Orientation-specific signalling by thrombopoietin receptor dimers

Judith Staerk^{1,2,6,7}, Jean-Philippe Defour^{1,2,6}, Christian Pecquet^{1,2}, Emilie Leroy^{1,2}, Hélène Antoine-Poirel³, Ian Brett⁴, Miki Itaya⁴, Steven O Smith⁴, William Vainchenker⁵ and Stefan N Constantinescu^{1,2,*}

¹Ludwig Institute for Cancer Research, Brussels, Belgium, ²de Duve Institute, Université Catholique de Louvain, Brussels, Belgium, ³Cliniques Universitaires St-Luc, Université Catholique de Louvain, Brussels, Belgium, ⁴Center for Structural Biology, Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY, USA and ⁵INSERM, U1009, Université Paris-Sud, Institut Gustave Roussy, Villejuif Cedex, France

Ligand binding to the thrombopoietin receptor is thought to stabilize an active receptor dimer that regulates megakaryocyte differentiation and platelet formation, as well as haematopoietic stem cell renewal. By fusing a dimeric coiled coil in all seven possible orientations to the thrombopoietin receptor transmembrane (TM)–cytoplasmic domains, we show that specific biological effects and *in vivo* phenotypes are imparted by distinct dimeric orientations, which can be visualized by cysteine mutagenesis and crosslinking. Using functional assays and computational searches, we identify one orientation that represents the inactive dimeric state and another similar to a physiologically activated receptor. Several other dimeric orientations are identified that induce proliferation and *in vivo* myeloproliferative and myelodysplastic disorders, indicating the receptor can signal from several dimeric interfaces. The set of dimeric thrombopoietin receptors with different TM orientations may offer new insights into the activation of distinct signalling pathways by a single receptor and suggests that subtle differences in cytokine receptor dimerization provide a new layer of signalling regulation that is relevant for disease.

The EMBO Journal (2011) 30, 4398–4413. doi:10.1038/emboj.2011.315; Published online 2 September 2011

Subject Categories: signal transduction

Keywords: dimerization; hematopoiesis; JAK2; myeloproliferation; TpoR

Introduction

The thrombopoietin (Tpo) receptor (TpoR, c-Mpl) is a key regulator of megakaryopoiesis (de Sauvage *et al*, 1994; Kaushansky, 1997), and of haematopoietic stem cell (HSC) renewal (Solar *et al*, 1998) and quiescence (Qian *et al*, 2007; Yoshihara *et al*, 2007). Binding of Tpo induces a conformational change of the receptor (Alexander *et al*, 1995), resulting in activation of the tyrosine kinases JAK2 and TYK2 (Drachman *et al*, 1995, 1999; Miyakawa *et al*, 1995; Watowich *et al*, 1996; Royer *et al*, 2005). Activated Janus kinases (JAKs) in turn phosphorylate tyrosine residues within the TpoR cytosolic domain that function as binding sites for downstream signalling molecules such as STAT3, STAT5 (the signal transducers and activators of transcription 3 and 5) and adaptors triggering phosphatidylinositol-3-kinase (PI-3-kinase) or Mitogen Activated Protein (MAP)-kinase activation (Miyakawa *et al*, 1995, 1996, 2001; Oda *et al*, 1996; Onishi *et al*, 1996; Drachman and Kaushansky, 1997).

Mutations that activate TpoR signalling in the absence of its ligand include single amino-acid substitutions by cysteine or asparagine in the extracellular and transmembrane (TM) domains, respectively (Alexander *et al*, 1995; Onishi *et al*, 1996; Ding *et al*, 2004), as well as mutations in the juxta-membrane (JM) region, on both the extracellular and intracellular sides (Pardani *et al*, 2006; Pikman *et al*, 2006; Staerk *et al*, 2006; Malinge *et al*, 2008). Furthermore, truncation of part of the extracellular domain and fusion on the amino-terminus with a viral-derived sequence also activates TpoR signalling (Souyri *et al*, 1990). All these mutant receptors trigger cytokine-independent cell proliferation and induce myeloproliferative disorders presumably by ligand-independent dimerization in conformations productive for signalling (Alexander *et al*, 1995; Onishi *et al*, 1996; Ding *et al*, 2009). The precise dimeric interface(s) adopted by these active TpoR mutants remain however unknown.

Among cytokine receptors, TpoR is special as it plays essential roles at different stages of haematopoiesis. To carry out its multiple functions, different ligands and distinct cell types might induce changes in the active dimeric state of the receptor and its interaction with other cellular proteins. It is known that TpoR can respond to at least two ligands, Tpo (Kaushansky, 1997) and a human homologue of a fungal nuclear migration protein (hNUDC) (Chen *et al*, 2010). Very little is known about the dimeric structure of the activated TpoR TM and cytoplasmic domains. Whether the dimeric orientations adopted by ligand-activated TpoR differ in various cell types, megakaryocyte progenitors versus HSCs remains unclear. Different biologic effects of activated TpoR might result from interactions with other receptors or signalling proteins, which may be cell type specific. One hypothesis would posit that TpoR interacts with cell type-specific receptors and signalling proteins through different conformations of the TM and cytoplasmic domains of TpoR. This proposed mechanism for activation is quite different from that of

*Corresponding author. Ludwig Institute for Cancer Research, Avenue Hippocrate 74, UCL 7459, Brussels 1200, Belgium. Tel.: +32 2 764 7540; Fax: +32 2 764 6566; E-mail: stefan.constantinescu@bru.lir.org

⁶These authors are co-first authors

⁷Present address: Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA

Received: 4 November 2010; accepted: 4 August 2011; published online: 2 September 2011

the erythropoietin receptor (EpoR), where essentially one dimeric conformation appears to be required for promoting erythroid cell formation (Seubert *et al*, 2003).

To test whether the TpoR requires a specific dimeric conformation for signalling or whether it signals from many different dimeric conformations, we replaced the extracellular domain of the TpoR by a dimeric coiled coil. By engineering the junction of the coiled coil to the TpoR TM domain, we were able to impose the seven possible relative orientations on the TM and cytoplasmic domains of the TpoR. In this approach, we obtain seven individual dimers that adopt different interfaces in the TM and intracellular JM domains, which are due to the register of the coiled coil. The JM cytosolic region of cytokine receptors was suggested to be rigid by both functional assays (Constantinescu *et al*, 2001; Huang *et al*, 2001) and by single particle electron microscopy (Skinotis *et al*, 2008; Lupardus *et al*, 2011). The different orientations of the TM–JM region are thus likely to be transmitted to the cytosolic domain and influence signalling. Furthermore, in the case of the growth hormone receptor, subunit rotation in a receptor dimer was shown to suffice for activation (Brown *et al*, 2005). To show that the rotation induced by the coiled coil is transmitted, as predicted, to the JM region, we used two approaches, cysteine mutagenesis in combination with crosslinking and alanine insertions at the intracellular boundary.

We tested the biologic effects of the seven possible TpoR dimers in cell lines and *in vivo* in mouse bone marrow reconstitution experiments. This allowed us to use functional blood formation as a read-out in order to identify the precise dimer conformation that most faithfully resembles the effect of Tpo on platelet formation (*cc-TpoR-I*). To our surprise, five other dimeric conformations of the TpoR also led to proliferation of cytokine-dependent cells, with differences in cell morphology and signalling. *In vivo*, these orientations induce different effects, with two being associated with mixed myelodysplastic/myeloproliferative phenotypes, and others with myeloproliferation. We propose the model that different dimeric TpoR interfaces induce different biological outcomes and that different dimeric receptors could possibly favour different myeloproliferative and myelodysplastic disorders. Importantly, we show that this orientation-dependent signalling specificity requires the KWQFP motif in the JM region, which maintains the unliganded TpoR inactive (Staerk *et al*, 2006).

Results

Fusion of the Put3 coiled coil to the TpoR TM and cytosolic domains

The C-terminal 28 amino acids of Put3, a soluble transcriptional activator protein from *Saccharomyces cerevisiae*, associate in a stable left-handed coiled-coil dimer (Swaminathan *et al*, 1997; Walters *et al*, 1997). Fusion of the C-terminal residue of the Put3 sequence to the N-terminal residue of the TM sequence of the TpoR induces dimerization of the TpoR TM domain, which is known to be α -helical through F510 based on polarized infrared spectroscopy of membrane reconstituted peptides corresponding to the TM and JM domains (Supplementary Figure S1). Fusion of the Put3 coiled-coil domain imposes its structure on the α -helical residues downstream in the TM domain and enables us to

identify the amino acids that are present in the interface of the active TpoR dimer (Figure 1A and B; Swaminathan *et al*, 1997; Walters *et al*, 1997). Position of heptad repeats characteristic of left-handed coiled coils are denoted *a*–*g* or *a'*–*g'*. The last residue of the Put3 coiled coil is in a *b* position. As a result, the first fused residue of the TpoR TM domain is in a *c* position (Figure 1B). By designing seven different junctions between the coiled coil and the TpoR TM domain, all possible dimeric receptor orientations can be imposed (Mattoon *et al*, 2001; Seubert *et al*, 2003), due to the registry and tight dimerization of the 28 amino acid Put3 coiled coil (Swaminathan *et al*, 1997; Walters *et al*, 1997).

We constructed seven TpoR fusion proteins where the Put3 coiled coil was fused to the TM and cytoplasmic domains of the TpoR, denoted *cc-TpoR-0–VI*. By varying the junction between the Put3 coiled coil and the TpoR TM and cytoplasmic sequences, all seven possible registers could be obtained (Figures 1A, B and 7A). All fusion proteins were expressed at the expected size of 27 kDa in Ba/F3 cells. In order to confirm the orientation of our *cc-TpoR* fusion proteins, we introduced a cysteine residue at position 505 (L505C) for crosslinking studies and eliminated all other cysteine residues by truncation of the cytosolic domain at position 553 in all *cc-TpoRs*. All the truncated fusion proteins were expressed in BOSC cells at the expected size of 12 kDa. Consistent with our hypothesis that only *cc-TpoR-I*, *cc-TpoR-II* and *cc-TpoR-V* should have L505C in the dimeric interface, we only visualized formation of *N,N'*-1,2-phenylenedimaleimide (o-PDM) crosslinked dimers for those three receptors (Figure 1C). Indeed, treatment of cells with o-PDM enables formation of intermolecular bonds maintaining stable dimers in denaturing conditions. Crosslinking on live cells, therefore, validates the orientation imposed by the coiled coil up to the outset of the cytosolic JM domain.

Cell surface expression levels of the *cc-TpoR-0–VI* proteins were measured by flow cytometry using an anti-HA tag antibody. Levels of *cc-TpoR* cell surface localization were between 20 and 40%, as compared with the wtTpoR levels, which were taken as 100% for the same level of GFP (Figure 1D).

Proliferation mediated by the *cc-TpoR-0–VI* fusion proteins

Ba/F3 cells were infected with retroviruses coding for the seven *cc-TpoR* fusion proteins and sorted for equal GFP levels. Cells were washed, grown in the absence of any cytokines and monitored by cell counting at different intervals. Four out of six active conformations induced maximal proliferation, *cc-TpoR-0*, -I, -III and -IV, while two, *cc-TpoR-V* and -VI, were able to promote growth of Ba/F3 cells, but to a lower extent (Figure 2A). *cc-TpoR-II* was inactive.

We next ensured that dimerization of the TpoR TM and cytoplasmic domains mediated by the Put3 coiled-coil fusion was required for the proliferative effects of the *cc-TpoR-0–VI* fusion proteins. We, therefore, substituted the lysine at position 86 within the Put3 amino-acid sequence by a proline (KtP). This mutation has been shown to block dimerization of the Put3 coiled coil (Swaminathan *et al*, 1997). The Put3-KtP substitution disabled cytokine-independent growth mediated by the *cc-TpoR* fusion proteins (Figure 2B), showing that dimerization mediated by the coiled-coil Put3 was crucial for the activity of *cc-TpoR* fusion proteins.

Figure 1 Design, validation and expression of seven fusion proteins that contain the Put3 coiled coil fused in all possible orientations to the TpoR TM–cytoplasmic domains. **(A)** Design of the *cc-TpoR* fusion proteins. The coiled-coil segment of Put3 was fused to the TM and cytoplasmic domains of TpoR. **(B)** By varying the junction between the Put3 coiled coil and the downstream TpoR TM α -helical domain, all seven possible registers could be imposed. All fusion proteins contain an HA tag at their N-terminus. The position of heptad repeats characteristic of coiled coils is denoted *a*–*g* or *a'*–*g'*. The last residue of the Put3 coiled-coil is in a *b* position, while the first fused residue will be in a *c* position. The dimeric interface contains the *a*, *d*, *d'* and *a'* positions. **(C)** Crosslinking studies on live cells. *Left panel*: BOSC cells were transiently transfected with cDNA coding for seven truncated coiled-coil TpoR cysteine mutants (L505C). Cells were lysed after crosslinking with *N,N'*-1,2-phenylenedimaleimide (+ o-PDM), or in the absence of crosslinking (– o-PDM). Lysates were analysed on 12–14% Bis-Tris Gel followed by immunoblotting with an anti-HA antibody. *Right panel*: Helical representation of the different *cc-TpoR* interfaces and the putative localization of the Cys505 residue. **(D)** HA staining for cell surface expression of the *cc-TpoR* fusion proteins. The wtTpoR cell surface expression was set as 100%. Percent expression of the *cc-TpoR* fusion proteins was related to that of the wtTpoR. Shown is one experiment with data collected for 10 000 cells of three independent determinations; variations were <10%.

Ba/F3 cells expressing the active *cc-TpoR* fusion proteins showed remarkable differences in their morphology. When cells expressed *cc-TpoR-IV*, large clusters were observed (Figure 2C). In contrast, cells that expressed *cc-TpoR-I* or *cc-TpoR-III* did not induce such strong cell-to-cell interactions, although they induced proliferation at similar levels (Figure 2A). Interestingly, *cc-TpoR-IV* induced cell-to-cell adhesion in many cell lines and in primary bone marrow haematopoietic progenitors, both in suspension and in methylcellulose assays (not shown). To examine the ability of the wtTpoR to induce such cell-to-cell interactions, we stimulated wtTpoR expressing Ba/F3 cells (Ba/F3-TpoR)

using Tpo concentrations between 5 and 500 ng/ml. Ba/F3-wtTpoR stimulated with extremely high Tpo concentrations (500 ng/ml) formed the same cell clusters (Figure 2C, right panel), as observed with *cc-TpoR-IV*, whereas at lower doses (5 ng/ml) only small clusters could be seen (Figure 2C, left panel). Thus, in the dimeric conformation adopted by *cc-TpoR-IV*, signalling events appear to be similar to those induced by very high Tpo levels via the wtTpoR. Such high levels may be attained in the bone marrow endosteal niches, close to HSC reservoirs.

Our results using the seven *cc-TpoR* fusion proteins suggest that the TpoR can signal from multiple dimeric conforma-

Figure 2 Cell proliferation induced by the seven *cc-TpoR* fusion proteins. (**A**, **B**) Proliferation assays were performed with Ba/F3 cells expressing the wtTpoR or the indicated *cc-TpoR*-0–VI fusion proteins (**A**), or K86P (KtoP) mutants thereof (**B**) at equal GFP levels in the absence of any cytokines or stimulated with 5 ng/ml Tpo as indicated. Cell numbers (averages of triplicates $\pm$ s.d.) were counted at day 6. One representative experiment out of four is shown. (**C**) Morphology of Ba/F3 cells expressing *cc-TpoR*-IV or *cc-TpoR*-I. Only *cc-TpoR*-IV induced cell-to-cell adhesion that lead to tight clusters (upper panel). Ba/F3 cells expressing the wtTpoR were stimulated with 5 or 500 ng/ml Tpo. At very high Tpo concentrations, cells formed clusters similar to those induced by *cc-TpoR*-IV (lower panel). Scale bar, 50 μ m. (**D**) Proliferation assays were performed with UT7 cells expressing the wtTpoR or the *cc-TpoR*-0–VI fusion proteins at equal infection efficiency (GFP levels) in the absence of any cytokines or stimulated with 5 ng/ml Tpo, as indicated. Cell numbers were counted at day 8 and shown are averages of triplicates $\pm$ s.d. (**E**) Induction of CD41 expression of UT7 retrovirally transduced with the seven *cc-TpoR* fusion proteins or with the wtTpoR. Initiation of megakaryocyte differentiation of UT7 cells was assayed by flow cytometry analysis of the induction of cell surface CD41. The induction of CD41 cell surface localization by 5 ng/ml Tpo on cells expressing the wtTpoR was taken as 100%.

tions (except for that of *cc-TpoR*-II, and in certain cells of *cc-TpoR*-VI), but that different orientations induce different effects. We took advantage of the UT7 cell line (Komatsu

et al, 1991), first to test whether *cc-TpoR* fusion proteins induce similar proliferative effects as in Ba/F3 cells, and second, to explore the abilities of the *cc-TpoR* proteins to

promote commitment towards the megakaryocyte lineage. UT7 cells are strictly dependent on GM-CSF or Epo for growth (Komatsu *et al*, 1991). We introduced all seven TpoR fusion proteins in parental UT7 cells maintained in GM-CSF, sorted for equivalent GFP expression and then tested proliferation after withdrawal of GM-CSF (Figure 2D). One of these weaker conformations in Ba/F3 cells, *cc-TpoR-VI*, was not able to mediate growth of UT7 cells, similarly to *cc-TpoR-II*, which was inactive in both cell lines (Figure 2A and D). Whereas UT7 cells that expressed *cc-TpoR-II* and *-VI* did not survive or proliferate in the absence of ligand, UT7 cells expressing *cc-TpoR-III* proliferated to a lower extent compared with the proliferation level in Ba/F3 cells (Figure 2A and D). One explanation for this observation is that *cc-TpoR-III* induces senescence, as it was recently shown for late megakaryocytes and UT7-Mpl cell lines treated with Tpo (Besancenot *et al*, 2010); another possibility is that *cc-TpoR-III* induces higher levels of megakaryocyte commitment.

Stably transfected UT7-TpoR cells respond to Tpo by induction of the expression of the megakaryocyte-specific differentiation antigen CD41 (gpIIb) (Porteu *et al*, 1996), and such cells have been the standard for exploring signals leading to megakaryocyte commitment. We then selected stably transduced UT7 cells for growth factor-independent proliferation. We analysed the expression of the CD41 in these cells and found that *cc-TpoR-0* and *-III*, as well as *cc-TpoR-I*, were able to induce CD41 expression, while *cc-TpoR-IV* and *-V* were very weak inducers of CD41 expression (Figure 2E). On the basis of these data, we predict that one or several of the *cc-TpoR* fusion proteins that induce CD41 expression in UT7 cells might induce *in vivo* physiologic platelet formation.

Transcriptional activity mediated by the *cc-TpoR* fusion proteins

We examined the transcriptional activity in Ba/F3 and in JAK2-deficient γ 2A cells (Kohlhuber *et al*, 1997) expressing the different coiled-coil fusion proteins using the reporter genes for STAT5, STAT3, MAP-kinase, PI-3-kinase, as described in Materials and methods. For all pathways tested, *cc-TpoR-0*, *-I* and *-IV* activated signalling via STAT5, STAT3 and MAP-kinase very strongly (Figure 3A and B). As expected, signalling via PI-3-kinase resulted in inhibition of transcriptional activity of the 6xDBE-Luc FoxO reporter, with *cc-TpoR-0*, *-I* and *-IV* being strong activators of this pathway (Supplementary Figure S2). Since *cc-TpoR-I* was one of the active orientations not promoting cell-to-cell adhesion, these results rule out the possibility that formation of cell clusters is simply a consequence of the quantitative level of signalling activation and suggests that different dimeric orientations may attract different adaptors or signalling proteins. While in Ba/F3 cells, *cc-TpoR-III* mediates proliferation to the same extent as *cc-TpoR-0* and *-IV*, *cc-TpoR-III* induces a slightly lower level of STAT5 and STAT3 activity, when compared with *cc-TpoR-0*, *-I*, *-IV* and it only weakly induces MAP-kinase activation (Figure 3A and B). While *cc-TpoR-III* and *-V* displayed a very weak MAP-kinase activity, *cc-TpoR-II* and *-VI* failed to induce any detectable MAP-kinase response. Noteworthy *cc-TpoR-II*, *-V*, and *-VI* are located on the same face in the helical representation (Figure 1C) and correspond to a low JAK/STAT, low MAP-kinase, and low PI-3-kinase interface (Supplementary Figure S4). On the opposite face,

cc-TpoR-0, *-I*, *-IV* activate very strongly JAK/STAT, MAP-kinase and PI-3-kinase (Figure 1C; Supplementary Figures S2 and S4; Table I).

Orientation-dependent activation of JAK2 and TYK2 signalling

Tpo binding to TpoR leads to activation of two members of the JAK family, JAK2 and TYK2. These kinases exhibit similar potency to promote traffic stability of TpoR at the cell surface (Royer *et al*, 2005). JAK2, however, is much more potent to mediate downstream signalling (Drachman *et al*, 1999; Royer *et al*, 2005). We asked whether differently oriented TpoR dimers would preferentially signal via JAK2 versus TYK2. To this end, we compared STAT5 or STAT3 transcriptional activity of *cc-TpoR* fusion proteins in JAK2-deficient γ 2A cells in the presence of JAK2 or TYK2 (Royer *et al*, 2005). As shown in Figure 3B (left panel), *cc-TpoR-0*, *-I* and *-IV* induced strong STAT5 signalling in the presence of JAK2. In contrast, in the presence of TYK2 (right panel), *cc-TpoR-III* and *cc-TpoR-VI* induced the highest STAT5 and STAT3 activation. Thus, signalling via JAK2 and TYK2 may be induced via different interfaces (with *cc-TpoR-VI* being specific for TYK2 and *cc-TpoR-III* supporting both JAK2 and TYK2 activations; Table I). Further studies are required to determine whether, in blood progenitors, TpoR could signal via JAK2–TYK2 heterodimers, and whether a dimeric interface, such as that of *cc-TpoR-III*, could actually assemble such JAK2–TYK2 heterodimers.

We then asked whether the differences observed in transcriptional activities of *cc-TpoRs* could also be obtained if rotations were introduced at the end of the TM domain. As a proof of principle, insertions of one or more alanines after the TM domain of *cc-TpoRs* rotate the receptor clockwise. Addition of two alanines to the strongest coiled coil via JAK2 (*cc-TpoR-IV* + 2A) diminished the STAT transcriptional activity, rendering *cc-TpoR-IV* similar to *cc-TpoR-II*, as predicted. Addition of one alanine to the weakest coiled coil via JAK2 (*cc-TpoR-II* + 1A) enhanced the STAT transcriptional activity, to the levels of *cc-TpoR-I*, as predicted (Figure 3C). This rotational effect can also be demonstrated by measuring the signalling downstream TYK2 with *cc-TpoR-V* + 2A being a phenocopy of the powerful *cc-TpoR-III* via TYK2 (Figure 3D). These results indicate that the TM α -helix is a continuum and that rotations at the C-terminus are transmitted to the JM domain in the same manner as those induced by the coiled coil at the N-terminus.

Early downstream signalling and traffic of *cc-TpoR* fusion proteins

Having determined the biologic activities of *cc-TpoRs* in Ba/F3 cells, and their transcriptional activities, we aimed to determine the immediate signalling events at the receptor cytosolic domain. We immunoprecipitated the receptors and tested the phosphorylation level of the main signalling tyrosine residue, Y626 (Drachman and Kaushansky, 1997), using an antibody specifically recognizing this phosphorylated residue (Pecquet *et al*, 2010). *cc-TpoR-V*, *-VI* and *-II* presented very low phosphorylation levels of Y626 compared with *cc-TpoR-0*, *-I*, *-III* and *-IV* (Figure 4A). We validated these results by analysing the activity of some downstream pathways, like STAT1 or STAT3. Indeed, *cc-TpoR-II* shows no activation of these pathways (Figure 4B). *cc-TpoR-V* and *-VI*

Figure 3 Transcriptional activity mediated by the wild-type (wt) TpoR or *cc-TpoR-0-VI* with and without alanine insertions. (A) Ba/F3 cells were selected in Tpo or without cytokines and starved overnight in RPMI/1 mg/ml BSA. To measure the transcriptional activity of STAT5 (left panel), STAT3 (middle) or MAP-kinase (MAPK) (right panel) pLHRE-luc, pGL3bPpr2-luc and pSRE-luc were used, respectively. pRL-TK encoding the renilla luciferase was co-electroporated for normalization of luciferase values. Cells were stimulated with 50 ng/ml Tpo or mock treated, as indicated. The results shown reflect averages of triplicate values $\pm$ s.d. Similar results were obtained performing three independent experiments. (B) γ 2A cells were transfected with cDNAs coding for the *cc-TpoR* fusion proteins, JAK2 (left panel) or TYK2 (right panel), STAT5/STAT3 pGRR5-luc luciferase reporter and pRL-TK. The results represent averages of triplicate values $\pm$ s.d. of one representative experiment out of at least three. (C, D) γ 2A cells were co-transfected with pGRR5-luc and pRL-TK luciferase reporter along with cDNAs coding for *cc-TpoRs* in which alanines were inserted at the end of the TM region, as indicated, and for JAK2 (C) or TYK2 (D).

presented low activation of STAT1 while *cc-TpoR-0*, -I, -III and -IV were fully active (Figure 4B). Thus, the strength of signalling and biologic effects appear to correlate with levels of Y626 phosphorylation.

We next analysed the internalization rate of the fusion proteins as well as their recycling. Cell surface biotinylation followed by cell surface immunoprecipitation enables us to assess the internalization over a certain period of time, but

Table 1 Summary of biologic effects and signalling of *cc-TpoR* fusion proteins in Ba/F3 cells

	Proliferation	Cell clustering	JAK2/STATs activation	TYK2/STATs activation	MAP-kinase activation	PI-3-K/AKT activation
<i>cc-TpoR-0</i>	+++	—	+++	—	+++	++
<i>cc-TpoR-I</i>	+++	+	+++	—	+++	++
<i>cc-TpoR-II</i>	—	—	—	—	—	—
<i>cc-TpoR-III</i>	+++	—	++	+++	+	+
<i>cc-TpoR-IV</i>	+++	+++	+++	+	+++	++
<i>cc-TpoR-V</i>	+	+	++	—	+	+
<i>cc-TpoR-VI</i>	+/-	—	+	++	—	+

Figure 4 Early signalling and traffic of *cc-TpoR* fusion proteins. **(A)** Immunoprecipitation of the indicated fusion proteins. Induction of phosphorylation of cytosolic Y626 was assessed using an anti-pY626 TpoR antibody. The anti-HA antibody was used to analyse the expression level of the *cc-TpoR* fusion protein. **(B)** Western blot analysis performed on Ba/F3 cells expressing the fusion proteins by using the indicated antibodies. **(C)** Recycling assay on Ba/F3 cells expressing *cc-TpoR-I*, *cc-TpoR-II*, *cc-TpoR-III* or *cc-TpoR-IV*. Cells were subjected to cell surface biotinylation, allowed to internalize the receptor, and then were maintained in culture in presence of JAK inhibitor I, in order to block JAK2 signalling, for the indicated time. Cell surface immunoprecipitation was performed on the cells followed by lysis in NP-40 buffer. Immunoprecipitates were analysed by immunoblot with an anti-biotin/HRP and an anti-HA.

also the recycling. For the latter, we blocked the activity of the receptors with a JAK2 inhibitor, JAK inhibitor I (Thompson *et al*, 2002). All fusion proteins tested (*cc-TpoR-I*, -II, -III or -IV) were internalized after 30 min in Ba/F3 cells, with higher

levels of internalization being correlated with signalling strength. Importantly, after treatment with JAK inhibitor I, recycling was observed for the tested fusion proteins, *cc-TpoR-I*, -II, -III or -IV (Figure 4C), suggesting that traffic

Figure 5 *In vivo* expression of *cc-TpoR* fusion proteins. (A, B) Mice were reconstituted with bone marrow cells retrovirally transduced with the indicated *cc-TpoR* fusion proteins. Peripheral blood counts were measured 6–8 weeks after reconstitution. (A) Platelets counts are the average $\pm$ s.d. of six mice per construct. (B) The peripheral number of platelets is shown for the indicated *cc-TpoR* fusion proteins; *n*, number of mice. Three independent reconstitutions are shown for TpoR wt, *cc-TpoR-I* and *cc-TpoR-V*, and two independent reconstitutions are shown for *cc-TpoR-III* and *cc-TpoR-IV*. (C) Peripheral blood was isolated from GFP-positive mice 6 weeks after reconstitution and blood smears were performed. Cover slides were stained using a May-Grunwald stain and analysed for qualitative or quantitative abnormalities in the blood. Bone marrow cytologic examination was performed after May Grunwald staining. The magnification was $\times 50$. Scale bar, 50 μm .

and especially recycling are not the determinants of their different biologic activities.

Expression of different TpoR fusion proteins in bone marrow progenitors induced specific myeloid disorders in bone marrow transplanted mice

We next investigated the *in vivo* effects of the *cc-TpoR*-0–VI fusion proteins. Bone marrow cells from 6- to 8-week-old C57/B6 mice were isolated and infected with retroviruses encoding the wtTpoR or the active *cc-TpoR* fusion proteins. In all, 0.5×10^6 cells were injected intravenously into lethally irradiated mice. Peripheral blood examined 3 weeks after reconstitution by FACS analyses showed that $>90\%$ of peripheral blood cells were GFP positive. A high level of platelet formation was induced *in vivo* by *cc-TpoR-I* (Figure 5A and B). Cytology examination on May-Grunwald-Giemsa stained bone marrow showed normal cellularity, with correct maturation of the myeloid lineages and excess of megakaryocytes of small to normal size that did not exhibit phenotypic changes of the myeloproliferative disorders. Interestingly, the *cc-TpoR-I* conformation has S498 (equivalent to S505 in human TpoR) in the interface (Figure 7A), suggesting that this particular orientation might reflect the active wtTpoR interface. Another fusion protein that contains S498 in the interface, *cc-TpoR-V*

(Figure 7A), induced a different phenotype with circulating erythroblasts, and dyserythropoietic picture with microcytic red cells overlapping in size with platelets, which gave an artificially high count for platelets. In the marrow, *cc-TpoR-V* induced a picture of dysplasia of the erythroid and granulocytic lineages (not shown). Adopting a different orientation (Figure 7A), *cc-TpoR-III* induced granulocytosis with normal platelet counts, 2% erythroblast peripheral counts with normal red blood cell counts, while in the marrow cytologic examination showed large megakaryocytes with emperipoiesis (dysmegakaryopoiesis), dyserythropoiesis and enhanced granulocytogenesis (Figure 5). *cc-TpoR-IV* is the one orientation that induced strong clustering activity in cell lines and primary bone marrow cells (Figure 2C), which led to variable levels of reconstitution (not shown); this made the analysis difficult. An inducible system would be necessary to avoid factoring in the effects of adhesion during bone marrow reconstitution. Mice reconstituted with *cc-TpoR-VI* (adopting a conformation resembling that of *cc-TpoR-III*; Figure 7A) had a high degree of granulocytosis, at higher levels than *cc-TpoR-III*, with also immature granulocytic precursors in the peripheral blood, high levels of erythroblasts (15%), reticulocytosis and normal platelets. In the marrow, *cc-TpoR-VI* induced a major increase in the granulocytic lineage (very high granulopoietic ratio),

with decreased megakaryocyte numbers and dysmegakaryopoiesis.

No orientation-dependent signalling is induced by the $\Delta 5cc$ -TpoR fusion proteins

In all of our *cc*-TpoR fusion proteins, the JM KWQFP motif is present at the junction between the TpoR TM and cytosolic domains. This domain, in particular the Trp residue, is required to maintain the unliganded TpoR inactive (Staerk *et al*, 2006). Amino-acid substitutions of W515 result in oncogenic receptors, and are the basis of a fraction of human myeloproliferative neoplasms (MPNs) (Pardanani *et al*, 2006; Pikman *et al*, 2006; Pecquet *et al*, 2010). We, therefore, addressed whether this motif affects signalling by the different dimeric conformations of the TpoR. Ba/F3 or UT7 cells were infected with retroviruses coding for the seven $\Delta 5cc$ -TpoR fusion proteins (Figure 6A) and sorted for equal GFP levels as described above.

Strikingly, when the KWQFP motif was deleted, all seven $\Delta 5cc$ -TpoR fusion proteins promoted maximal proliferation of Ba/F3 or UT7 cells (Figure 6B and C). These results indicate that the KWQFP motif played an important role in orientation-dependent proliferation obtained by the wtTpoR. None of the $\Delta 5cc$ -TpoR fusion proteins could induce formation of cell-to-cell adhesion or cell clusters (Figure 6D, compared with Figure 2C). Furthermore, the inhibitory conformation induced by *cc*-TpoR-II was changed by the deletion of the KWQFP motif and the $\Delta 5cc$ -TpoR-II became active (Figure 6B and C). The specificity of TYK2 activation by only *cc*-TpoR-III and -VI fusion proteins was lost, with all $\Delta 5cc$ -TpoR fusion proteins activating TYK2 (Figure 6E) and JAK2 (not shown) to the same high extent.

Reconstitution of mice with $\Delta 5cc$ -TpoR fusion proteins showed in general a uniform increase in granulocytes and platelets in peripheral blood, without the specific effects seen for the *cc*-TpoR fusion proteins (Figure 6F). For $\Delta 5cc$ -TpoR-I, -III and -VI all three had granulocytosis, while that was not the case for *cc*-TpoR-I. Platelet numbers were increased for $\Delta 5cc$ -TpoR-I, -III, and for the latter this was in direct contrast with *cc*-TpoR-III. Functionally, $\Delta 5cc$ -TpoR-VI resembles the most its *cc*-TpoR-VI counterpart, especially also showing erythroblastosis (10%); however, the platelet numbers increased, which was not the case for *cc*-TpoR-VI. Overall, the differences noted between individual *cc*-TpoR fusion proteins were mostly lost for the $\Delta 5cc$ -TpoR counterparts, with the exception of the erythroblastosis for $\Delta 5cc$ -TpoR-VI.

Introduction of the equivalent of the W515K mutation in the *cc*-TpoR fusion proteins (W508C for the murine TpoR sequence) led to active proteins for all orientations, when signalling was tested via JAK2. This included the inactive *cc*-TpoR-II, which became active upon W508K mutation (Supplementary Figure S3). These results are in agreement with the activating effect induced by deletion of the KWQFP motif.

Computational searches of the TpoR TM dimer interface

The *cc*-TpoR fusion proteins identify several orientations for the TM helices of the TpoR that induce strong receptor activity, as well as orientations that inhibit receptor activity. The choice of Put3 as a fusion partner was dictated by its ability to function as an extracellular domain, and importantly that it forms a left-handed (LHCC) dimeric coiled coil.

Structural studies using solid-state NMR spectroscopy on membrane reconstituted peptides that include the TM and JM sequence of the TpoR up to the Box 1 region show that the sequence up to F510 is helical (Staerk *et al*, 2006; Supplementary Figure S1).

The helical wheel diagrams for dimers packing with an LHCC geometry have a spacing of 3.5 residues-per-turn. The heptad repeat is formed by two helical turns. The positions of amino acids relative to the dimer interface are denoted *a*–*g* with the *a* and *d* positions corresponding to those residues that are directly in the dimer interface. The diagrams for the seven Put3 coiled-coil dimers are shown in Figure 7A. In order to evaluate which of these orientations may correspond to low-energy dimer conformations, computational searches were performed using the program CHI (Adams *et al*, 1996; see Materials and methods). For the EpoR TM domain (Seubert *et al*, 2003), using this approach, we were able to identify two stable dimer conformations that corresponded well with active and inactive orientations imposed by the Put3 coiled coil. For the murine TpoR, computational searches were carried out on residues I485–L506 corresponding to the TM domain, and on residues I485–P511 corresponding to the TM domain with the KWQFP motif.

Figure 7B presents the results of a typical computational search on the TpoR TM domain (residues I485–L506) where possible dimer structures are generated by rotating each helix through rotation angles ϕ_1 and ϕ_2 from 0° to 360° with a sampling size of 45° and performing molecular dynamics simulations for each orientation. Favourable orientations are identified by structures that fall into a ‘cluster’, where a cluster is defined as a minimum of five structures with a root mean square (r.m.s.) deviation of 1 Å or less. An average molecular structure is calculated and the energy is minimized for each cluster. The clusters with the lowest energy are shown for each search.

There are three clusters of structures located along the diagonal of the search plot in Figure 7B. Since symmetric orientations of the TM helices (where $\phi_1 = \phi_2$) are induced by the Put3 constructs, these are the clusters of interest that represent the natural tendencies of the TM residues to assume symmetric dimers. The two lowest energy clusters found in the searches correspond to *cc*-TpoR-II and *cc*-TpoR-VI. *cc*-TpoR-II is inactive in both Ba/F3 and UT7 cells. *cc*-TpoR-VI is generated by a 50° counterclockwise rotation from *cc*-TpoR-II. *cc*-TpoR-VI is inactive in UT7 cells and is the least active of the dimers other than *cc*-TpoR-II in Ba/F3 cells. These results suggest that ligand-independent dimerization of the wild-type TpoR via TM domain interactions would result in an inactive receptor. The lowest energy active dimeric orientation that results in cell proliferation in both Ba/F3 and UT7 cells is *cc*-TpoR-V. For this structure, the TM dimer is stabilized by interhelical hydrogen bonding of S498. Higher energy dimer structures are found for the other *cc*-TpoR orientations in the computational searches, including *cc*-TpoR-I.

Computational searches of TM domain along with the five residues KWQFP insert typically yield clusters of *cc*-TpoR-II, *cc*-TpoR-IV and *cc*-TpoR-V. The average molecular structures of the clusters corresponding to the *cc*-TpoR-II and the *cc*-TpoR-V dimers are shown in Figure 7C and D, respectively. In the *cc*-TpoR-II dimer, K507 and W508 are oriented away from the dimer interface. The dimer structure is largely

Figure 6 Deletion of the juxtamembrane KWQFP motif eliminates differences in biologic effects between the seven *cc-TpoR* dimers. (A) Design of the *cc-TpoR* and *Δ5cc-TpoR* fusion proteins. The coiled-coil segment of Put3 was fused to the TM and cytoplasmic (CP) domains of the wtTpoR or the *Δ5TpoR*. (B, C) Proliferation assays were performed with Ba/F3 (A) and UT7 (B) cells expressing the *cc-TpoR* fusion proteins where the KWQFP motif was deleted (*Δ5cc-TpoR-0–VI*). Cell numbers (averages of triplicates $\pm$ s.d.) were counted at day 6 (A, B). (D) Cell morphology of Ba/F3 cells expressing *Δ5cc-TpoR-IV* or *Δ5cc-TpoR-I*. No clusters were induced by *Δ5cc-TpoR-IV*, which lacks the KWQFP motif. For all pictures shown, Ba/F3 cells were plated in 24-well plates and pictures were taken at day 4. (E) $\gamma 2A$ cells were transfected with cDNAs coding for the *Δ5cc-TpoR* fusion proteins, and TYK2, STAT3 and STAT5/STAT3 pGRR5-luc luciferase reporter and pRL-TK. The results represent averages of triplicate values $\pm$ s.d. of one representative experiment out of at least three. (F) Peripheral blood and bone marrow were isolated from mice expressing the indicated *Δ5cc-TpoR* fusion proteins. Coverslips were stained using a May-Grunwald stain and analysed for qualitative or quantitative abnormalities in the blood. Bone marrow was examined by haematoxylin–eosin staining. The magnification was $\times 50$. Scale bar, 50 μ m.

stabilized by an interhelical hydrogen bond between the side chain amide of Q509 and the backbone carbonyl of Q509 on the opposing helix. van der Waals interactions between V488, L492, L498, A499, G502 and L506 also contribute to stabilizing this orientation of the TM helices.

One major question we asked was whether the *cc-TpoR-I* conformation (the fully active conformation) could be adopted spontaneously by the TpoR TM domain having the KWQFP JM insert. In our searches with the KWQFP insert, tryptophan at position 508 prevented dimer formation for

Figure 7 Computational searches of the TpoR transmembrane dimer. (A) Helical wheel predictions of the seven dimeric interfaces of the TM domain of the TpoR imposed by the Put3 coiled-coil fusion. Residue S498 of the TM domain is placed in the interface in *cc-TpoR-I* and *cc-TpoR-V*. (B) Computational search using the TpoR TM sequence from I485 to L506. The axial separation between the TM helices was set to be 10 Å. Computational searches with axial separations of 9.5–10.5 Å gave similar results. (C, D) Average molecular structures of the helix dimer corresponding to *cc-TpoR-II* and *cc-TpoR-V*. The helices have left-handed crossing angles and the dimer is viewed down the helix axis from the C-terminal end of the dimer. In *cc-TpoR-II* (C), the hydrogen bonding of Q509 stabilizes the helix dimer. The side chain of K509 contacts the aromatic ring of W508 characteristic of a stabilizing cation- π interaction. In *cc-TpoR-V* (D), the TM helices have rotated 50° counterclockwise, and hydrogen bonds form between indole NH of W508 and the amide side chain of Q509. The active *cc-TpoR-I* fusion protein adopts a conformation rotated by another 50° from *cc-TpoR-V*, where W508 residues would be predicted to clash.

orientations of TM helices corresponding to *cc-TpoR-I*. One can easily visualize the steric clash of W508 that would occur in *cc-TpoR-I* by rotating the helices in Figure 7D counterclockwise by 50°. A 50° rotation converts the *cc-TpoR-V* structure shown in Figure 7D into *cc-TpoR-I*, with both dimers having S498 in the interface (Figure 7A). This might explain why the KWQFP motif impairs activation of TpoR in the absence of Tpo (Staerk *et al*, 2006), and why adopting this structure needs either coiled-coil fusion or ligand binding to the extracellular domain of the receptor, both events requiring strong conformational changes.

Discussion

Our main observation is that the TpoR can signal from six out of seven possible symmetric dimeric orientations (Table I) leading to cell proliferation and different differentiation outputs, such as different abilities to promote megakaryocyte differentiation, cell-to-cell adhesion and myeloproliferative

and myelodysplasia phenotypes *in vivo*. These results are relevant for the mechanisms by which TpoR can function at different stages of differentiation from HSCs to mature platelets in the course of physiologic haematopoiesis. Besides its ligand Tpo, the extracellular domain of TpoR was recently shown to bind a human homologue of a fungal nuclear migration protein (hNUDC) (Chen *et al*, 2010), a novel regulator of haematopoiesis (Miller *et al*, 1999; Gocke *et al*, 2000). It is, thus, conceivable that subtle differences in dimeric conformations are induced by different ligands or by the same ligand in different cell types. On the other hand, our data indicate that signalling by differently oriented TpoR dimers induces *in vivo* myeloproliferative and myelodysplastic disorders. While TpoR has already been discovered to be mutated in Essential Thrombocythemia and Myelofibrosis neoplasms (Pardanani *et al*, 2006; Pikman *et al*, 2006), it is not yet known whether it plays a role in myelodysplasia or mixed myelodysplasia/myeloproliferative disorders. TpoR is one of the three receptors that bind to JAK2 V617F, a mutant

associated with the vast majority of BCR-ABL-negative MPNs (Vainchenker *et al*, 2008). The basis for the observed orientation-dependent signalling of TpoR is represented by the recently identified JM KWQFP motif, which contains the W515, the very residue that maintains the unliganded TpoR inactive (Staerk *et al*, 2006) and which is mutated to Leu, Lys, Ala residues in MPNs (Pardanani *et al*, 2006; Pikman *et al*, 2006; Pecquet *et al*, 2010).

In addition to the W515 mutations in MPNs, several activating mutations in the TM and JM domains of TpoR signalling have been described in patients or cell lines from leukaemia patients (Onishi *et al*, 1996; Malinge *et al*, 2008). Substitutions of serine at position 498 (mouse) or 505 (human) by asparagine activate TpoR signalling in the absence of the ligand (Onishi *et al*, 1996; Ding *et al*, 2004). This residue is in the interface of *cc-TpoR-I* and *cc-TpoR-V* (Figure 7A); however, the phenotype induced by these two fusion proteins *in vivo* was remarkably different. *cc-TpoR-I* induced high numbers of platelets, without cytological features of myeloproliferation in the marrow, and *cc-TpoR-V* induced a picture of myelodysplasia, with small red blood cells and variable levels of thrombocytosis (Figure 5C). Again, subtle differences in dimeric conformation are translated into important differences in biologic effects.

We investigated the orientation requirements for TpoR-mediated signalling and identified several fusion proteins (*cc-TpoR-0*, *-I*, and *-IV*) that induced maximal proliferation of Ba/F3 and UT7 cells, two fusion proteins that supported weaker proliferation and one conformation that was inactive (*cc-TpoR-II*). This situation is different from that of the EpoR where essentially one dimeric orientation is permissive for signalling (Seubert *et al*, 2003). Self-association and packing density of TM sequences were shown to promote signalling by the active EpoR dimeric interface (Kubatzky *et al*, 2001, 2005; Becker *et al*, 2008). That EpoR activation is stringently controlled by dimeric orientation is also supported by the report that only rare anti-EpoR monoclonal antibodies are agonistic (Elliott *et al*, 1996). In contrast, tagged versions of TpoR can be activated by treatment with anti-tag antibodies (Millot *et al*, 2004). While antibodies and other small molecules may activate TpoR signalling in cell lines, our data suggest that careful studies would be necessary in order to establish exactly which biologic function of the TpoR would be induced by agonistic molecules.

Although six out of seven *cc-TpoR* fusion proteins induced cell proliferation, they induced unique effects depending on their dimeric conformation (Figures 2 and 5; Supplementary Figure S4; Table I). *cc-TpoR-IV* induced strong cell-to-cell adhesion (clusters), but was not permissive to induce commitment to the megakaryocyte lineage, as measured by CD41 expression in UT7 cells (Figure 2E). *cc-TpoR-IV*-induced clustering is reminiscent of the effects of high levels of Tpo ligand in TpoR expressing Ba/F3 cells (Figure 2D) or in UT-7-TpoR cells. High local levels of Tpo might exist in niches, such as the osteoblastic niche, where Tpo was reported to maintain quiescence of HSCs (Qian *et al*, 2007; Yoshihara *et al*, 2007). On the other hand, the effect of Tpo was initially described to induce HSC renewal (Alexander *et al*, 1996b) and early haematopoiesis (Solar *et al*, 1998), or to amplify the immediate progenies of HSCs (Abkowitz and Chen, 2007). More recently, we described a senescence-inducing effect of Tpo in late-stage post-mitotic megakaryocytes (Besancenot *et al*,

2010). Such distinct and apparently opposed actions of TpoR might be the result of different dimeric orientations adopted by the receptor in response to various ligand levels, ligand presentation or cell type. Signalling by TpoR was previously shown to require phosphorylation of Y626, which couples the receptor to several signalling pathways, especially to the STAT5/3 and MAP-kinase pathways (Gurney *et al*, 1995; Alexander *et al*, 1996a; Drachman and Kaushansky, 1997; Pecquet *et al*, 2010). The same TpoR cytosolic Y626 residue is absolutely required for induction of myeloproliferative disorder by the TpoR W515A mutant via excessive activation of the STAT3 and MAP-kinase pathways. The strength of signalling by *cc-TpoR* fusion proteins also appears to correlate with levels of phosphorylation of Y626 (Figure 4A). The *in vivo* phenotype induced by *cc-TpoRs* is milder than that of TpoR W515 mutants (Pecquet *et al*, 2010). It will be of interest to determine the precise adaptors associated with phosphorylated Y626, as a function of dimeric orientation (*cc-TpoRs*) or in the case of TpoR W515 mutants.

Transplantation assays in mice with bone marrow cells expressing the active *cc-TpoR* fusion proteins confirmed our hypothesis that different dimeric orientations induce effects that resemble the physiological function of TpoR (*cc-TpoR-I*), or that induce different degrees of myeloproliferative and myelodysplasia disorders (*cc-TpoR-III*, *cc-TpoR-VI* and *cc-TpoR-V*; Figure 5). All seven fusion proteins were equally able to support cell proliferation but lost orientation-specific signalling upon deletion of the KWQFP motif ($\Delta 5cc-TpoR$ fusion proteins). For example, $\Delta 5cc-TpoR-II$ was active after deletion of the KWQFP motif, while *cc-TpoR-II* was not. In addition, $\Delta 5cc-TpoR-IV$ did not induce cell-to-cell adhesion.

These data support our hypothesis that the TpoR could indeed signal through various orientations. One possible explanation for TpoR signalling from multiple dimeric interfaces is the utilization of two JAKs for signal transmission: JAK2 and TYK2. Expression of TYK2 alone, in the absence of JAK2, allows only *cc-TpoR-III* and *cc-TpoR-VI* to activate STAT5 and STAT3 signalling, while expression of JAK2 promotes STAT signalling via multiple interfaces, represented by *cc-TpoR-0*, *-I*, *-III*, and *-IV* (Figure 3). It needs to be further investigated whether the TpoR is signalling *in vivo* via heteromeric JAK2-TYK2 complexes. Of interest, *cc-TpoR-III* and *cc-TpoR-VI* have some common signalling and biologic features: (i) activation of TYK2 signalling in addition to JAK2, (ii) induction of granulocytosis and of different degrees of erythroid and megakaryocytic dysplasia, (iii) lower levels of MAP-kinase activation. A tight control and limitation of the levels of MAP-kinase activation were described to be necessary for normal granulocyte differentiation and maintenance of granulocytic progenitors (Baumann *et al*, 2005; Geest *et al*, 2009).

Coiled coils are symmetric dimers and the models that emerge after the use of coiled-coil fusion proteins have a bias towards symmetric left-handed parallel dimers of α -helices, while membrane proteins may also adopt asymmetric geometries. Further structural studies are required to establish whether the TpoR dimer is symmetric or asymmetric. Cysteine crosslinking (Figure 1C) and alanine insertion mutagenesis (Figure 3C and D) confirmed that the coiled-coil fusion to successive TM residues at the N-terminus of the TM domain enables to dial-in the predicted dimeric

conformations at the outset of the JM cytosolic domain. To gain insight into the dimerization mechanism(s) of the TpoR, we performed computational searches of the TM and JM sequences. In this approach, stable dimer conformations are observed as clusters of structures. A comparison of the computational searches in Figure 7B–D shows that the orientation of the TM helices in *cc-TpoR-II* generates a stable structure. This construct has the only orientation that results in an inactive TpoR dimer. Thus, the natural tendency of the TpoR TM dimer is to adopt the inactive conformation. In this inactive structure, K507 and W508 are oriented as far as possible away from the dimer interface. Rotation of the TM helices in either direction by 50° (leading to *cc-TpoR-V* or *cc-TpoR-VI*) results in partial activity. Functional data indicate that *cc-TpoR-I* represents the closest conformation to the physiologically activated TpoR by Tpo. Although dimeric clusters representing *cc-TpoR-I* are identified by our searches, they are among the higher energy dimer structures (less stable). Thus, this dimeric orientation is unlikely to be adopted spontaneously. Interestingly, in the computational search that includes the KWQFP motif, we could not identify a cluster corresponding to *cc-TpoR-I*. Therefore, it is very likely that the KWQFP motif actively prevents the formation of a dimer corresponding to the *cc-TpoR-I*, and is doing so via a clash between interfacially oriented W508 residues (W515 in the human TpoR). Taken together, functional and computational data indicate that the W508 of the KWQFP motif is crucial for preventing TpoR activation and is required for orientation-specific signalling. Noteworthy, the TM domain of the human TpoR, in the absence of the JM RWQFP motif, was found by bacterial TOXCAT and cysteine cross-linking assays to promote dimerization in three rotationally related orientations, possibly corresponding to inactive, active and partially active states of the receptor (Matthews *et al*, 2011). The proposed interfaces correspond to our coiled coil-induced inactive *cc-TpoR-II* and active orientations.

In conclusion, our data show that several dimeric conformations of the TpoR can support cell proliferation and that specific dimeric orientations imposed by the KWQFP JM motif differ in their signalling output and biologic effects. While one dimeric orientation having S498 in the interface emerges as the best model for the physiologically activated TpoR, and a different one corresponds to the inactive dimer, other orientations induce different degrees of myeloproliferative and myelodysplastic disorders. The system of differently dimerized TpoRs may offer new insights with respect to apportioning a particular signalling pathway to a biologic effect, such as megakaryocytic differentiation or the establishment of myeloid disorders.

Materials and methods

cDNA constructs

The wtTpoR and mutants thereof (TpoR mutants where the KWQFP insert was deleted ($\Delta 5$) or mutated (W508K), the *cc-TpoR* fusion proteins (*cc-TpoR-0-VI*) and the *cc-TpoR* fusion proteins where the KWQFP insert was deleted ($\Delta Sc-TpoR-0-VI$) were subcloned in pMX-IRES-GFP and MSCV-IRES-GFP vectors, as described previously (Staerk *et al*, 2006). All TpoRs and *cc-TpoRs* contain an HA tag at the amino-terminus, after the cleavage site of the signal peptidase (Huang *et al*, 2001). For the crosslinking experiment, the seven cysteine-free coiled-coil receptor backbones were created by introducing a stop codon after amino acid 553, and Leu505 was mutated into Cys. To alter the helical register of the seven murine *cc-TpoR* fusion

proteins and as a proof of principle, alanines were inserted at the end of the TM region. We also mutated Trp508 (mouse equivalent of Trp515 in the human TpoR) into Lys, introducing in the seven *cc-TpoRs*, the pathological W515K mutation found in MPN patients. All constructs were verified by sequencing.

Crosslinking studies

Six-well plates of human BOSC cells were transfected (LipofectamineTM 2000, Invitrogen, Carlsbad, CA) with cDNAs encoding the seven cysteine *cc-TpoR* mutants. Cells were harvested 24 h after transfection and checked for GFP expression prior to crosslinking experiments.

Cells were washed with phosphate-buffered saline and resuspended in 1 ml of phosphate-buffered saline containing 1 mM MgCl₂ and 0.1 mM CaCl₂. Crosslinking was then performed for 10 min at room temperature with a final concentration of the crosslinker *N,N'*-1,2-phenylenedimaleimide (o-PDM) dissolved in DMSO of 100 μ M. Cells were lysed with 1 $\times$ NP-40 lysis buffer containing 2% of β -mercaptoethanol in order to quench excess crosslinker (Kubatzky *et al*, 2005). Lysate samples were analysed by SDS-PAGE using a 4–12% gradient NuPage[®] NOVEX Bis-Tris Gel (Invitrogen) and then transferred onto nitrocellulose membrane. After blocking with 1% milk PBS, membranes were incubated with a 1:500 diluted mouse anti-HA antibody (HA.11, Covance, Berkeley, CA) followed by a 1:2500 diluted peroxidase-conjugated anti-mouse antibody (GE Healthcare UK Ltd, Buckingham, UK). Detection was then performed using enhanced chemiluminescence.

Generation of cell lines

Ba/F3 and UT7 cell lines were transduced with *cc-TpoR* constructs using ecotropic (Liu *et al*, 2000) or amphotropic viral supernatant, respectively (Chagraoui *et al*, 2002; Staerk *et al*, 2006). Cell populations were sorted for equal GFP expression.

Assay for Tpo-independent growth

Transduced and sorted Ba/F3 cells were expanded in IL-3, washed and subsequently placed in RPMI 10% fetal bovine serum without cytokines or Tpo as indicated. UT7 cells expressing the *cc-TpoR* constructs were washed with α -MEM medium (Invitrogen) to remove GM-CSF and placed in cytokine-free α -MEM medium containing 10% FBS or in α -MEM medium containing 10% FBS and Tpo as specified. Cell numbers were counted at the indicated time points.

Immunoprecipitation and immunoblots

Ba/F3 cells were washed with PBS and starved for 2 h in RMPI with 0.1% BSA. Cells were stimulated with 50 ng/ml rmTpo (R&D) or PBS and lysed in NP-40 buffer as described previously (Staerk *et al*, 2006; Pecquet *et al*, 2010). Immunoprecipitation was performed with anti-HA antibodies (Roche Diagnostics, Indianapolis, IN), incubated with protein G agarose (Invitrogen) overnight. Samples were analysed by western blot using anti-pY626 TpoR (Cell Signaling Technology, Danvers, MA) and anti-HA.

Activation of downstream signalling pathways was analysed using anti-p-STAT1, anti-STAT1, anti-p-STAT3, anti-STAT3, or anti-p-Erk1,2, anti-Erk1,2 (Cell Signaling Technology), anti- β -actin (Sigma, St Louis, MO) and anti-HA antibodies (Roche Diagnostics), as described previously (Pecquet *et al*, 2010).

Receptor-recycling assay and cell surface biotinylation

In all, 3×10^7 Ba/F3 cells expressing the indicated *cc-TpoRs* were incubated with 10 mg/ml EZ link sulfo-NHS-Biotin (Thermo Scientific, Rockford, IL) at 4°C for 2 h. Cells were washed in 100 mM glycine-PBS solution to remove excess biotinylation reagent. After biotinylation, cells were maintained in culture for 30 min and then treated or not with 10 μ M JAK inhibitor I (Calbiochem, La Jolla, CA) for additional 30 min. For cell surface immunoprecipitation, cells were incubated with anti-HA antibodies (Roche Diagnostics) for 1 h at 4°C. Cells were then lysed in 1% NP-40 buffer as previously described and lysates were incubated overnight with protein G agarose at 4°C. The beads were washed in lysis buffer and subjected to western blotting by using an anti-biotin/HRP (New England Biolabs, Beverly, MA) and anti-HA antibodies (Roche Diagnostics).

Dual luciferase assays

Activation of STAT5 and STAT3 was analysed by assaying for luciferase production in cells transfected with pLHRE-Luc (Sotiropoulos *et al*, 1995; Storz *et al*, 1999), pGL3bPpr2 (Eyckerman *et al*, 1999) or pGRR5 constructs (Dumoutier *et al*, 2000). To assess MAPK activation we used the pSRE-luc construct (Treisman, 1995). The PI-3-kinase/Akt pathway negatively affects FoxO activity. PI-3-kinase/Akt activation was, therefore, indirectly measured using a 6 × DBE-Luc reporter, which is activated by FoxO family members (Kuiperij *et al*, 2005). The pRL-TK vector (Promega, Madison, WI) containing the renilla luciferase gene under control of the TK promoter was used as internal control. Ba/F3 cells expressing TpoR proteins were starved for 3 h in RPMI/1 mg/ml BSA. Luciferase assays were performed as described (Seubert *et al*, 2003). JAK2-deficient γ 2A fibrosarcoma cells (Kohlhuber *et al*, 1997) were transiently transfected with TpoR proteins and signalling proteins as described (Royer *et al*, 2005). Luminescence was recorded in cell lysates 24 h after using a Perkin-Elmer Victor X Light analyzer.

Surface expression of HA-tagged TpoR and HA-tagged cc-TpoR fusion proteins

Surface expression of HA-TpoR and HA-tagged cc-TpoR fusion proteins was measured by flow cytometry as described (Huang *et al*, 2001; Pecquet *et al*, 2010).

Bone marrow reconstitutions

Bone marrow was isolated from mice 72 h after 5-fluorouracil injection (Chagraoui *et al*, 2002; Pecquet *et al*, 2010). In all, 1×10^6 bone marrow cells transduced with cc-TpoRs or TpoR were injected into lethally irradiated, 6–8-week-old C57Bl6 mice. Reconstitution was assessed by GFP expression in peripheral blood cells using flow cytometry 3 weeks after transplantation. Peripheral blood cell numbers were counted using Melet Schloessing MS9-3. Histopathology analysis and blood smears were performed as described (Pecquet *et al*, 2010).

Computational searches

Low-energy dimer structures of the TM sequence of the TpoR were identified computationally using the program CHI (Adams *et al*, 1996). The program generates canonical helices from the TpoR sequence and constructs a matrix of helix dimers in which each helix is rotated through all possible orientations between 0° and 360° with a sampling size of 45°. For each starting structure, MD simulations with simulated annealing and energy minimization were performed within CHI using the program X-PLOR. The X-PLOR force field uses the TOPH19 and PARAM19 parameters sets with all polar hydrogens included. A non-bonded cutoff of 13 Å is set with a switching function between 10 and 12 Å for the van der Waals and electrostatic terms. Distance constraints are applied between the

backbone O_i and N_{i+4} atoms to maintain an α -helical conformation and between the helix directors to maintain a fixed axial separation. Different computational searches were performed for axial distances of 9.5, 10 and 10.5 Å, and for helices starting with left- and right-handed crossing angles. Both the helix rotation and the crossing angles are allowed to vary during the simulations, and the results are plotted as a function of the angles ϕ_1 and ϕ_2 , corresponding to rotational orientation of each helix in the dimer. The dimer structures migrate from their initial geometry and cluster into low-energy conformations. All calculations were performed *in vacuo*, approximating the low dielectric environment of the membrane. Separate computational searches were performed for TpoR sequence without (I485–L506) and without (I485–P511) the KWQFP insert.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Dr Jean-Marie Scheiff for histopathology diagnosis, Céline Mouton for expert technical assistance, André Tonon for expert flow cytometry expertise, Yan Yin and Julie Klein for technical and graphics assistance. This work was supported by grants from *La Ligue Nationale contre le Cancer* (équipe labellisée 2010, WV), by NIH grant (GM-46732) to SOS, the *Fondation contre le Cancer*, the de Hovre Foundation, the Salus Sanguinis Foundation, the *Action de Recherche Concertée* MEXP31C1 of the *Université catholique de Louvain*, the PAI Program BCHM61B5, Belgium and the Atlantic Philanthropies, New York, and the FNRS (*Mandat d'impulsion*), Belgium (to SNC). JS was supported by fellowships from Daimler-Benz, Exchange Marie Curie, Télévie and Salus Sanguinis Foundations. J-PD is supported by an FRIA PhD fellowship of the FRS-FNRS Belgium. CP was supported by a postdoctoral fellowship of the de Duve Institute, by the PAI Program BCHM61B5 and by the FRS-FNRS. WV is supported by an interface contract between INSERM and IGR. SNC is a Research Associate of the FNRS.

Author contributions: JS, J-PD and SNC planned and performed experiments, interpreted data and wrote paper; CP, EL and IB performed experiments and interpreted data; SOS and WV planned experiments and interpreted data; MI and SOS performed molecular dynamics simulations; HAP interpreted data.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Abkowitz JL, Chen J (2007) Studies of c-Mpl function distinguish the replication of hematopoietic stem cells from the expansion of differentiating clones. *Blood* **109**: 5186–5190
- Adams PD, Engelman DM, Brunger AT (1996) Improved prediction for the structure of the dimeric transmembrane domain of glycoporphin A obtained through global searching. *Proteins* **26**: 257–261
- Alexander WS, Maurer AB, Novak U, Harrison-Smith M (1996a) Tyrosine-599 of the c-Mpl receptor is required for Shc phosphorylation and the induction of cellular differentiation. *EMBO J* **15**: 6531–6540
- Alexander WS, Metcalf D, Dunn AR (1995) Point mutations within a dimer interface homology domain of c-Mpl induce constitutive receptor activity and tumorigenicity. *EMBO J* **14**: 5569–5578
- Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D (1996b) Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood* **87**: 2162–2170
- Baumann M, Frye T, Naqvi T, Gomez-Cambronero J (2005) Normal neutrophil maturation is associated with selective loss of MAP kinase activation by G-CSF. *Leuk Res* **29**: 73–78
- Becker V, Sengupta D, Ketteler R, Ullmann GM, Smith JC, Klingmuller U (2008) Packing density of the erythropoietin receptor transmembrane domain correlates with amplification of biological responses. *Biochemistry* **47**: 11771–11782
- Besancenot R, Chaligne R, Tonetti C, Pasquier F, Marty C, Lecluse Y, Vainchenker W, Constantinescu SN, Giraudier S (2010) A senescence-like cell-cycle arrest occurs during megakaryocytic maturation: implications for physiological and pathological megakaryocytic proliferation. *PLoS Biol* **8**: e1000476
- Brown RJ, Adams JJ, Pelekanos RA, Wan Y, McKinstry WJ, Palethorpe K, Seiber RM, Monks TA, Eidne KA, Parker MW, Waters MJ (2005) Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. *Nat Struct Mol Biol* **12**: 814–821
- Chagraoui H, Komura E, Tulliez M, Giraudier S, Vainchenker W, Wendling F (2002) Prominent role of TGF- β 1 in thrombopoietin-induced myelofibrosis in mice. *Blood* **100**: 3495–3503
- Chen WM, Yu B, Zhang Q, Xu P (2010) Identification of the residues in the extracellular domain of thrombopoietin receptor involved in the binding of thrombopoietin and a nuclear distribution protein (human NUDC). *J Biol Chem* **285**: 26697–26709
- Constantinescu SN, Huang LJ, Nam H, Lodish HF (2001) The erythropoietin receptor cytosolic juxtamembrane domain con-

- tains an essential, precisely oriented, hydrophobic motif. *Mol Cell* **7**: 377–385
- de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, Darbonne WC, Henzel WJ, Wong SC, Kuang WJ, Oles KJ, Hultgren B, Solberg Jr LA, Goeddel DV, Eaton DL (1994) Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature* **369**: 533–538
- Ding J, Komatsu H, Iida S, Yano H, Kusumoto S, Inagaki A, Mori F, Ri M, Ito A, Wakita A, Ishida T, Nitta M, Ueda R (2009) The Asn505 mutation of the c-MPL gene, which causes familial essential thrombocythemia, induces autonomous homodimerization of the c-Mpl protein due to strong amino acid polarity. *Blood* **114**: 3325–3328
- Ding J, Komatsu H, Wakita A, Kato-Uranishi M, Ito M, Satoh A, Tsuboi K, Nitta M, Miyazaki H, Iida S, Ueda R (2004) Familial essential thrombocythemia associated with a dominant-positive activating mutation of the c-MPL gene, which encodes for the receptor for thrombopoietin. *Blood* **103**: 4198–4200
- Drachman JG, Griffin JD, Kaushansky K (1995) The c-Mpl ligand (thrombopoietin) stimulates tyrosine phosphorylation of Jak2, Shc, and c-Mpl. *J Biol Chem* **270**: 4979–4982
- Drachman JG, Kaushansky K (1997) Dissecting the thrombopoietin receptor: functional elements of the Mpl cytoplasmic domain. *Proc Natl Acad Sci USA* **94**: 2350–2355
- Drachman JG, Millett KM, Kaushansky K (1999) Thrombopoietin signal transduction requires functional JAK2, not TYK2. *J Biol Chem* **274**: 13480–13484
- Dumoutier L, Van Roost E, Colau D, Renauld JC (2000) Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor. *Proc Natl Acad Sci USA* **97**: 10144–10149
- Elliott S, Lorenzini T, Yanagihara D, Chang D, Elliott G (1996) Activation of the erythropoietin (EPO) receptor by bivalent anti-EPO receptor antibodies. *J Biol Chem* **271**: 24691–24697
- Eyckerman S, Waelput W, Verhee A, Broekaert D, Vandekerckhove J, Tavernier J (1999) Analysis of Tyr to Phe and fa/fa leptin receptor mutations in the PC12 cell line. *Eur Cytokine Netw* **10**: 549–556
- Geest CR, Buitenhuis M, Groot Koerkamp MJ, Holstege FC, Vellenga E, Coffey PJ (2009) Tight control of MEK-ERK activation is essential in regulating proliferation, survival, and cytokine production of CD34+ -derived neutrophil progenitors. *Blood* **114**: 3402–3412
- Gocke CD, Reaman GH, Stine C, Zhang MY, Osmani SA, Miller BA (2000) The nuclear migration gene NudC and human hematopoiesis. *Leuk Lymphoma* **39**: 447–454
- Gurney AL, Wong SC, Henzel WJ, de Sauvage FJ (1995) Distinct regions of c-Mpl cytoplasmic domain are coupled to the JAK-STAT signal transduction pathway and Shc phosphorylation. *Proc Natl Acad Sci USA* **92**: 5292–5296
- Huang LJ, Constantinescu SN, Lodish HF (2001) The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. *Mol Cell* **8**: 1327–1338
- Kaushansky K (1997) Thrombopoietin: more than a lineage-specific megakaryocyte growth factor. *Stem Cells* **15** (Suppl 1): 97–102; discussion 102–103
- Kohlhuber F, Rogers NC, Watling D, Feng J, Guschin D, Briscoe J, Witthuhn BA, Kutenko SV, Pestka S, Stark GR, Ihle JN, Kerr IM (1997) A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. *Mol Cell Biol* **17**: 695–706
- Komatsu N, Nakauchi H, Miwa A, Ishihara T, Eguchi M, Moroi M, Okada M, Sato Y, Wada H, Yawata Y, Suda T, Miura Y (1991) Establishment and characterization of a human leukemic cell line with megakaryocytic features: dependency on granulocyte-macrophage colony-stimulating factor, interleukin 3, or erythropoietin for growth and survival. *Cancer Res* **51**: 341–348
- Kubatzy KF, Liu W, Goldgraben K, Simmerling C, Smith SO, Constantinescu SN (2005) Structural requirements of the extracellular to transmembrane domain junction for erythropoietin receptor function. *J Biol Chem* **280**: 14844–14854
- Kubatzy KF, Ruan W, Gurezka R, Cohen J, Ketteler R, Watowich SS, Neumann D, Langosch D, Klingmuller U (2001) Self assembly of the transmembrane domain promotes signal transduction through the erythropoietin receptor. *Curr Biol* **11**: 110–115
- Kuiperij HB, van der Horst A, Raaijmakers J, Weijzen S, Medema RH, Bos JL, Burgering BM, Zwartkruis FJ (2005) Activation of FoxO transcription factors contributes to the antiproliferative effect of cAMP. *Oncogene* **24**: 2087–2095
- Liu X, Constantinescu SN, Sun Y, Bogan JS, Hirsch D, Weinberg RA, Lodish HF (2000) Generation of mammalian cells stably expressing multiple genes at predetermined levels. *Anal Biochem* **280**: 20–28
- Lupardus PJ, Skinotis G, Rice AJ, Thomas C, Fischer S, Walz T, Garcia KC (2011) Structural snapshots of full-length Jak1, a transmembrane gp130/IL-6/IL-6Ralpha cytokine receptor complex, and the receptor-Jak1 holocomplex. *Structure* **19**: 45–55
- Malinge S, Ragu C, Della-Valle V, Pisani D, Constantinescu SN, Perez C, Villeval JL, Reinhardt D, Landman-Parker J, Michaux L, Dastugue N, Baruchel A, Vainchenker W, Bourquin JP, Penard-Lacronique V, Bernard OA (2008) Activating mutations in human acute megakaryoblastic leukemia. *Blood* **112**: 4220–4226
- Matthews EE, Thévenin D, Rogers JM, Gotow L, Lira PD, Reiter LA, Brissette WH, Engelman DM (2011) Thrombopoietin receptor activation: transmembrane helix dimerization, rotation and allosteric modulation. *FASEB J* **25**: 2234–2244
- Mattoon D, Gupta K, Doyon J, Loll PJ, DiMaio D (2001) Identification of the transmembrane dimer interface of the bovine papillomavirus E5 protein. *Oncogene* **20**: 3824–3834
- Miller BA, Zhang MY, Gocke CD, De Souza C, Osmani AH, Lynch C, Davies J, Bell L, Osmani SA (1999) A homolog of the fungal nuclear migration gene nudC is involved in normal and malignant human hematopoiesis. *Exp Hematol* **27**: 742–750
- Millot GA, Vainchenker W, Dumenil D, Svinarchuk F (2004) Differential signalling of NH2-terminal flag-labelled thrombopoietin receptor activated by TPO or anti-FLAG antibodies. *Cell Signal* **16**: 355–363
- Miyakawa Y, Oda A, Druker BJ, Kato T, Miyazaki H, Handa M, Ikeda Y (1995) Recombinant thrombopoietin induces rapid protein tyrosine phosphorylation of Janus kinase 2 and Shc in human blood platelets. *Blood* **86**: 23–27
- Miyakawa Y, Oda A, Druker BJ, Miyazaki H, Handa M, Ohashi H, Ikeda Y (1996) Thrombopoietin induces tyrosine phosphorylation of Stat3 and Stat5 in human blood platelets. *Blood* **87**: 439–446
- Miyakawa Y, Rojnuckarin P, Habib T, Kaushansky K (2001) Thrombopoietin induces phosphoinositol 3-kinase activation through SHP2, Gab, and insulin receptor substrate proteins in BAF3 cells and primary murine megakaryocytes. *J Biol Chem* **276**: 2494–2502
- Oda A, Miyakawa Y, Druker BJ, Ishida A, Ozaki K, Ohashi H, Wakui M, Handa M, Watanabe K, Okamoto S, Ikeda Y (1996) Crkl is constitutively tyrosine phosphorylated in platelets from chronic myelogenous leukemia patients and inducibly phosphorylated in normal platelets stimulated by thrombopoietin. *Blood* **88**: 4304–4313
- Onishi M, Mui AL, Morikawa Y, Cho L, Kinoshita S, Nolan GP, Gorman DM, Miyajima A, Kitamura T (1996) Identification of an oncogenic form of the thrombopoietin receptor MPL using retrovirus-mediated gene transfer. *Blood* **88**: 1399–1406
- Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadleigh M, Steensma DP, Elliott MA, Wolanskyj AP, Hogan WJ, McClure RF, Litzow MR, Gilliland DG, Tefferi A (2006) MPLW515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood* **108**: 3472–3476
- Pecquet C, Staerk J, Chaligne R, Goss V, Lee KA, Zhang X, Rush J, Van Hees J, Poirel HA, Scheiff JM, Vainchenker W, Giraudier S, Polakiewicz RD, Constantinescu SN (2010) Induction of myeloproliferative disorder and myelofibrosis by thrombopoietin receptor W515 mutants is mediated by cytosolic tyrosine 112 of the receptor. *Blood* **115**: 1037–1048
- Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, Cuker A, Wernig G, Moore S, Galinsky I, Deangelo DJ, Clark JJ, Lee SJ, Golub TR, Wadleigh M, Gilliland DG, Levine RL (2006) MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med* **3**: e270
- Porteu F, Rouyez MC, Cocault L, Benit L, Charon M, Picard F, Gisselbrecht S, Souyri M, Dusanter-Fourt I (1996) Functional regions of the mouse thrombopoietin receptor cytoplasmic domain: evidence for a critical region which is involved in differentiation and can be complemented by erythropoietin. *Mol Cell Biol* **16**: 2473–2482

- Qian H, Buza-Vidas N, Hyland CD, Jensen CT, Antonchuk J, Mansson R, Thoren LA, Ekblom M, Alexander WS, Jacobsen SE (2007) Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell* **1**: 671–684
- Royer Y, Staerk J, Costuleanu M, Courtoy PJ, Constantinescu SN (2005) Janus kinases affect thrombopoietin receptor cell surface localization and stability. *J Biol Chem* **280**: 27251–27261
- Seubert N, Royer Y, Staerk J, Kubatzky KF, Moucadel V, Krishnakumar S, Smith SO, Constantinescu SN (2003) Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer. *Mol Cell* **12**: 1239–1250
- Skiniotis G, Lupardus PJ, Martick M, Walz T, Garcia KC (2008) Structural organization of a full-length gp130/LIF-R cytokine receptor transmembrane complex. *Mol Cell* **31**: 737–748
- Solar GP, Kerr WG, Zeigler FC, Hess D, Donahue C, de Sauvage FJ, Eaton DL (1998) Role of c-mpl in early hematopoiesis. *Blood* **92**: 4–10
- Sotiropoulos A, Moutoussamy S, Binart N, Kelly PA, Finidori J (1995) The membrane proximal region of the cytoplasmic domain of the growth hormone receptor is involved in the activation of Stat 3. *FEBS Lett* **369**: 169–172
- Souyri M, Vigon I, Penciolelli JF, Heard JM, Tambourin P, Wendling F (1990) A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. *Cell* **63**: 1137–1147
- Staerk J, Lacout C, Smith SO, Vainchenker W, Constantinescu SN (2006) An amphipathic motif at the transmembrane-cytoplasmic junction prevents autonomous activation of the thrombopoietin receptor. *Blood* **107**: 1864–1871
- Storz P, Doppler H, Horn-Muller J, Groner B, Pfizenmaier K, Muller G (1999) A cellular reporter assay to monitor insulin receptor kinase activity based on STAT 5-dependent luciferase gene expression. *Anal Biochem* **276**: 97–104
- Swaminathan K, Flynn P, Reece RJ, Marmorstein R (1997) Crystal structure of a PUT3-DNA complex reveals a novel mechanism for DNA recognition by a protein containing a Zn2Cys6 binuclear cluster. *Nat Struct Biol* **4**: 751–759
- Thompson JE, Cubbon RM, Cummings RT, Wicker LS, Frankshun R, Cunningham BR, Cameron PM, Meinke PT, Liverton N, Weng Y, DeMartino JA (2002) Photochemical preparation of a pyridone containing tetracycline: a Jak protein kinase inhibitor. *Bioorg Med Chem Lett* **12**: 1219–1223
- Treisman R (1995) Journey to the surface of the cell: Fos regulation and the SRE. *EMBO J* **14**: 4905–4913
- Vainchenker W, Dusa A, Constantinescu SN (2008) JAKs in pathology: role of Janus kinases in hematopoietic malignancies and immunodeficiencies. *Semin Cell Dev Biol* **19**: 385–393
- Walters KJ, Dayie KT, Reece RJ, Ptashne M, Wagner G (1997) Structure and mobility of the PUT3 dimer. *Nat Struct Biol* **4**: 744–750
- Watowich SS, Wu H, Socolovsky M, Klingmuller U, Constantinescu SN, Lodish HF (1996) Cytokine receptor signal transduction and the control of hematopoietic cell development. *Annu Rev Cell Dev Biol* **12**: 91–128
- Yoshihara H, Arai F, Hosokawa K, Hagiwara T, Takubo K, Nakamura Y, Gomei Y, Iwasaki H, Matsuoka S, Miyamoto K, Miyazaki H, Takahashi T, Suda T (2007) Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell* **1**: 685–697