-Meg cultures of bone marrow cells derived from transgenic aimals are TPO-dependent (data not shown).

mpl locus	***TG***	***n***	**total number of cells/ femur (x 10^6)**	*CFU-G* ***per 5 x 10^4 cells***	*CFU-GM* ***per 5 x 10^4 cells***	*CFU-M* ***per 5 x 10^4 cells***	*BFU-E* ***per 5 x 10^4 cells***
+ / +	none	3	26.7 ± 4.5	4.3 ± 0.7	3.3 ± 0.9	56 ± 5.8	5.3 ± 1.6
ko / ko	none	3	31.1 ± 3.2	2.5 ± 0.8	1.5 ± 0.3	52 ± 3.4	3.5 ± 0.8
ko / ko	mpl	3	18.5 ± 1.4	12.0 ± 3.1	6.0 ± 1.2	147 ± 20.6	9.7 ± 3.2

Table 3. Bone marrow cells form different mouse strains were cultured as described under Materials and Methods. Colony forming units (CFUs) for neutrophil (G), neutrophil-macrophage (GM), and macrophage (M) as well as burst forming units for erythrocytes (E) were scored. Bone marrow cells from three mice were analyzed for each genotype.

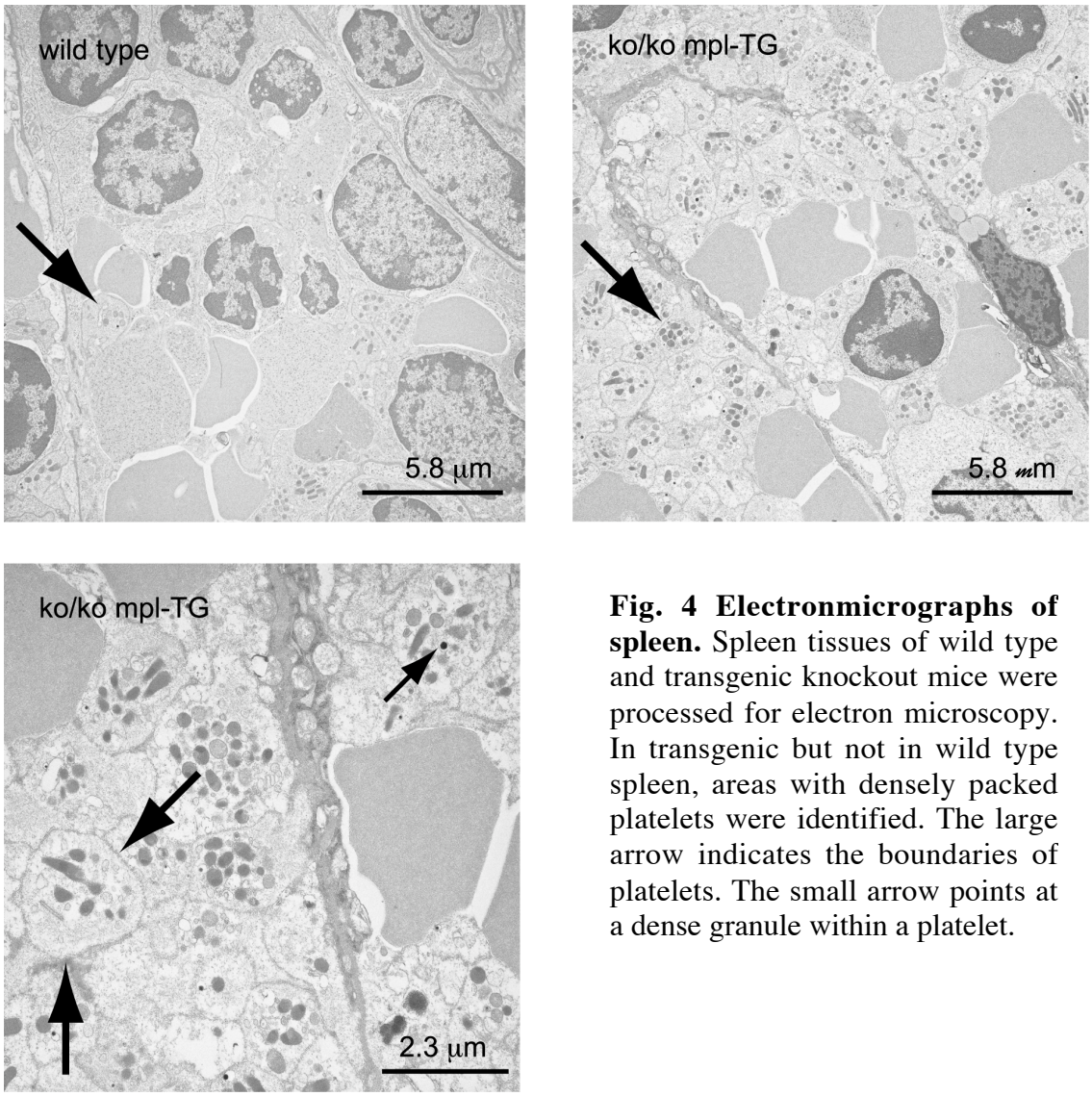

Fig. 4 Electronmicrographs of spleen. Spleen tissues of wild type and transgenic knockout mice were processed for electron microscopy. In transgenic but not in wild type spleen, areas with densely packed platelets were identified. The large arrow indicates the boundaries of platelets. The small arrow points at a dense granule within a platelet.

Endogenous mpl locus restores platelet count to wild type levels. Mice expressing full-length mpl as a transgene in a mpl knockout background lack all alternate mpl isoforms. To address the question whether the observed thrombocythemia is caused by the absence of alternative mpl isoforms, we reintroduced the endogenous mpl locus into the thrombocythemic mice, which are homozygous for the mpl knckout allele. The resulting transgenic mice, which are heterozygous for the endogenous locus, show platelet numbers similar to their non-transgenic littermates (Fig. 6A). Along the same lines, no difference exists between the number of megakaryocytic precursors of transgenic and non-transgenic ko/+ mice, as assessed by CFU-Meg assays (Fig. 6B). These results suggest that the endogenous mpl locus exerts a dominant-negative effect over the mpl-TG.

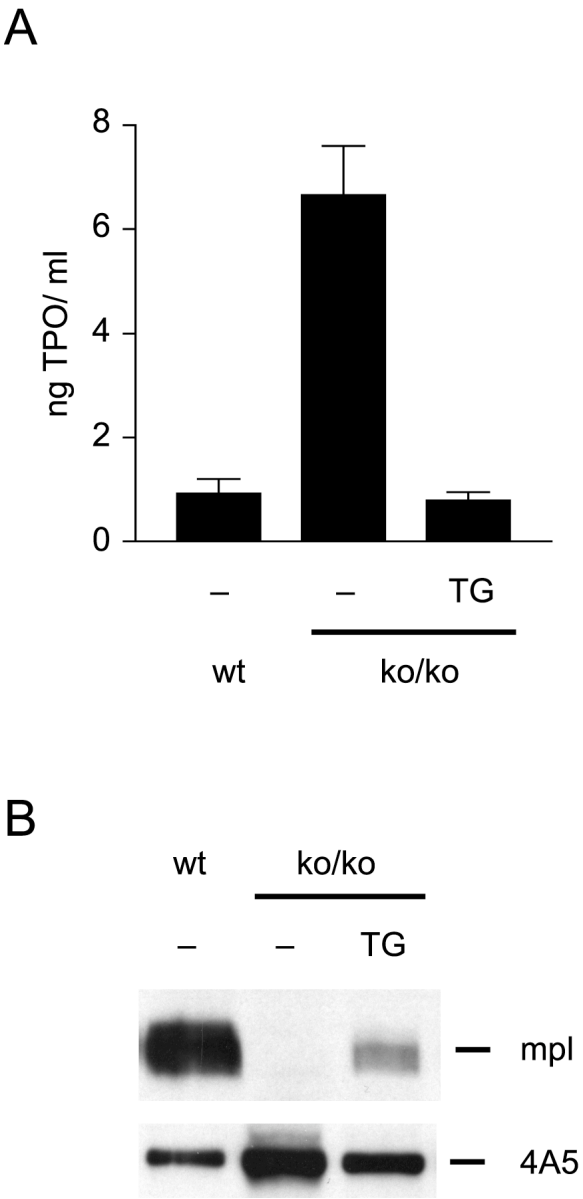

Fig. 5 TPO serum levels are normal in transgenic mice. (A) Serum TPO levels were determined as described in Materials and Methods. Because platelets sequester and thereby diminish the concentration of TPO through binding of TPO to mpl protein, TPO serum levels are elevated in mpl knockout mice. However, transgenic mice, albeit having five times higher platelet numbers compared to wild type mice, show TPO levels that are similar to wild type mice. **(B)** Immunoblotting for mpl protein shows that thromobocythemic transgenic mice (ko/ko TG-mpl) express mpl protein at much lower levels than wild type mice (wt).

Lack of mpl-tr is not sufficient to cause thrombocythosis. Two murine alternate mpl isoforms have been identified: mpl-tr [74] and mpl-II [80]. We have recently characterized one of these variants, mpl-tr, as a dominant-negative regulator of mpl function (Coers et al, *in press*). One possible way to explain the observed thrombocythemic phenotype is the absence of mpl-tr. To test this hypothesis, we took advantage of the existence of the mpl allele, Δ60. This allele was generated using the knock-in approach to replace exons 11 and 12 encoding the intracellular region of c-mpl with a cDNA fragment encoding only the first half of the cytoplasmic signaling domain [154] (Fig. 7A).

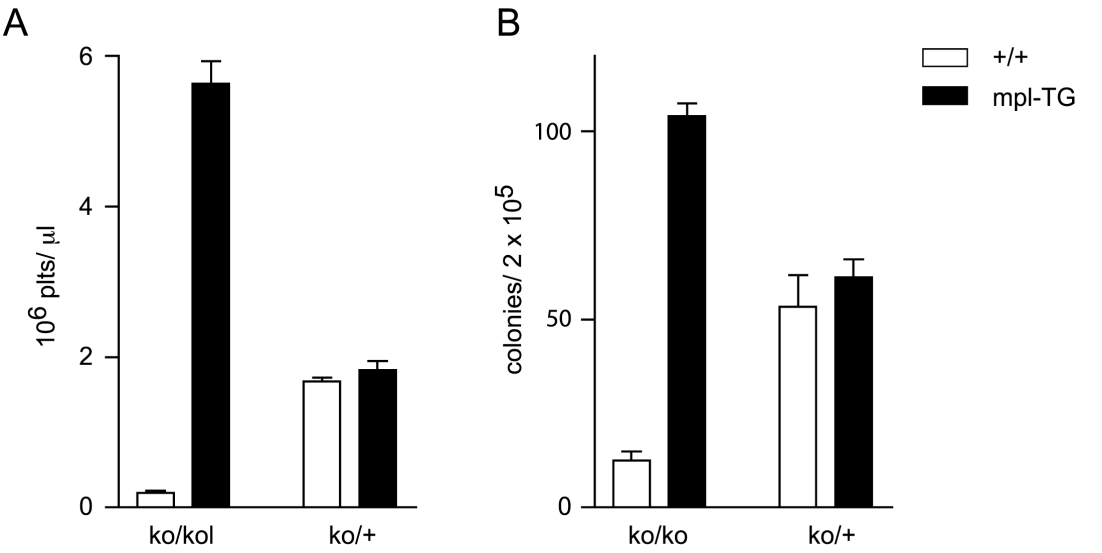

Fig. 6 Endogenous *mpl* locus reverts thrombocythemic phenotype in transgenic mice. (A) The platelet counts of non-transgenic (white boxes) and transgenic (black boxes) mice in the absence (ko/ko) and presence (ko/+) of the endogenous mpl locus are shown. Platelet counts were determined of at least 5 mice for each genotype. **(B)** The same mice were analyzed for the number of megakaryocytic precursors in the bone marrow, as determined in CFU-Meg assays, as described in Materials and Methods.

Mice homozygous for Δ60 have, by and large, normal platelet counts, showing that the last 60 C-terminal amino acids of mpl are not essential for normal mpl function *in vivo* [154]. Because the splice acceptor for the generation of mpl-tr is removed in the Δ60 allele, mpl-tr is expected to be absent in mice homozygous for Δ60 (Fig 7A). Using 3' RACE, we could confirm lack of mpl-tr (data not shown). We also confirmed the expression of the mpl-II variant in Δ60 homozygous mice (data not shown). Next, transgenic ko/ko mice were backcrossed with mice homozygous for Δ60 to obtain transgenic and non-transgenic mice with a ko/Δ60 mpl genotype. Like the wild type mpl alelle, the Δ60 allele reduced platelet counts of mpl-TG animals by about 70 % (Fig. 7B), demonstrating that a dominant-negative mpl isoform different from mpl-tr must exist.

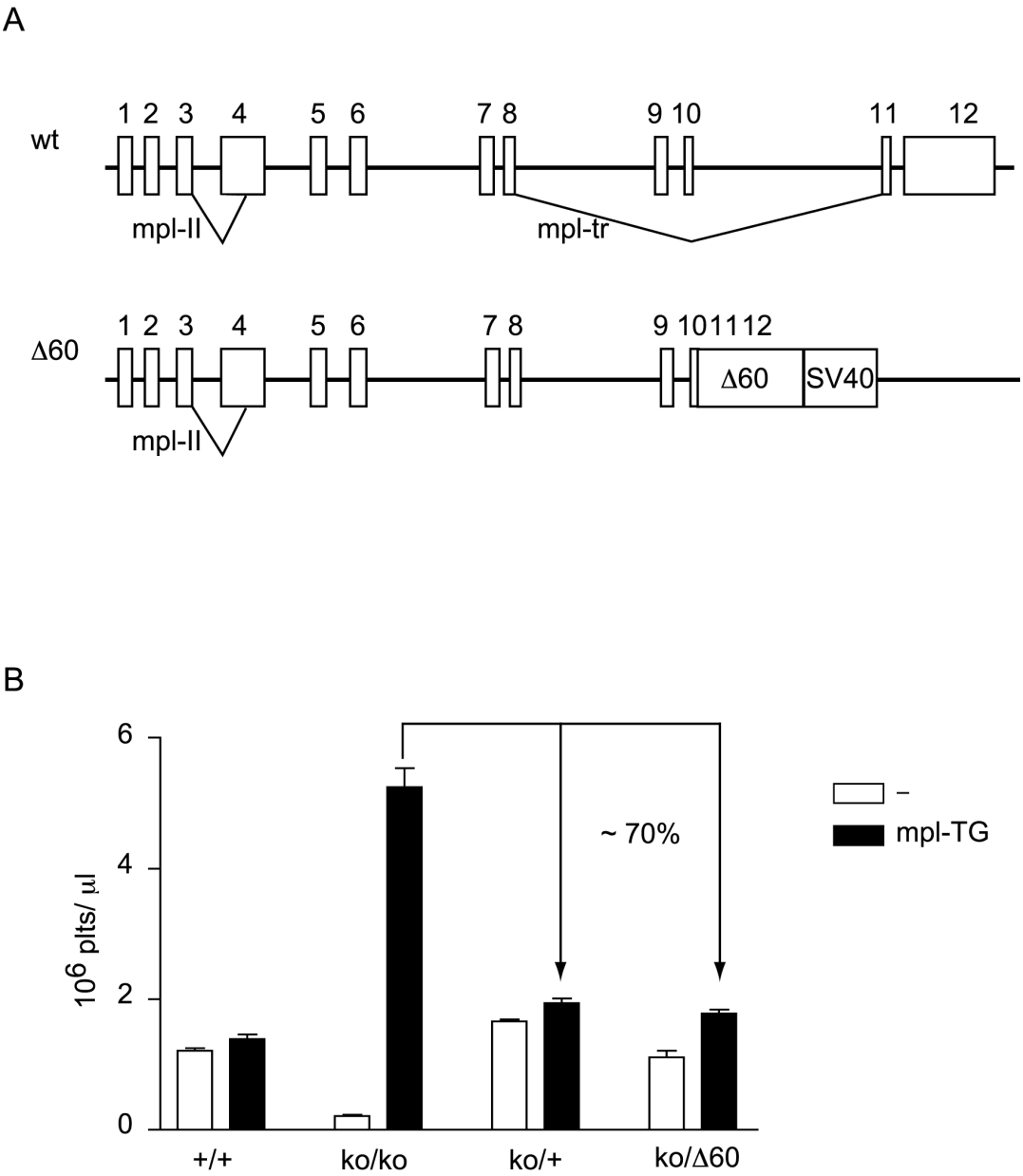

Fig. 7 Absence of mpl-tr does not cause thrombocythemia. (A) Schemata of the 3' region of the mpl locus: the boxes represent exons. A line connecting exon 8 with exon 7 indicates the splicing event generating the dominant negative variant mpl-tr. In the Δ60 allele, exons 10 and 11 are removed. As consequence, mpl-tr is not generated from this allele. **(B)** Platelet numbers are shown of non-transgenic (white boxes) and transgenic (black boxes) mice expressing different combinations of mpl alleles, as indicated. As shown, platelet numbers are five times the wild type counts in the absence of the endogenous mpl (ko/ko). When the wild type allele (ko/+) or the Δ60 (ko/Δ60) allele is backcrossed into these mice, platelet numbers decrease by about 70% to numbers similar to platelet counts in wild type mice (+/+).

Discussion

The precise mechanism by which hematopoietic progenitor cells proliferate, mature and enter blood circulation is not known. Cytokines can stimulate cell proliferation [93-95] or instruct cell fate determination [91,92]. The cytokine thrombopoietin and its receptor c-mpl are the principal regulators of megakaryopoiesis [74,155-157]. Mice deficient in TPO or its receptor continue to produce functional platelets, albeit at much lower levels [82,83,131], suggesting that c-mpl mainly controls quantitative aspects of megakaryopoiesis. Consistently, studies done in knock-in animals, where the intracellular domains of c-mpl and G-CSF-R have been interchanged, also indicate that TPO supports thrombopoiesis primarily by promoting proliferation of megakaryocytic progenitors [94]. Because hyperproliferation of the megakaryocytic lineage is often observed in myeloproliferative disease, it is of particular interest to understand how the TPO signal is attenuated. One negative regulator of megakaryopoiesis is transfoming growth factor-beta [158], which reduces TPO-induced STAT5 and ERK1/ERK2 activity [159]. Additionally, interferon-alpha directly inhibits TPO induced cell growth, possibly by inducing SOCS-1 expression and inhibiting JAK2 [160]. However, the *in vivo* importance of these hormone in regulating thrombopoiesis in largely undetermined.

Alternatively spliced cytokine receptor variants have emerged as important regulators of cytokine signaling [36-38]. Recently, we have characterized the alternate mpl isoform mpl-tr as a dominant-negative regulator of TPO dependent proliferation and survival signaling. To test for the importance of alternate mpl isoform *in vivo*, we generated transgenic mice, which express only the full-length mpl isoform. Interestingly, these mice show an expansion of the number of megakaryocytic progenitors and have five times elevated platelet counts. We have found that the reintroduction of the endogenous mpl allele restored platelet counts to normal, demonstrating that the mpl allele contains a dominant-negative regulator of thrombopoiesis.

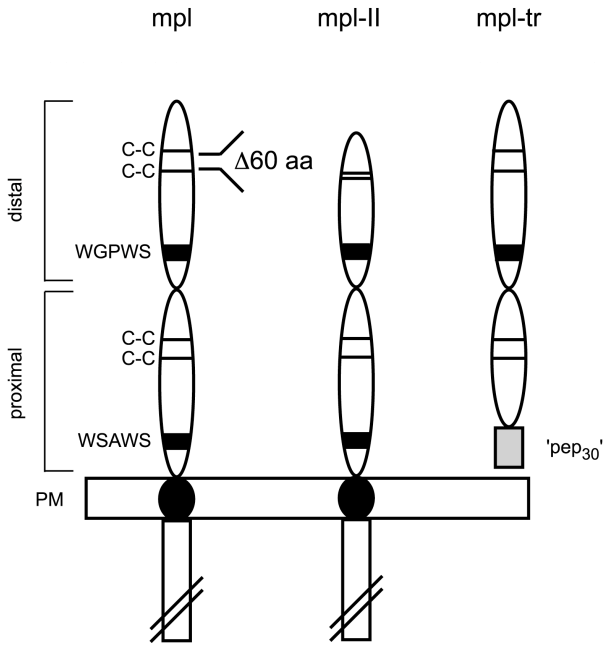

Fig. 8 The three known mouse mpl isoforms are shown in a schematic drawing. The full-length mpl receptor consists of two cytokine receptor homology modules (CRH) in the extracellular portion, a transmembrane domain and an intracellular signaling domain. An alternative splice acceptor site in exon 4 generates the isoform mpl-II with an in-frame deletion of 60 amino acids between the two conserved cysteine pairs in the distal CRH. The deleted region is probably important in ligand binding. The third isoform, mpl-tr, is due to alternative splicing of exon 8 directly to exon 11, eliminating the juxtamembrane WSXWS motif, the transmembrane domain, and the intracellular domain. Due to a frame-shift at the splicing site, the C-terminus of mpl-tr consists of a peptide of unique sequence, 30 amino acids in length. This peptide is required for the anti-proliferative response mediated by mpl-tr.

Two different mpl knockout alleles exist. Alexander et al. generated a genuine mpl knockout allele, removing a 2.6 kb fragment was removed bearing exons 1 to 5 and the core mpl promoter [83]. Expression of the mpl TG in mice homozygous for this mpl null allele causes severe thrombocythosis (Kaushansky, personel communication), in agreement with our observation. The knockout allele used in this study has a *neo*-cassette inserted in mpl exon 3 [82]. It is unlikely that the chromatin organisation in the vicinity of the mpl gene is drastically altered by either the *neo*-cassette insertion or by the deletion of five mpl exons. However, both knockout alleles lack components that restrict the expansion of the megakaryocytic lineage. Therefore, the inhibitory regulator of thrombopoiesis must be expressed by the endogenous mpl locus. We propose that dominant-negative mpl isoforms constitute this inhibitory effect on megakaryopoiesis. In this study, we used the mpl knock-in allele Δ60, which does not give rise to the mpl-tr isoform (Fig. 7A). We show that the Δ60 allele, like the endogenous mpl allele, contains an element inhibiting megakaryocytic proliferation. This result suggests that an additional dominant-negative mpl isoform other than mpl-tr must exist. An alternative splice

acceptor site in exon 4 generates the isoform mpl-II, which is expressed by both the endogenous mpl and the Δ60 knock-in allele (Fig. 7A). The mpl-II isoform has an in-frame deletion of 60 amino acids between the two conserved cysteine pairs in the distal cytokine receptor homology domain (CRH) of mpl (Fig. 8) [80]. This region constitutes the dimer interface homology region [161]. It has been shown that the distal CRH is required for TPO binding but not for receptor dimerization and signaling [162]. The importance of the deleted portion of mpl in receptor activation is supported by the finding that the introduction of cytosine residues in this region renders mpl constitutively active [161]. We propose that this recently described isoform acts as a dominant-negative by binding to full-length mpl, thus forming a heterodimer, which fails to bind ligand. This concept will require experimental proof in the future.

Interestingly, the increased platelet levels in the thrombocythemic mice are accompanied by decreased mpl protein expression on platelets. It has been hypothesized that TPO levels in circulation are mainly controlled by platelet [84,163-165] mass through c-mpl mediated uptake and metabolism. According to this model the highly elevated platelet levels would lead to TPO plasma levels below the detection limit of the ELISA. However, TPO levels in the thrombocythemic mice were normal. It is therefore not only the platelet and megakaryocyte mass but also the c-mpl expression level on platelets that regulates the TPO serum concentration. It is to date not clear, why the transgenic protein is less stable in platelets, especially because expression levels in megakaryocytes are normal. The combination of a thrombocytosis accompanied with normal TPO levels in circulation and reduced c-mpl expression on platelets has been observed in 17 out of 17 patients with ET [153] and less frequently in sporadic cases of polycythemia vera and chronic idiopathic myelofibrosis [1]. With our mpl knockout mice expressing mpl-TG we have an animal model at hand that allows us to study the basis of the decreased c-mpl expression on platelets and which may also allow the testing of future ET therapies.

RESULTS II:

Cloning of a GATA-1 regulated gene encoding a novel SIAH-interacting protein

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Running title: Identification of a novel GATA-1 regulated gene

Key words: GATA-1, SIAH, GASIP, thrombopoietin, platelets

Abstract

A few common hematopoietic progenitor cells produce many vastly different cell types. The diversification of cell types is controlled through the use of both lineage-restricted and more widely expressed transcriptional regulators and the combinatorial actions of these regulators specify gene expression. The differentiation of megakaryocyte precursors is dependent on the proper function of the GATA-1 transcription factor. Mice lacking GATA-1 selectively in megakaryocytes have dramatically fewer platelets but more megakaryocytes, altered platelet size and shape and prolonged bleeding times. Further, GATA-1-null megakaryocytes hyperproliferate *in vitro*, suggesting that GATA-1 is both a differentiation factor and negative regulator of megakaryocyte cell proliferation. However, GATA-1 regulated genes which are responsible for this growth inhibition are presently unknown. The cytokine thrombopoietin and its cognate receptor c-mpl are the main stimulators of megakaryocyte proliferation. In this study we have identified a novel gene, GASIP, which is dramatically downregulated in mpl-transfected hematopoietic cell lines, identifying mpl as a negative regulator of GASIP expression. The presence of juxtaposed GATA and Ets-binding *cis*-elements in the GASIP promoter are typical for a megakaryocytic gene. Indeed GASIP expression in platelets is robust and correlates with mRNA levels of GATA-1, but not GATA-2 or –3, identifying GATA-1 as a positive regulator of GASIP expression. The finding that mpl and GATA have opposite effects on both proliferation and on GASIP expression, make GASIP a candidate GATA-1 target gene involved in growth inhibition. To investigate the potential role of GASIP in growth regulation, we screened for potential protein binding partners. Interestingly, we identified the p53-inducible tumor suppressor seven in absentia homologe (SIAH) as a GASIP interacting protein. We speculate that GASIP may contribute to the anti-proliferative effect of SIAH.

Introduction

The hematopoietic system consists of eight distinct cell lineages that are derived from a common precursor in an ongoing process that involves the selective activation of gene expression programs characteristic of individual cell types. Several transcriptional regulators are involved in hematopoietic gene regulation. Phenotypes of c-Myb-, AML-1-, GATA-2- and SCL-1-deficient mice reveal a profound failure of definite hematopoiesis [166-170]. In contrast, GATA-1, PU.1, E2A, C/EBP□, C/EBP□, and C/EBP□knockouts have more selective phenotypes [171-179]. One theme to emerge from gene targeting experiments and complementary studies in cell differentiation *in vitro* is that cell-restricted expression of lineage specific genes is achieved through the use of both lineage restricted and more widely expressed transcriptional regulators. Several advances have highlighted the particular importance of the erythro-megakaryocytic transcriptional regulator of the GATA family of zinc-finger proteins, GATA-1, in megakaryocyte and platelet differentiation.
The rat platelet factor 4 and human glycoprotein IIB genes are regulated in part through isolated GATA sites or the combination of juxtaposed GATA and Ets-binding *cis*-elements [180,181]. Additionally, virtually every examined gene with elevated expression in megakaryocytes reveals functional GATA and Ets *cis*-elements [182,183]. This holds true for the c-mpl gene, which encodes the thrombopoietin receptor, the most important regulator of platelet homeostasis [74,82,155-157]. To a large extent, c-mpl expression in megakaryocytes is regulated by GATA- and Ets-family transcription factors [78,184]. Mice lacking GATA-1 selectively in megakaryocytes [185] and humans with critical GATA-1 point mutations [186,187] have defects that reveal many of the requirements for this factor in thrombopoiesis. Absent or impaired GATA-1 function is associated with thrombocytopenia and defective platelets as a result of a unique megakaryocyte differentiation arrest [185-188]. These and other findings indicate that GATA-1 must regulate a portion of the program of gene expression that regulates cell replication, drives cytoplasmic maturation in megakaryocytes, and coordinates the development of platelet organelles. Other features, including proplatelet formation and regulation of platelet size and numbers,

may either be directly under GATA-1 control or simply reflect the failure of the cells to progress beyond an early differentiation block.

The cytokine thrombopoietin and its cognate receptor c-mpl are the key regulators of the quantitative aspects of thrombopoiesis. Accumulating evidence also points to an important function of mpl in hematopietic stem cell (HSC) self-renewal and expansion [85]. It has recently been shown that TPO regulates the expression of the homeobox transcription factor Hoxb4 [189], which has been demonstrated to function in regulating the HSC pool [190-193]. The goal of our study was to identify other genes which are transcriptionally regulated by mpl. We cloned a gene that is downregulated in hematopietic cell lines when mpl is expressed. The expression of this gene, which has been named GASIP for GATA-1 regulated SIAH Interacting Protein, appears to be under the transcriptional control of GATA-1 *in vivo*. Contrarily to mpl, GATA-1 is negative regulator of megakaryocyte cell proliferation. The finding that mpl and GATA have opposite effects on both proliferation and on GASIP expression, make GASIP a candidate GATA-1 target gene involved in growth inhibition To gain insights into the potential function of GASIP protein, we performed Yeast-Two-Hybrid screens. Notably, we identified the tumor suppressor SIAH as a binding partner of GASIP protein and we speculate that GASIP may contribute to the anti-proliferative effect of SIAH. Additionally, we identified cytoskeletal proteins, the RNA binding protein hnRBP E1 and the E3 ubiquitin ligase and tumor supressor SIAH as GASIP interacting proteins. The interactions of GASIP with filamin, actinin and a kinesin suggest a potential function of GASIP in cytoskeletal rearrangements.

Materials and Methods

Cloning of murine and human GASIP cDNA

The fragment obtained by differential display was used to screen a mouse cDNA library. 5 independent clones were obtained and sequenced. A genomic clone was isolated from a bacterial artificial chromosome (BAC) library (Genome systems, St. Louis). The clone address is 212: A20. The murine GASIP sequence was used to search the cDNA clone database at the Deutsches Ressourcenzentrum für Genomforschung (www.rzpd.de) and several clones were obtained. The IMAGp998E0411679 clone was found to have an insert encoding the entire hGASIP cDNA.

Expression analyses

Blood samples were processed as described elsewhere [1]. Northern blot analysis was performed as described by standard textbooks. The probe was amplified from the sequence fragment out of the DD-RT-PCR screen using TCAGCAAAAACACACCATAC (sense) and ATTCGTTTCCAGAAGCATTG (antisense). For quantitative polymerase chain reaction (Q-PCR) RNA was extracted from cell lines by means of Trizol reagent (Life technologies, Rockville, MD). RNA was treated with RNase-free Dnase (Promega, Madison, WI) for 90 minutes at 37°C, heat-inactivated and then purified with RNAeasy (Qiagen, Germany) according to manufactures protocol. For reverse-transcriptase-polymerase chain reaction (RT-PCR), 1□g of RNA was reverse-transcribed after random hexamer priming in a 30-□L reaction mix containing 100 U of omniscript RT (Qiagen, Germany). Differential quantification was performed on an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA), as described [1]. The following primers were used for SYBR-PCR: mGASIP: GATCATGGCTCGTCTGCAAGA (sense), GACACTGATGCTGACGTGGAA (antisense); hGASIP: GACCAATGGAGATAAGCTCCGA (sense), GGCACCCGGGCTAGGTTA (antisense); human MPL: AGCCCTGAGCCCGCC (sense), TCCACTTCTTCACAGGTATCTGAGA (antisense); GATA-1: GCCCAAGAAGCGCCTGAT (sense), TGGTCGTCTGGCAGTTGGT (antisense); GATA-2: CCTCTACTACAAGCTGCACAATGTTAA (sense), CCGAGTCTGGATCCCTTCCT (antisense); GATA-3: GGCTCTACTACAAGCTTCACAATATTAACA (sense), TTGCTAGACATTTTTCGGTTTCTG (antisense). To prevent influence from genomic DNA amplification, all primers were designed across exon-intron junctions. The GASIP primers were designed to detect both of two putative splice variants. The □C_T values were derived by subtracting the threshold cycle (C_T) values for GASIP, MPL and GATA-1 to -3 from the C_T value for mouse or human ribosomal protein L19 (RPL19), which serves as an internal control [1,108]. All reactions were run in duplicates.

Sequence analyses

Primers specific for the mGASIP cDNA were used to identify the location of introns. Using long range PCR, the sizes of the introns were determined except for intron 2. The size of intron 2 was determined by sequencing portions of the mGASIP genomic BAC clone and constructing a contig. For the alignment of the sequences “Sequencer" software (Gene Codes Inc., Ann Arbor, MI) was used.. Exons of hGASIP were identified by a Blast [194] search with the human GASIP cDNA sequence in the human genome database at the NCBI (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html). Intron sizes given by the AceView analysis (http://www.ncbi.nlm.nih.gov/AceView/) were compared with the numbers obtained by a Blast search of Celera's assembled sequence of the human genome (http://public.celera.com). Sequences of the putative promoter regions of GASIP were obtained by sequencing of the mGASIP genomic BAC clone and by identifying the region upstream of the first hGASIP exon in the human genome database. Potential binding site of transcriptional regulator were identified using the softwares TFSEARCH [195] and TESS (http://www.cbil.upenn.edu/tess).

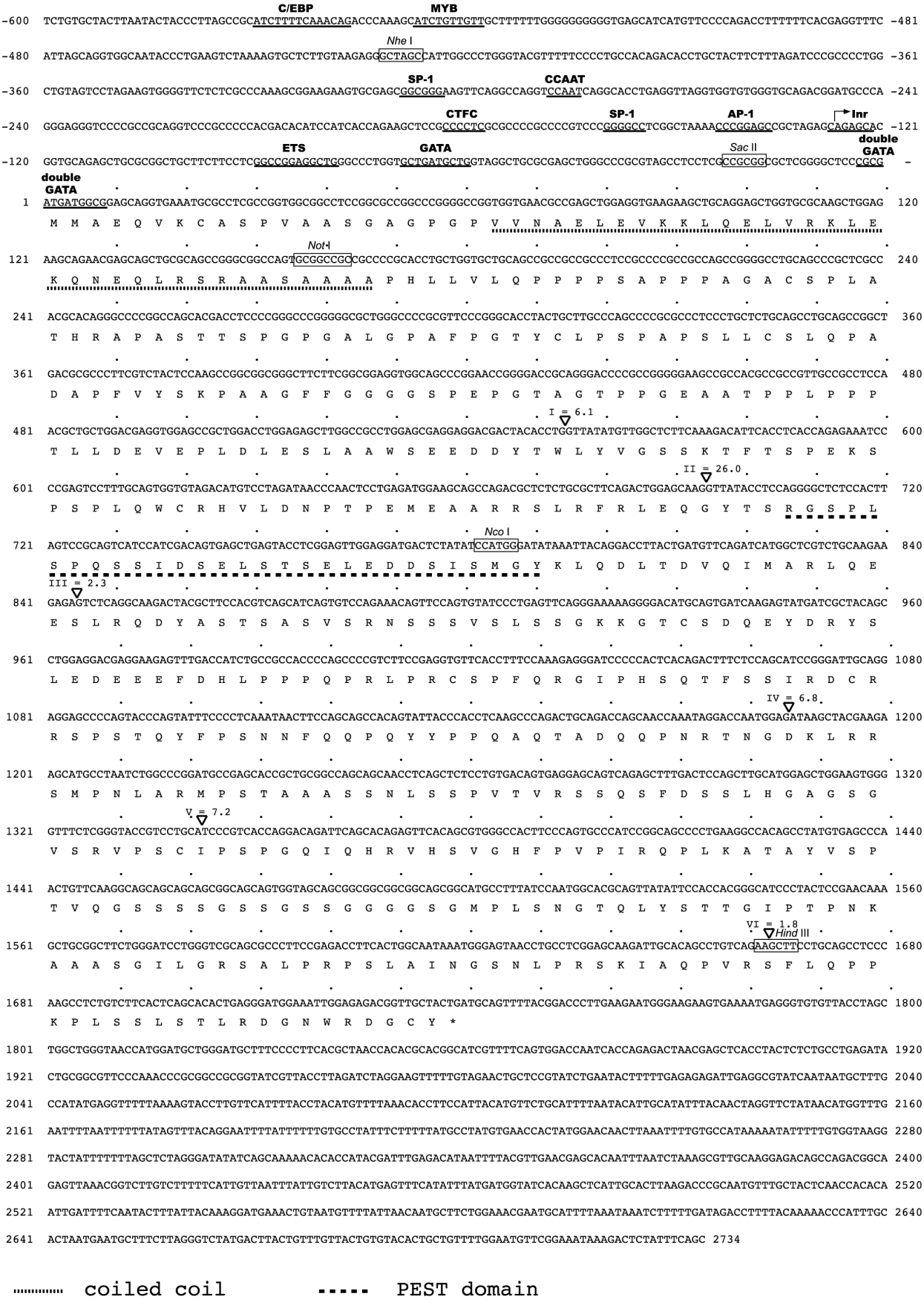
Analyses of GASIP protein interactions

A human bone marrow cDNA library was screened with the indicated portions of GASIP as a bait using the Matchmaker system III (Clontech), following the manufacturer's instructions. Transformants were obtained under high stringency conditions using growth media lacking Ade/His/Leu/Trp. The specificity of the interaction was confirmed using a □-galactosidase-DNA binding domain hybrid (G4-DB-hybrid) of the laminin protein as bait and two □-galactosidase activation domain hybrids as prey for negative controls. Selected clones were individually streaked out onto new plates and prey plasmids were isolated, amplified in *E. coli* and retransformed into yeast strains. The original interaction was reconfirmed by natural mating, followed by growth on selective media as well as screening for □-galactosidase activity, as described in the manufacturer’s manual. HA-tagged GASIP and myc-tagged SIAH-2 were [196] methionine labeled by transcription and translation using the TNT kit (Promega Corp., Madison, WI). After synthesis, the lysate was diluted with 87.5 µl incubation buffer (10% glycerol, 100 mM KCl, 20 µM Tris/HCl, pH 7.9, 0.5% NP40, and 1% BSA without EDTA and dithiothreitol). The reactions were incubated with anti-myc, anti-HA antibodies or no antibodies, respectively, at 4°C O/N and then 20□l of protein A/G agarose beads (Promega Corp., Madison, WI) were added. After one hour of incubation, the beads were washed three times with the incubation buffer. SDS-PAGE lysate buffer was added and the reaction was bioled for minutes. The protein complexes were analyzed by electrophoresis through a 10% polyacrylamide gel containing. Gels were dried and exposed to film.

Results

Cloning of mouse GASIP (GATA-1 regulated SIAH Interacting Protein). To identify mpl-regulated genes, we generated stable transfections of the murine IL-3-dependent BaF3 cell line with cDNAs encoding murine c-mpl or, as a control, human IL-4R. Cell clones were cultured in the presence of TPO or either IL-4 or IL-3, and mRNA expression of cells grown in the presence of these different cytokines was compared by the differential display method. The screen yielded four differentially regulated sequence fragments after confirmation by Northern blot analysis (data not shown). One of the four sequences corresponds to the mast cell carboxypeptidase A gene, which has previously been shown to be differentially regulated by individual cytokines [196,197]. Of the three remaining sequences, two are regulated by IL-4R signals and only one is specifically regulated by c-mpl. The sequence of this fragment shares 100% homology with the cDNA clone MGC:32254 (accession number BC025223). Next, by screening a mouse cDNA library, cDNA clones were obtained. The gene product encoded by these cDNA clones will be referred to as GASIP (GATA-1 regulated SIAH Interacting Protein) from now on. A genomic clone was isolated from a BAC library. Sequence analysis of the murine genomic GASIP clone identified seven exons spanning ~ 54 kb of mouse genomic DNA, the size of the individual introns and the genomic sequence surrounding the transcriptional start site (Fig. 1). The mouse GASIP (mGASIP) cDNA is identical the transcript represented by the TIGR consensus sequence TC255155, which is localized to the piebald deletion region of mouse chromosome 14 [198].

Downregulation of GASIP in mpl transfected hematopoietic cell lines. Next, we confirmed the c-mpl mediated downregulation of GASIP by Q-PCR. The expression of GASIP is reduced by more than 10 ΔC_T values, which is equivalent to a 2^{10} fold reduction in GASIP mRNA expression, in BaF3 cells expressing either murine or human TPO-R (Fig. 2A). Interestingly, five c-mpl expressing BaF3 clones downregulated GASIP in the absence of TPO and three cell clones


C/EBP
MYB
Nhe I
SP-1
CCAAT
CTFC
SP-1
AP-1
Inr
ETS
GATA
Sac II
double GATA
double GATA
Not I
I = 6.1
II = 26.0
Nco I
III = 2.3
IV = 6.8
V = 7.2
VI = 1.8
Hind III
coiled coil
PEST domain

required TPO for a transient reduction in GASIP mRNA (data not shown). To examine whether downregulation of GASIP mRNA following ectopic expression of a cytokine receptor is specific to mpl, we assessed GASIP expression in a number of clones transfected with cDNAs encoding mGCSF-R, mIL-4R, mOB-R or mEPO-R, respectively. Downregulation of GASIP was not found in any of these cell lines (Fig. 2A and data not shown). Interestingly, a chimeric receptor consisting of the intracellular portion of mpl and the extracellular domain of GCSF-R triggered a reduction in GASIP expression. This is in contrast to a second chimeric receptor with the intracellular domain of GCSF-R and the extracellular portion of mpl, which did not affect GASIP expression (Fig. 2A), suggesting that the downregulation of GASIP is dependent on the intracellular domain of mpl. We were further able to observe ligand independent downregulation of GASIP mRNA following ectopic expression of TPO-R in FDCP-1 cells, a second IL-3 dependent cell line (Fig. 2B), thus excluding mpl-dependent GASIP regulation as a BaF3 specific phenomenon. In the human cell line UT7/TPO, which expresses endogenous mpl and which is responsive to TPO, stimulation with TPO triggers only a moderate and transient reduction in GASIP mRNA expression by two to three fold (data not shown).

Sequence analysis of GASIP protein. We decided to study GASIP in more detail for two reasons. First, the reduction in GASIP expression in the presence of mpl might indicate that GASIP is inhibitory to the proliferation or differentiation of megakaryocytes, and secondly, the unusual mode of regulation suggests an alternative ligand-independent mechanism of mpl function. The cDNA of

Fig. 1. (opposite page) Composite genomic and cDNA sequence for mouse GASIP. The sequence shown is composed of genomic sequence (position – 600 to +554) and of cDNA sequence (position –128 to +2734; the polyA-tail is not shown). The sequence of the overlapping region from –128 to +554 is identical. Potential binding sites for transcription factors surrounding the transcription initiator site (Inr) are indicated. Important restriction sites are included in this diagram. The positions of introns are indicated by open triangles and the intron sizes in kb are stated above the triangles. The amino acid sequence for the identified longest ORF is shown. Indicated are amino acid sequence stretches for putative coiled coil and PEST domains.

mGASIP is 2862 nucleotides long (Fig. 1), which is in the same size range as the 2.9 kb mRNA detected by Northern blot analysis (data not shown). The longest ORF encodes a protein of 579 amino acids with a calculated molecular weight of 61 kDa. It is characterized by a high proportion of serine and threonine residues (20%) and proline residues (12%). The N-terminus contains a stretch of 30 amino acids, which are predicted to form a coiled coil. The central region of the protein has a predicted PEST domain (Fig. 1). However, in spite of three proline rich clusters, GASIP shows no particular domain or sequence motif or homology to any characterized protein.

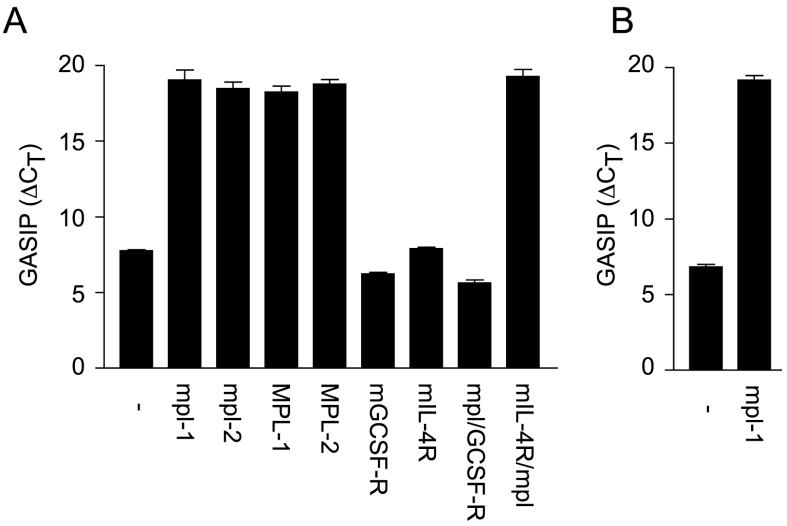
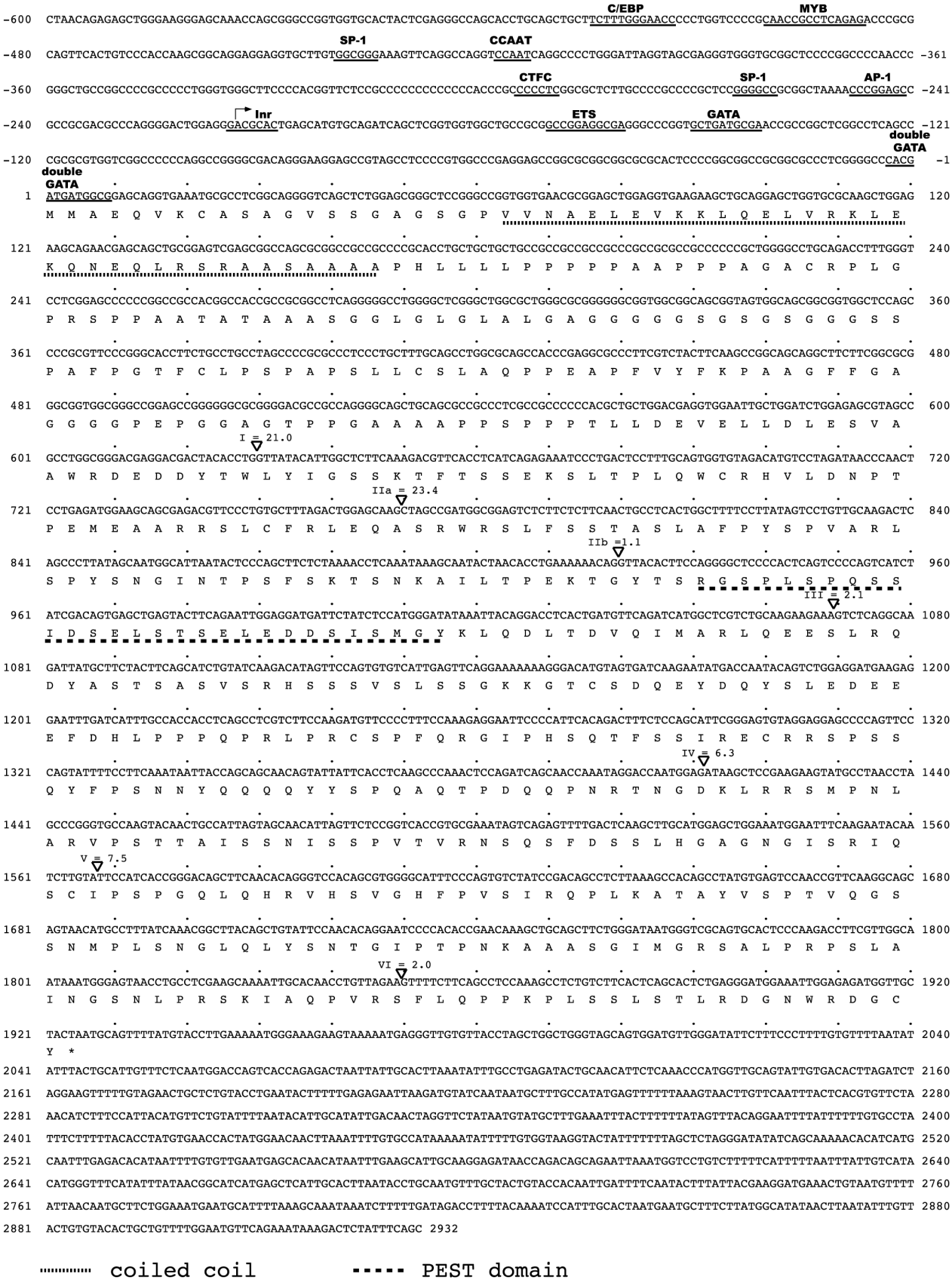


Fig. 2. Expression of mGASIP in BaF3 and in FDCP-1 cells transfected with the indicated cDNAs. (A) BaF3 cell clones transfected with mouse (mpl-1 and –2) and human (Mpl-1 and –2) TPO-R and control constructs are shown. Expression levels of GASIP are in given as ΔC_T values. High ΔC_T values indicate low expression. For instance, the GASIP (ΔC_T) value in parental BaF3 cells is about 8, but in mpl-transfected BaF3 cells the GASIP (ΔC_T) value goes up to 18. This equals a decrase in GASIP expression by 2^{10} fold in mpl expressing cells. **(B)** Shown are the GASIP (ΔC_T) values in parental FDCP-1 cells and in mpl transfected FDCP-1 cells.

Human and mouse GASIP are highly conserved. The human ortholog was cloned as described in Materials and Methods. The human GASIP (hGASIP) cDNA is 3146 nucleotides long (Fig. 3) and its longest ORF encodes a protein, which is 641 amino acids in length. Human and murine GASIP are highly


C/EBP
MYB
SP-1
CCAAT
CTFC
SP-1
AP-1
Inr
ETS
GATA
double GATA
double GATA
I = 21.0
IIa = 23.4
IIb =1.1
III = 2.1
IV = 6.3
V = 7.5
VI = 2.0
coiled coil
PEST domain

conserved with over 80% identity on protein level (data not shown). Genomic sequence of hGASIP, which encompasses ~ 65 kb and maps to chromosome 13q22.3, was obtained by searching the public database of the human genome.

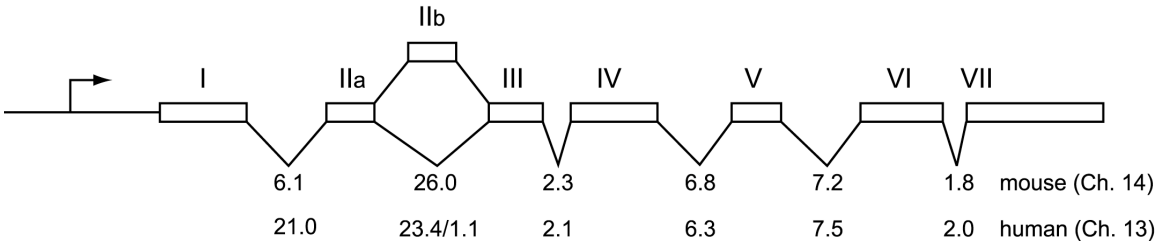


Fig. 4. Intron/ exon structure of mouse and human GASIP. The additional exon, found in the cDNA of human GASIP is termed 2b. The GASIP loci map to the piebald deletion region of mouse chromosome 14 and the orthologous region of human chromosome 13. The length of mouse and human GASIP introns in kb is shown.

The intron/ exon structure of hGASIP is very similar to its murine ortholog (Fig. 3) with the exception of one additional exon which we termed exon 2b (Fig. 4). This is probably due to alternative splicing, because the exon 2b is also found in the murine genomic GASIP locus and a mouse EST clone (GenBank Acc: BE656090) containing this sequence exists. Searches in the human and mouse EST databases did not identify any GASIP cDNAs extending at the 5' end of the sequence shown here, suggesting that we have obtained the complete human and mouse GASIP cDNAs.

GASIP and GATA-1 expressions correlate. Next, we investigated the effect of human MPL on GASIP expression *in vivo*. We determined GASIP expression in platelets obtained from healthy human individuals. Here, expression levels of GASIP mRNA correlated positively with the amount of MPL mRNA: individuals

Fig. 3 (Opposite page). Composite genomic and cDNA sequence for human GASIP. The sequence shown is composed of genomic sequence (position – 600 to +629) and of cDNA sequence (position –207 to +2932; the polyA-tail is not shown). The sequence of the overlapping region from –207 to +629 is identical. Potential binding sites for transcription factors surrounding the transcription initiator site (Inr) are indicated. Important restriction sites are included in this diagram. The positions of introns are indicated by open triangles and the intron sizes in kb are stated above the triangles. The amino acid sequence for the identified longest ORF is shown. Indicated are amino acid sequence stretches for putative coiled coil and PEST domains.

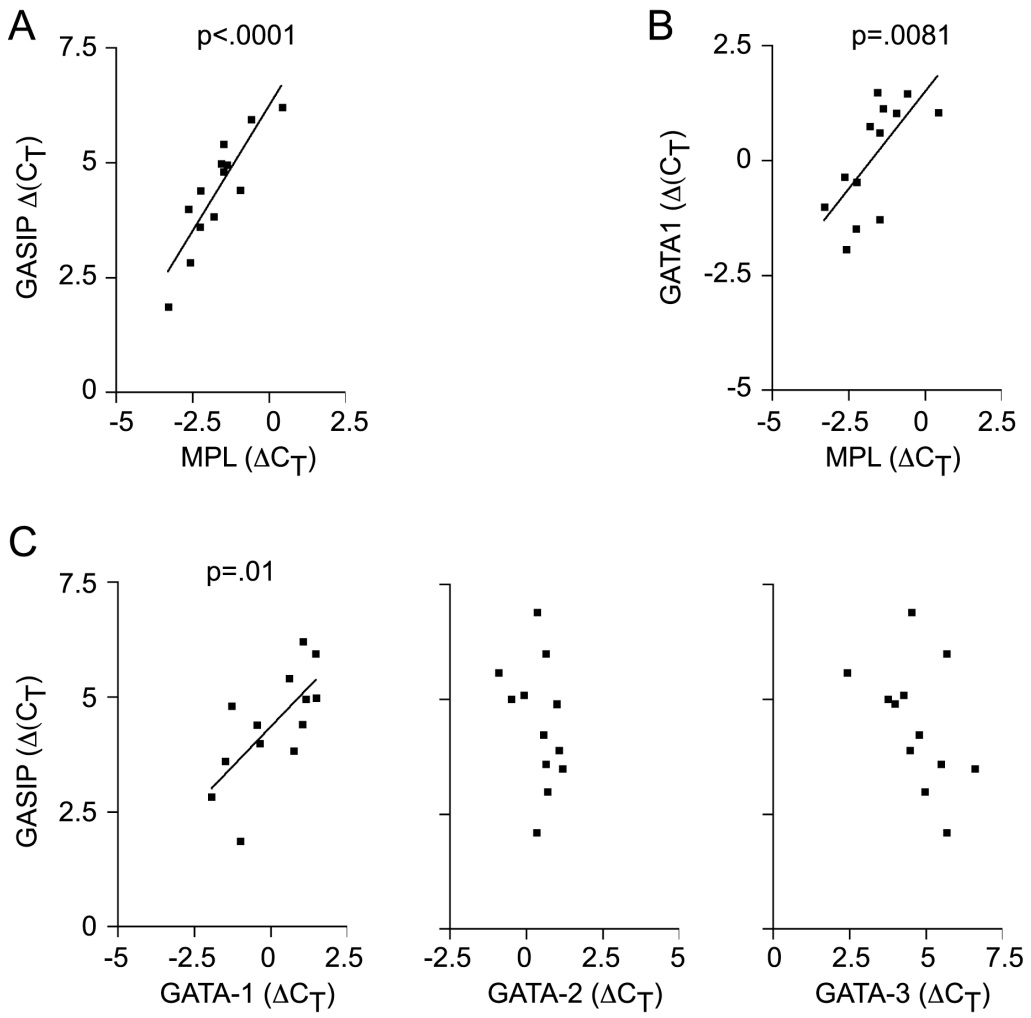


Fig. 5 Correlation of GASIP and GATA-1 mRNA expression. (A) Expression of hGASIP and MPL mRNA positively correlate in platelets. Expression levels are shown as ΔC_T values. High ΔC_T values indicate low expression. Each dot represents the values of one healthy human individual. **(B)** Mpl expression is dependent on the transcription factor GATA-1, which explains why GATA-1 and MPL mRNA levels positively correlate in platelets. Expression levels are shown as ΔC_T values and each dot represents the values of one healthy human individual. **(C)** Similar to MPL mRNA expression, the GASIP mRNA levels are higher the more GATA-1 is expressed, suggesting transcriptional control by GATA-1. In contrast, GATA-2 and GATA-3 expression levels show no significant correlation with the amount of GASIP mRNA.

with high expression of MPL (low ΔC_T value) also showed high expression of GASIP and individuals with low expression of MPL (high ΔC_T value) had accordingly less GASIP mRNA (Fig. 5A). This result appeared to conflict with our *in vitro* data. To clarify whether TPO-R protein expression has a positive effect on GASIP expression in the megakaryocytic lineage *in vivo*, we looked at GASIP and MPL expression in patients who suffer from sporadic

myeloproliferative disease (MPD). We chose MPD patients, who have drastically diminished MPL protein expression but normal MPL mRNA expression in platelets [1]. In these patients GASIP mRNA expression still positively correlates with MPL mRNA levels (data not shown), suggesting that MPL protein expression and thus signaling through TPO-R is not responsible for the observed correlated expression of MPL and GASIP. An alternative explanation for the observed linear relationship between GASIP and MPL expression would suggest that GASIP could positively regulate MPL transcription. However, overexpression of GASIP in UT7/TPO cells had no effect on MPL expression (data not shown). We thus speculated that TPO-R and GASIP are transcriptionally regulated in a similar fashion. Alignment of the sequences surrounding the transcriptional start sites of both murine and human GASIP allowed us to identify conserved putative binding motifs for transcription factors. This analysis revealed a conserved CAAT box and potential binding motifs for members of the GATA, Ets, and C/EBP families of transcription factors amongst others (Fig. 2 and 3). Particularly, the combination of juxtaposed GATA and Ets-binding *cis* elements found in the GASIP promoter is characteristic of megakaryocytic genes like mpl. Indeed, the expression of MPL mRNA correlates with the expression levels of GATA-1 in platelets of healthy individuals (Fig. 5B). Similarly, the mRNA expression of GASIP correlates with the expression level of GATA-1, but not GATA-2 or –3 (Fig. 5C), suggesting GATA-1 to be a regulator of GASIP transcription. To compare the expression of GASIP in different hematopoietic cell lineages, the blood from healthy donors was fractionated into platelets, adherent and non-adherent mononuclear cells and granulocytes. The lowest expression of GASIP is found in macrophages and lymphoid cells, which make up most of the non-adherent mononuclear cells (Fig. 6A). In platelets the average GASIP (ΔC_T) value is ~ 4 compared to ~7.5 in the lymphiod compartment and ~8.5 in macrophages (Fig. 6A), which means that the expression of GASIP is about 10 - 20 times higher in platelets than in mononuclear cells. In granulocytes the average GASIP (ΔC_T) value is ~ 2, which translates into a GASIP expression of about 50 to 100 times that found in mononuclear cells (Fig. 6A).

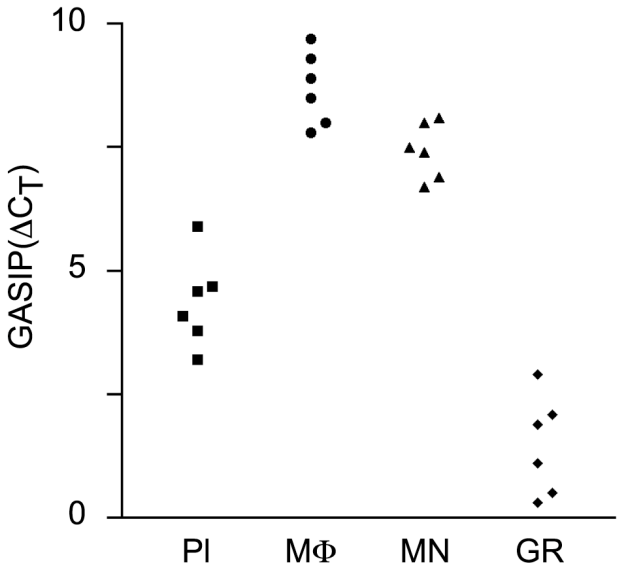


Fig. 6 GASIP expression in blood lineages. The blood from six healthy individuals was separated into granulocyets (GR), mononuclear cells (MN) and platelets (Pl), as described [1]. Macrophages (M□) were isolated form the mononuclear cell fraction by allowing them to adhere to tissue culture dishes for two hours. RNA from the different cell fraction was isolated and Q-PCR was performed. Each dot represents the □C_T value of the indicated cell type from one healthy human individual.

GASIP interacts with seven in absentia homolog (SIAH). Finally, to gain a better understanding of potential biological functions of GASIP, we screened a human bone marrow expression library for protein-protein interaction with GASIP in a Yeast-Two-Hybrid screen. One GASIP 'bait' construct for the N-terminus and two 'bait' constructs for the C-terminus were used (Fig. 7). To avoid false positives, the screen was performed with two independent selection markers. Additionally, clones were screened for lacZ expression as a third maker. The obtained 'prey' plasmids were isolated out of the yeast strain, amplified in *E. coli*, and sequenced. The purified plasmids were then re-transformed into yeast and tested for unspecific activation of a G4-DB-laminin control 'bait' construct and interaction with different GASIP constructs (Table 1). This approach allowed the identification of true positives and a summary of the results of the Two-Hybrid screens is shown in Fig. 7 and Table 1. We identified three different cytoskeleton-associated proteins, filamin, actinin alpha, and the kinesin KIF4, the RNA binding proteins hnRNP E1 and the E3 ubiquitin ligase SIAH-1 and -2. This is in agreement with the finding that SIAH interacting proteins are able bind to both family members due to the high degree of homology between SIAH-1 and –2. To further verify the interaction of GASIP with SIAH, tagged versions of both proteins were transcribed and translated *in vitro*. Immunoprecipitation of GASIP precipitates SIAH and GASIP is co-immunoprecipitated with SIAH (Fig. 8), confirming GASIP as a SIAH interacting protein.

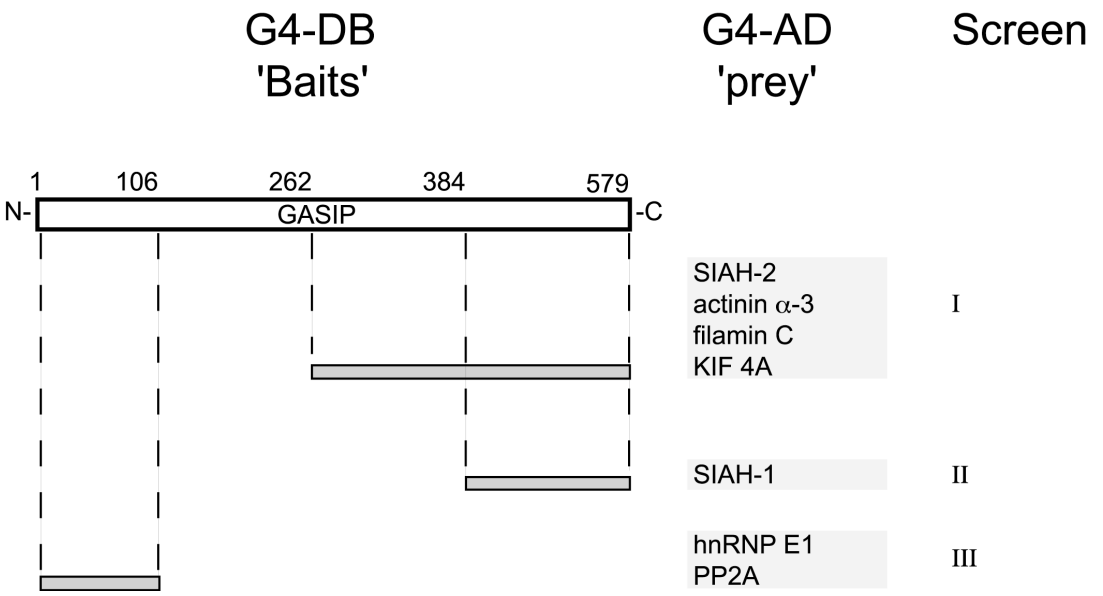


Fig. 7 Summary of the results obtained from the Yeast-Two-Hybrid screen. Three 'bait' constructs were used consisting of a GAL4-DNA-binding domain (G4-DB) fused to the indicated portions of GASIP protein. These constructs were screened against a human bone marrow cDNA library expressed as GAL4-activation domain (G4-AD) fusion proteins. When a protein-protein interaction occurs, the G4-AD activates expression of reporter genes. In screens with the three different 'baits' proteins were identified as GASIP interacting proteins as listed.

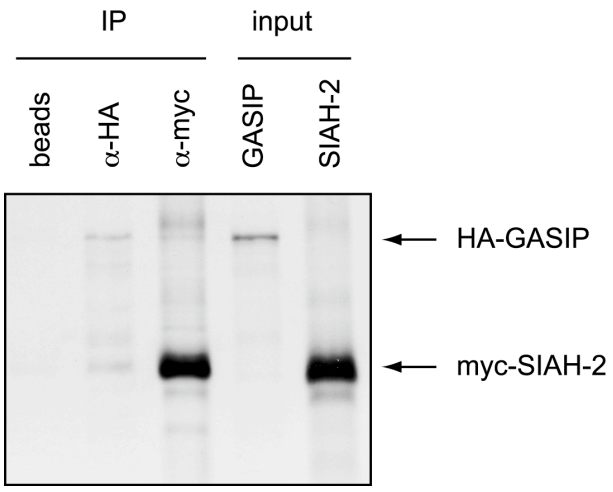


Fig. 8 *In vitro* translated radioactively labelled HA-tagged GASIP and myc-tagged SIAH-2 fusion proteins were tested for protein interaction. The two fusion proteins were mixed in binding buffer and immunoprecipitated (IP) with either anti-myc or anti-HA antibodies. Protein A/G agarose beads alone (beads) did not result in any protein precipitation. HA-GASIP and myc-SIAH-2 co-immunoprecipitate in the presence of either anti-HA or anti-myc antibody. The amounts of *in vitro*-translated proteins added to the binding reaction are shown in the lanes marked 'input.'

G4-DB-hybrid **'bait'**	**G4-AD-hybrid 'prey'**	**Growth on LWHA-plates**	**-Gal staining**
laminin	SIAH-2 (146-324)	-	-
GASIP (262-579)	SIAH-2 (146-324)	+	+++
laminin	SIAH-2 (1-324)	-	-
GASIP (211-579)	SIAH-2 (1-324)	+	+++
GASIP (211-346)	SIAH-2 (1-324)	-	-
GASIP (262-579)	SIAH-2 (1-324)	+	+++
GASIP (262-386)	SIAH-2 (1-324)	-	-
GASIP (262-346)	SIAH-2 (1-324)	-	-
GASIP (348-579)	SIAH-2 (1-324)	+	+++
GASIP (384-579)	SIAH-2 (1-324)	+	+++
laminin	SIAH-1 (1-313)	-	-
GASIP (262-579)	SIAH-1 (1-313)	+	+++
GASIP (211-579)	SIAH-1 (1-313)	+	+++
GASIP (262-386)	SIAH-1 (1-313)	-	-
GASIP (384-579)	SIAH-1 (1-313)	+	+++
SIAH-2 (1-324)	GAL4	-	-
laminin	GASIP (1-579)	-	-
SIAH-2 (1-324)	GASIP (1-579)	+	++
laminin	actinin -3 (1-901)	-	-
c13 (262-579)	actinin -3 (1-901)	+	++
c13 (211-579)	actinin -3 (1-901)	+	+++
c13 (262-386)	actinin -3 (1-901)	-	-
c13 (262-346)	actinin -3 (1-901)	-	-
actinin -3 (1-901)	GAL4	+	+++
laminin	filamin (2174-2692)	-	-
GASIP (262-579)	filamin (2174-2692)	+	++
GASIP (211-579)	filamin (2174-2692)	+	++
GASIP (262-386)	filamin (2174-2692)	-	-
GASIP (262-346)	filamin (2174-2692)	-	-
GASIP (348-579)	filamin (2174-2692)	+	++
laminin	KIF 4 (776-1232)	-	-
GASIP (262-579)	KIF 4 (776-1232)	+	++
GASIP (211-579)	KIF 4 (776-1232)	+	++
GASIP (211-346)	KIF 4 (776-1232)	-	-
GASIP (262-386)	KIF 4 (776-1232)	-	-
GASIP (262-386)	KIF 4 (776-1232)	-	-
GASIP (262-346)	KIF 4 (776-1232)	-	-
GASIP (348-579)	KIF 4 (776-1232)	-	-
laminin	hnRNP E1 (21-356)	-	-
GASIP (1-106)	hnRNP E1 (21-356)	+	+++
laminin	PP2A (1-590)	-	-
GASIP (1-106)	PP2A (1-590)	+	++

Table 1. (Opposite page) A human bone marrow cDNA library was screened for GASIP protein interactions. Selected clones were individually streaked out onto new plates and prey plasmids were isolated, amplified in *E. coli* and retransformed into yeast strains. The original interaction was reconfirmed by natural mating, followed by growth on selective media lacking Ade/His/Leu/Trp (LWHA) as well as screening for □-galactosidase (□-Gal) activity, as described in the manufacturer's manual. Growth on selective plates is indicated (+). Very high (+++), high (++) or no (-) □-Gal activity was determined. The specificity of the interaction was confirmed using a □-galactosidase-DNA binding domain hybrid (G4-DB-hybrid) of the laminin protein (laminin) as bait and □-galactosidase activation domain hybrids (GAL4) as prey for negative controls. Additional relevant mating tests are listed. The numbers in brackets indicate the stretch of amino acid sequence that is encompassed by the specific construct. The SIAH-1-, one of the SIAH-2- and the actinin-constructs encode full-length proteins.

Discussion

Mechanisms of thrombopoiesis are of considerable interest in hematology and cell biology, in part because of the variety of human thrombocytopenia syndromes and because megakaryocyte differentiation encompasses many unusual attributes like polyploidy, a unique set of organelles and the regulated fragmentation of the cytoplasm to generate platelets.

Genetic studies have recently provided rich insights into the molecular and transcriptional regulation of megakaryocyte differentiation and thrombopoiesis. Three transcription factors, GATA-1, FOG-1, and NF-E2, are essential regulators of distinct stages in megakaryocyte differentiation, extending from the birth of early committed progenitors to the final step of platelet release.

Much of the attention in transcriptional regulation of megakaryocyte genes has focused on the GATA family of zinc-finger proteins, which activate transcription by engaging the DNA consensus sequence (A/T) GATA (A/G) in the *cis*-regulatory elements of many lineage-restricted genes. Mice lacking GATA-1 selectively in megakaryocytes [185] and critical GATA-1 point mutations in human disease [186,187] cause defects that reveal at least some of the requirements for this transcription factor in thrombopoiesis. First, platelet counts are about 15% of the normal, platelet size is increased at least twofold, platelet shape is spherical rather than discoid, and the bleeding time is prolonged [185-188]. Second, GATA-1-null megakaryocytes proliferate excessively *in vitro*, and their substantial accumulation *in vivo* appears to reflect primary growth dysregulation rather than a secondary response to thrombocytopenia [185,188]. This finding suggests that GATA-1 is a negative regulator of cell proliferation in early megakaryocyte progenitors.

In this study we have identified a novel gene, GASIP, which is downregulated in hematopoietic cell lines, which express the c-mpl. However, the expression level of GASIP positively correlates with the expression level of MPL in platelets of human individuals. The phenomenon can be explained by the presence of juxtaposed GATA and Ets-transcription factor binding sites in both the mpl and the GASIP promoters, suggesting that the two genes are

transcriptionally regulated in a similar fashion in megakaryocytes. It is known that GATA-1 binds to the mpl promoter and, in concert with FOG-1, activates mpl transcription. Indeed, the expression levels of MPL positively correlates with the expression of GATA-1 in platelets (Fig. 5) and the same is true for GASIP expression (Fig. 5), indicating that GASIP is regulated by GATA-1. Importantly, TPO-R stimulates the proliferation of megakaryocytic precursors, but appears to be dispensable for most aspects of megakaryocytic differentiation. In contrast, as described above, GATA-1 is a negative regulator for the proliferation of early megakaryocytic precursors, though GATA-1 regulated genes, which are responsible for this growth inhibition, are presently unknown. Possibly, GASIP is one of these genes, explaining why mpl transfected cell lines reduce GASIP expression. Using purified megakaryocytes, future work will clarify whether the mpl-dependent GASIP downregulation is TPO-dependent *in vivo.* We expect TPO to be required to transiently downregulate GASIP in megakaryocytes and foresee that this downregulation contributes to the proliferative signal that is mediated by TPO. The mRNA amounts in platelets are probably representative of megakaryocytic steady state mRNA levels. We think that the steady state expression of both GASIP and mpl mRNA is controlled by GATA-1, which would explain the positive correlation of GASIP and MPL expression in platelets.

Interestingly, we have identified the p53 regulated tumor suppressor SIAH [199-204] as a binding partner of GASIP protein. The biological significance of this interaction awaits clarification. Interestingly, the pool of myeloid progenitor cells is expanded in the recently described SIAH-2 knockout mouse [205]. Additional effects of GATA-1 malfunction include a vast increase in numbers of tissue megakaryocytes revealing an abnormally small and immature cytoplasm that harbors few platelet granules amid highly disorganized internal membranes [185,188]. Thus, absent or impaired GATA-1 results in a unique megakaryocyte differentiation arrest. This shows that, in addition to attenuating cell replication in precursors, GATA-1 also drives cytoplasmic maturation in megakaryocytes and co-ordinates development of platelet organelles. Other features, including proplatelet formation and regulation of platelet size and numbers, may either be directly under GATA-1 control or

simply reflect failure of the cells to progress beyond an early differentiation block. Synthesis of platelet-specific organelles is mediated by a variety of regulators of intracellular vesicle membrane fusion, and platelet release is co-ordinated through extensive and dynamic reorganization of the actin and microtubule cytoskeletons. The interactions of GASIP with actinin-□3, filamin and the kinesin Kif 4A suggest a possible role of GASIP in this process. Finally, we found the highest expression of GASIP in granulocytes. Notably, forced GATA-1 expression induces the formation of eosinophil granulocytes in hematopoietic progenitors from human cord blood [206] and GATA-1 is required for the differentiation of eosinophils *in vivo* [206,207]. Yu et al. describe a double GATA binding motif as a regulatory element in the GATA-1 promoter that selectively governs expression of GATA-1 in eosinophils [207]. Interestingly, the GASIP promoter also contains a double GATA motif (Fig. 1 and Fig. 3). This may explain the high expression of GASIP in granulocytes and suggests that GASIP is an important GATA-1 target gene with a possible role in eosinophil formation.

PERSPECTIVES

Many aspects of cell behavior, such as growth, motility, differentiation, and apoptosis, are regulated by signals which cells receive from their environment. Such signals are critical during many physiological processes like embryonal development, wound healing, hematopoiesis, and in the regulation of the immune response. Extracellular stimuli such as hormones, growth factors, and cytokines bind to and activate receptors at the cell surface. However, signal transduction cascades are not simple one-way streets. They are modulated at every step. Signals conveyed from receptors are relayed, amplified, and integrated, resulting in the expression of target genes in the nucleus and subsequent biological responses, which also depend on the activity of other signaling events and/or the 'history' of a cell. In the first part of my thesis, I studied the importance of alternate cytokine receptor isoforms as regulators of signaling exemplified by the thrombopoietin receptor. In a second project, I studied the role of mpl signaling in regulating the expression of a novel gene with a potential function in cell differentiation and proliferation.

Aberrations of cytokine signaling in disease

A number of pathological conditions have been identified with mutations in cytokine receptors or associated signaling molecules. As could be expected on the basis of the knockout models, mutations leading to a complete loss of receptor function often give rise to severe disease phenotypes. Examples are deleterious mutations in the leptin receptor, causing a syndrome characterized by obesity and various forms of severe combined immunodeficiency (SCID).

An important issue to be addressed is to what extent cytokine receptor signaling contributes to tumorigenesis. Thus far, several reports link aberrations in the JAK/STAT pathways to malignant phenotypes. In mammals, JAKs and STATs are known to be constitutively activated in

hemopoietic cells transformed by diverse oncogenic tyrosine kinases [208,209] and in a variety of lymphomas and leukemias [210-212]. Expression of a constitutively active STAT3 molecule in immortalized fibroblasts causes cellular transformation [213]. Further, JAK2 and STAT5B have been identified as fusion partners in translocations in leukemias. In the Tel-JAK2 fusion protein the kinase domain of JAK2 is constitutively activated leading to constitutive activation of STAT proteins [214]. STAT5B has been found to be fused to the retinoic receptor alpha gene in patients with acute promyelocytic-like leukemia [215]. Together these data are indicative of a role for constitutive activation of the JAK-STAT pathways in leukemogenesis. It has been shown that transforming mutations can also occur in cytokine receptors. Moreover, several mutations in the EPO-R have been identified as the molecular lesion resulting in human erythroid malignancies [216,217]. Last but not least, the thrombopoietin receptor was originally identified as a homolog of the viral oncogene v-mpl [73], emphasizing the oncogenic potential cytokine receptors in general.

Cytokine receptor splice forms and hematopoietic malignancies

In my thesis work, I have charaterized the alternate mpl isoform mpl-tr as a dominant-negative regulator of TPO signaling and provided circumstantial evidence that a second alternative mpl variant, mpl-II, is an additional dominant-negative isoform. Dominant-negative cytokine receptor isoforms have also been identified for other cytokine receptors, but their importance *in vivo* is not well defined, and most spliced isoforms have not been studied at all. The importance of the mpl isoforms in regulating platelet counts suggests a major regulatory function for alternate cytokine receptor isoforms in general. Notably, many cancer-associated gene are regulated by alternative splicing [138]. Although the functions of most spliced isoforms are not well defined, some have antagonistic activities associated with cell death mechanisms. More recently, it has become apparent that carcinogenesis induces changes in the ratio of alternatively spliced isoforms that might affect cellular behavior and tumorigenesis. *Bcl-x*, a member of the *Bcl-2* family of apoptotic regulators,

produces two alternatively spliced isoforms, Bcl-xL and Bcl-xS, with anti-apoptotic and pro-apoptotic activities, respectively. The Bcl-xS protein is upregulated in cells undergoing apoptosis and is downregulated in tumor cells, whereas the Bcl-xL protein is predominantly expressed in many lymphoma cells [218]. Another example of the involvement of alternative splicing regulation in cancer is the CD44 family of surface glycoproteins, which has a role in cell adhesion and migration as well as in cell-matrix interactions. In many human malignancies, certain CD44 splice variants predominate and in the serum of gastric carcinoma patients, elevated levels of CD44v5 were found to correlate with increased tumor invasion and metastasis [219]. Differences in the levels of alternatively spliced transcripts were suggested to arise from differences in the mechanism regulating alternative splicing. The possibility that changes in the regulation of alternative splicing might lead to disease was studied in a mouse model for mammary gland tumorigenesis [220]. In this model, tumorigenesis was accompanied by changes in the relative levels of CD44 transcripts, and a profound change in the repertoire and levels of several splicing factors from the SR protein family. These results indicate that changes in splicing regulation might have a significant role as a genetic modifier of tumorigenesis. Interestingly, it has been shown that many alternate cytokine receptor splice forms are differentially expressed during cell differentiation and in cancerous cells [110,123,221-223], suggesting disruptions in proper cytokine receptor splicing as a possible cause of hematopoietic malignancies.

A mouse model of essential thrombocythemia

The mouse model analyzed in this work emphasizes the importance of splice isoforms as inhibitory regulators *in vivo*, because the deletion of the dominant-negative mpl isoforms causes thrombocytosis. The mouse phenotype resembles in many ways human essential thrombocythemia (ET). For instance, the increased platelet levels in the thrombocythemic mice are accompanied by decreased mpl protein expression on platelets and normal TPO levels. The combination of a thrombocytosis accompanied with normal TPO levels in circulation and reduced c-mpl expression on platelets has been observed in ET [153] and less frequently in sporadic cases of polycythemia vera

and chronic idiopathic myelofibrosis [1]. We have therefore an animal model at hand that allows us to study the basis of the decreased c-mpl expression on platelets and which may also allow the testing of future ET therapies.

Fig. 1 Alignment of the C-terminus of mpl-tr with a short peptide sequence, 10 amino acids in length, contained in the extracellular domain of the protein Kremen. The unique sequenc at the C-terminus of mpl-tr is due to a shift of the reading frame at the alternate splice junction of exon 8/11. The mouse mpl-tr petide is terminated after 30 and the human form after 21 amino acids. The sequence in Kremen homologous to mpl-tr is conserved amongst the species rat, mouse (m) and human (h). A consensus sequence is depicted.

Receptor isoform-mediated proteolysis as a novel regulatory mechanism

Mechanistically, dominant-negative receptor variants can form non-functional heterodimers with the full-length receptor or, when expressed as secreted ‘soluble’ isoforms, compete with the receptor for ligand binding [63]. In this work I have discovered a third mechanism, in which the mpl isoform mpl-tr mediates protein degradation of the full-length receptor by a cathepsin-like cysteine protease activity, suggesting the lysozome as the degradative compartment. Future studies, using an array of different protease inhibitors and lysomotropic agents as well as inhibitors of protein trafficking and studies on the subcellular localization of mpl in dependence on mpl-tr expression should clarify the precise mode of mpl destruction.

I have also been able to demonstrate that the unique sequence at the C-terminus of mpl-tr is essential for its function. Interestingly, the wild type form of mpl-tr fails to be secreted [74]. In contrast, truncated mpl forms consisting of the extracellular domain only, therefore lacking the unique C-terminus of mpl-

tr, are readily secreted into the cell culture supernatant [74,75]. In one possible scenario, the C-terminus of mpl-tr prevents the secretion of mpl-tr and directs the putative mpl/mpl-tr dimer to traffic instead along the endosomal-lysosomal route. Increasing acidification of the endosomal vesicle would cause the dimer to disassemble allowing mpl-tr to be recycled. This model would explain why mpl-tr expression is apparently not affected by mpl protein. Preliminary fluorescent microscopy studies show that mpl-tr is located to the ER, the site of mpl-tr synthesis, but also to other unidentified vesicular structures. Interestingly, the C-terminal mpl-tr peptide motif appears to be present in the Kremen (Krm) protein (Fig. 1). Kremen functions in a sophisticated mechanism of Wnt signal inhibition, whereby an activating coreceptor, LDL-receptor-related protein 6 (LRP6), is segregated and removed by internalization[229]. LRP6 clearance was observed as a consequence of ternary complex formation in Krm- and LRP6-transfected cells provided with Dickkopf (Dkk) protein. After application of Dkk1, the membrane localization of tagged versions of LRP6 and Dkk1 diminished quickly. The proteins were instead subsequently enriched in intracellular vesicles, with a slight reduction in LRP6 levels. This decrease was interpreted as degradation and it will be important to verify this hypothesis with further studies. It will be especially interesting to determine whether Krm is being recycled or whether it is also being degraded. A functional role of the Kremen peptide sequence, which aligns with the mpl-tr C-terminus, would suggest that Kremen, in analogy to mpl-tr, is recycled. Further structure function analysis of the C-terminal sequence of mpl-tr will hopefully allow us to define a minimal functional peptide motif. It will be of interest to determine if such a peptide motif is also present in other receptor isoforms or proteins other than Kremen that mediate proteolysis.

Characterization of a novel mpl- and GATA-regulated gene

In the second part of my thesis work, I have characterized a novel gene, which has been named GASIP for GATA-1 regulated SIAH Interacting Protein. GASIP is downregulated in hematopietic cell lines when mpl is expressed. So far it is not clear, which signaling events initiated by mpl lead to GASIP repression. Moreover, downregulation of GASIP in mpl-expressing cell

lines can occur in both a TPO-dependent and –independent fashion and studies on primary megakarycocytes should clarify the role of mpl signaling in regulating GASIP. The expression of GASIP appears to be to some extent under the transcriptional control of GATA-1 *in vivo*. In contrast to mpl, GATA-1 is a negative regulator of megakaryocyte cell proliferation [185,188]. However, target genes of GATA-1, which mediate the anti-proliferative effect, have remained elusive. The finding that mpl and GATA have opposite effects on both proliferation and on GASIP expression, make GASIP a candidate GATA-1 target gene involved in growth inhibition. In one possible model, GATA-1 constitutively activates GASIP transcription in the megakaryocytic precursors and together with other factors GASIP would exert an anti-proliferative effect and/or play a role in differentiation. Contrarily, activation of TPO-R triggers the repression of anti-proliferative genes and GASIP could be such a target gene for repression. The potential function of GASIP in the megakaryocytic lineage will be tested by generating mice which express GASIP as a transgene under the control of the mpl promoter. To assure overexpression of GASIP, the 3' UTR of GASIP, which is AU-rich and probably exerts a negative effect on mRNA stability, has been removed in the transgenic construct (Fig. 2A).
The highest expression of GASIP was found in granulocytes. Interestingly, the assembly of transcription factor binding sites in the GASIP promoter resemble the composition of the arch-typical eosinophilic promoter of the eos47 gene. The promoter of the eos 47 gene (encoding EOS47, the avian ortholog of the mammalian melanotransferrin gene) consists of binding sites for Myb-, Ets-, C/EBP-, and GATA-type transcription factors [224,225], all elements also contained in the GASIP promoter (Fig.3). Further, the double GATA site in the GASIP promoter appears to be specific for eosinophil expression [207]. An available embryonic stem cell clone containing a gene-trap insertion in intron 2 of GASIP (Fig. 2B) will allow the generation of a mouse with a GASIP gene disruption. Such a mouse would be a valuable tool in studying a putative function of GASIP in eosinophilic granulocytes.

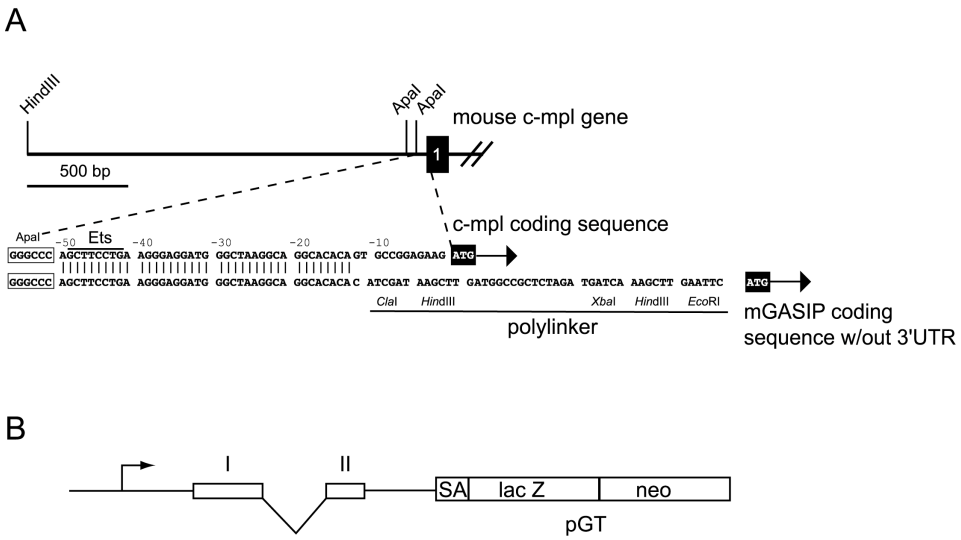


Fig. 2. Tools for the *in vivo* analysis of GASIP function. (A) The construct used for the over-expression of GASIP in the megakaryocytic lineage. A 2kb mpl promoter fragment has been cloned in front of mGASIP cDNA followed by a SV40-derived polyA signal (not shown). The AU-rich 3'UTR of GASIP, which probaly functions as a negative regulator of mRNA stability, has been removed. Several transgenic founders are available. **(B)** A gene-trap vector insertion in intron 2 of mGASIP has been identified in 3 independent mouse ES clones. These cells will allow the study of GASIP gene disruption in a mouse model. The presence of the lacZ gene in the vector insertion will be useful for GASIP expression studies.

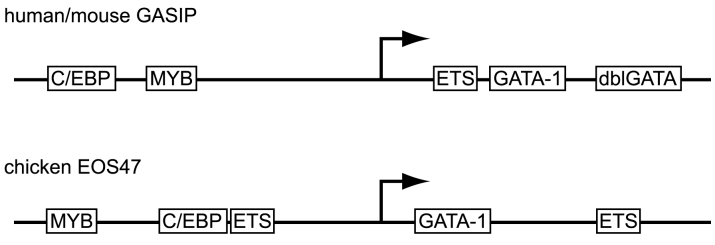


Fig. 3. Alignment of the GASIP and EOS47 promoter. The promoter of the eosinophilic EOS47 gene contains several transcription factor elements, which are also found in the GASIP promoter. Of particular importance in eosinophilic lineage commitment are members of the C/EBP family of transcription factors and GATA-1.

To gain insights into the potential function of GASIP protein, I performed Yeast-Two-Hybrid screens. Notably, the tumor suppressor SIAH was identified as a binding partner of GASIP protein and one could speculate whether GASIP could contribute to the anti-proliferative effect of SIAH. Additionally, cytoskeletal proteins and the RNA binding protein hnRNP E1 were found to be GASIP interacting proteins. The binding of GASIP to filamin, actinin and a kinesin suggests a potential function of GASIP in cytoskeletal

rearrangements with potential importance in the differentiation of both megakaryocytes and granulocytes.

Of particular interest is the interaction of GASIP with the heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1). The hnRNPs E1 and E2 proteins share over 80% homology, which makes it likely that GASIP also interacts with hnRNP E2. Both, hnRNPs E1 and E2, function as regulators of cytoplasmic mRNAs of key factors in erythroid and granulocytic differentiation. In erythroid-precursor cells, hnRNP E1 regulates translation of reticulocyte 15-lipoxygenase (LOX) mRNA specifically, through the differentiation-control element (DICE) in the 3' UTR of the LOX [226,227]. LOX is a key enzyme in erythroid-cell differentiation. It catalyses the dioxygenation of intact phospholipids in mitochondrial membranes and participates in their breakdown in the final steps of erythrocyte maturation.

By a similar mechanism, hnRNP E2 inhibits the translation of C/EBPalpha, the principal regulator of granulocytic differentiation. Importantly, the BCR-ABL oncoprotein induces expression of hnRNP E2, which blocks the production of C/EBP and causes an arrest in granulocytic differentiation [228]. Because GASIP interacts with hnRNP through its N-terminus and with the E3-ubiquitin ligase SIAH through its C-terminus, it is feasible that GASIP, hnRNP E1/2 and SIAH can form a complex. In a highly speculative model, GASIP would recruit hnRNP E1/2 for ubiquitin-mediated proteolysis and thus promote differentiation and inhibit proliferation

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Jörn Coers, Christina Ranft, Robert Kralovics, Sandra Ziegler, Radek Skoda
Symposium Swiss Society of Hematology (2003), oral presentation

Inhibitory effect of a truncated form of c-MPL on TPO dependent cell survival and proliferation
Jörn Coers, Christina Ranft, Sandra Ziegler, Radek Skoda
32nd Annual Meeting of the International Society for Experimental Hematology (2003), oral presentation

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