Supporting Information

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SI Substitutions at Positions 514, 516, 517, and 518 Do Not Induce Activation of TpoR

The RWQFP motif is largely conserved in thrombopoietin receptor (TpoR) (Fig. S1), suggesting that each residue in the motif plays a role in the structure or activation mechanism of the receptor. Fig. S2 shows the ability of additional mutations at positions 514, 516, 517, and 518 to constitutively activate the receptor. The mutants were expressed in γ-2A cells cotransfected with JAK2 and tested for ligand-dependent and -independent induction of STAT5-dependent transcriptional activity using the dual luciferase assay.

SI Constitutive Activation of TpoR by W515 Mutants Requires JAK2

In cells, TpoR signals mainly via JAK2 and to a lesser extent via TYK2 (1). We tested signaling of W515 mutants via STATs using the luciferase reporter assay in γ-2A cells, which are deficient in JAK2 but not TYK2 (2). Constitutive activation of STAT5 transcriptional activity is detected in γ-2A cells transfected with JAK2 for both the W515K and W515L mutations. In contrast, the level of ligand-induced and ligand-independent signaling is low in γ-2A cells transfected with TYK2. Data presented in Figs. S3 and S4 indicate that the TpoR W515K mutant is constitutively active only in the presence of JAK2 and not TYK2. Studies performed with engineered TpoR dimers indicate that the W515K mutation seems to induce a dimer interface that is not permissive for activation of TYK2 (Fig. S4). Thus, the dimer induced by the W515K must be distinct from the physiological dimer induced by Tpo.

We next compared signaling by one conformation that is inactive via TYK2 (cc-TpoR-I) in the presence of a constitutive active mutant of TYK2, TYK2 V678F, a mutant homologous to the activating V617F mutation in JAK2 (3). cc-TpoR-I becomes active in the presence of TYK2 V678F (Fig. S4*C*), suggesting that inactive dimeric conformations like cc-TpoR-I fail to trigger TYK2 activation, but once this step accomplished, the receptor dimer could support downstream signaling.

SI Comparison of the Association of TpoR with the Association of EpoR

Erythropoietin receptor (EpoR) and TpoR were fused in frame with Gluc1 and Gluc2 fragments and tested for *Gaussia princeps* luciferase complementation (Fig. S5).

SI Temperature Dependence of Deuterium MAS Side Bands in the TpoR TM Peptides Labeled at Leu512

The loss of NMR intensity in the deuterium magic angle spinning (MAS) spectra of wild-type TpoR transmembrane (TM) peptides suggests that the motion of the peptide interferes with averaging by MAS. To test this idea, the sample temperature was decreased to slow the rotational diffusion of the peptide in the membrane bilayer (Fig. S6).

SI Secondary Structure of the RWQFP Insert by Solution NMR Spectroscopy

Structural studies of TM peptides solubilized in detergent micelles can provide insights into peptide structure. We have undertaken solution NMR measurements of peptides corresponding to the TpoR TM–juxtamembrane (JM) sequence (residues 481–520) to complement our previous solid-state NMR measurements in membrane bilayers (4) and to complement the analytical ultracentrifugation measurements in this study of TpoR peptides solubilized in detergent micelles (Fig. S7). The peptides were expressed as an MBP-His-fusion protein as described by Itaya et al. (5). The MBP and His tags were removed by cleavage with Tobacco Etch Virus (TEV) protease, and the purified protein labeled with ^{13}C and ^{15}N was solubilized in deuterated SDS for NMR experiments.

1. 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(29):27251–27261.
2. Kohlhuber F, et al. (1997) A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. *Mol Cell Biol* 17(2):695–706.
3. Staerk J, Kallin A, Demoulin JB, Vainchenker W, Constantinescu SN (2005) JAK1 and Tyk2 activation by the homologous polycythemia vera JAK2 V617F mutation: Cross-talk with IGF1 receptor. *J Biol Chem* 280(51):41893–41899.
4. Kubatzky KF, et al. (2005) Structural requirements of the extracellular to transmembrane domain junction for erythropoietin receptor function. *J Biol Chem* 280(15):14844–14854.
5. Itaya M, Brett IC, Smith SO (2012) Synthesis, purification, and characterization of single helix membrane peptides and proteins for NMR spectroscopy. *Methods Mol Biol* 831: 333–357.


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Rabbit              VLSLSALLGLLLLKWQFPAHYRRLRHALWPSLPDLHRVLGQYLRDTAALSPSKATVT-DS 79
Mouse               VLSLSALLGLLLLKWQFPAHYRRLRHALWPSLPDLHRVLGQYLRDTAALSPSKATVT-DS 550
Cattle              VLSVSALLGLLLLRWQFPEHYRSLRHALWPSLPDLHRVLGQYLRDTAALSPPKAAVS-DV 557
Human               VLGLSAVLGLLLLRWQFPAHYRRLRHALWPSLPDLHRVLGQYLRDTAALSPPKATVS-DT 559
Crabeating monkey   VLGLSAVLGLLLLRWQFPAHYRRLRHALWPSLPDLHRVLGQYLRDTAALSPPKATVS-DT 551
Marmoset            VLGLSALLGLLLLRWKFPAHYRRLRHVLWPSLPDLHRVLGQYLRDTAAPSPPKATVS-DT 565
Guinea pig          VLGLSALLGLLLLRWKFPAHYRRLRHVLWPSLPDLHRVLGQYLRDTAAPSPPKATVS-DT 552
Rhesus monkey       VLGLSALLGLLLLRWKFPAHYRRLRHVLWPSLPDLHRVLGQYLRDTAAPSPPKATVS-DA 552
Squirrel monkey     VLGLSALLGLLLLRWKFPAHYRRLRHVLWPSLPDLHRVLGQYLRDTAAPSPPKATVS-DA 552
                                                    -BOX1-
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Fig. S1. Alignment of TpoR JM sequences (including W515, red) from different species. Box 1, conserved PxxPxP/x motif required for JAK binding in cytokine receptor type I superfamily.

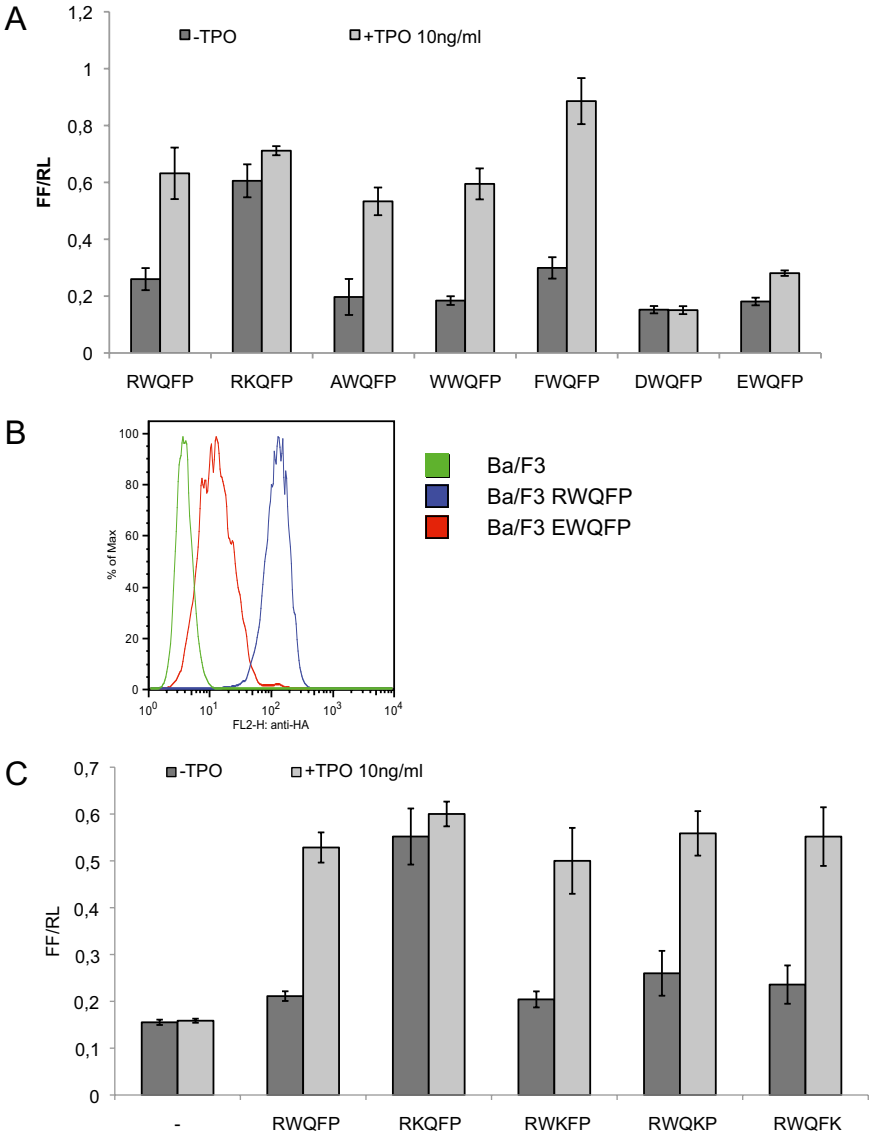

Fig. S2. (*A*) The first residue of the RWQFP motif is conserved as a positively charged arginine or lysine. Mutation of R514 to nonpolar amino acids with either a large (Phe, Trp) or small (Ala) side chain yields receptors that behave like wild-type TpoR (i.e., they do not exhibit constitutive signaling in the absence of Tpo ligand and are fully active in the presence of Tpo ligand). These results demonstrate that a positively charged residue at position 514 is not required for the inhibitory action of W515. Negative charges at position 514 (i.e., R514E) yield receptors that are defective in cell surface localization (*B*), as shown by flow cytometry of cells transduced with equal levels of receptor and stained for HA, the epitope tag placed at the amino terminus of TpoR. Positively charged residues typically define the cytoplasmic boundary of type I membrane proteins (the positive inside rule), and the conservation of Arg or Lys at position 514 may facilitate protein insertion into the endoplasmic reticulum (ER) membrane during synthesis. (*C*) The last three residues of the RWQFP motif are conserved as glutamine or lysine at position 516, phenylalanine at position 517, and proline at position 518. Individual mutations of W515, Q516, F517, and P518 to lysine show that only W515K induces constitutive signaling via JAK2 and STAT5. These results suggest that the occurrence of polar residues at these positions does not influence the ability of W515 to inhibit TpoR activity. With respect to the model proposed for TpoR activation, the presence of charged residues C-terminal to W515 does not influence the ability of the tryptophan side chain to partition into the membrane bilayer and prevent receptor dimerization.

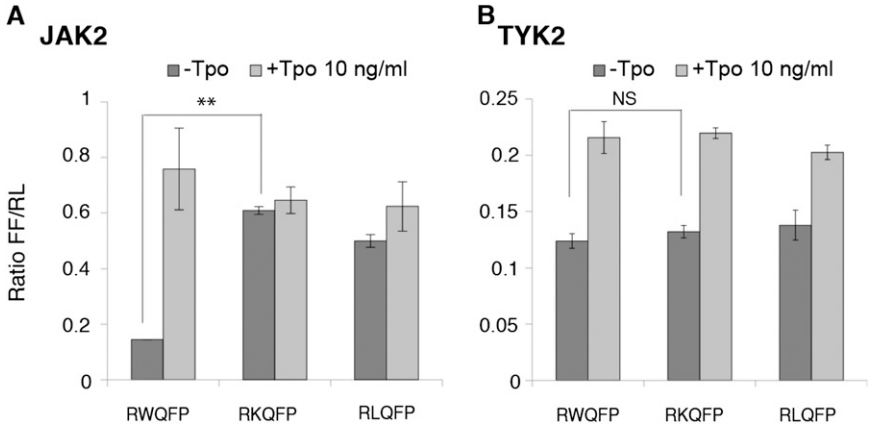

Fig. S3. (*A*) Transient transfection of wild-type TpoR or W515 mutants in γ-2A cells did not induce ligand-independent or -dependent signaling in the absence of JAK2. In contrast, the TpoR W515 mutants were constitutively active by cotransfecting with JAK2. (*B*) Overexpression of TYK2 in γ-2A cells did not allow constitutive activation by TpoR W515 mutants, whereas the Tpo-dependent signaling is still observed. Thus, W515 mutations constitutively activate signaling of TpoR via ligand-independent activation of JAK2.

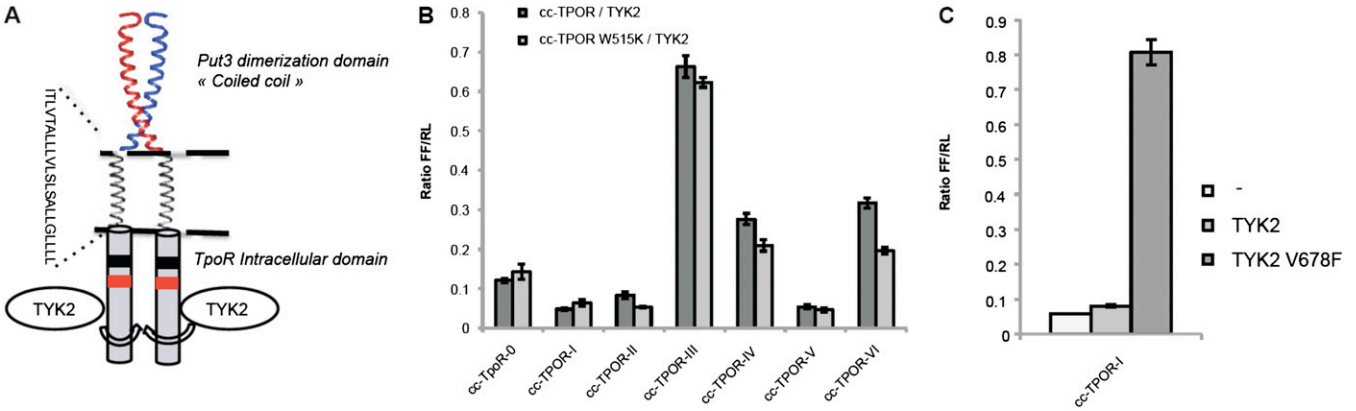

Fig. S4. Introduction of the W515K mutation in engineered coiled-coil TpoR dimers does not change signaling via TYK2. We previously used various engineered coiled-coil TpoR dimers (*cc*-TpoRs) (*A*) that mimic each of seven possible dimer orientations (1). This was accomplished by fusing the Put3 28 amino acid coiled coil with the TM domain of TpoR (cc-TpoR-0) or with progressive deletions of the N-terminal residues of the TpoR TM domain (cc-TpoR-I, II, III, IV, V, and VI). The seven fusion proteins adopt the predicted different dimeric orientations, as shown by cross-linking studies (1). By using these *cc*-TpoR dimers, we found that TYK2 activation can be triggered by only one orientation, represented by cc-TpoR-III and cc-TpoR-VI fusion proteins (1). In contrast, JAK2 could signal in all orientations, except one, cc-TpoR-II (1). Here we show that for TYK2 signaling, introduction of the W515K mutation (human receptor numbering, actually W508K in the mouse TpoR used for the cc-TpoR fusions) does not induce any change from the situation where the wild-type W515 residue is present (*B*, solid bars). Thus, again, the dimeric interface required for TYK2 signaling remains the same after introduction of the W515K mutation, and this is different from the case of JAK2 (1). These differences might be explained by the fact that JAK2 and TYK2 do not bind to the same cytosolic part of the receptor, and TYK2 is believed to bind a lower part of the cytosolic tail, below the Box1 motif (2). One of the dimeric orientations of TpoR that does not support wild type TYK2 signaling, cc-TpoR-I, can support signaling by the constitutive active TYK2 V678F mutant (3), (*C*, solid bars), indicating the defect concerns the activation step and not binding.

1. Staerk J, et al. (2011) Orientation-specific signalling by thrombopoietin receptor dimers. *EMBO J* 30(21):4398–4413.
2. 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(29): 27251–27261.
3. Staerk J, Kallin A, Demoulin J.-B, Vainchenker W, Constantinescu SN (2005) JAK1 and Tyk2 activation by the homologous polycythemia vera JAK2 V617F mutation: Cross talk with IGF1 receptor. *J Biol Chem* 280(51):41893–41899.

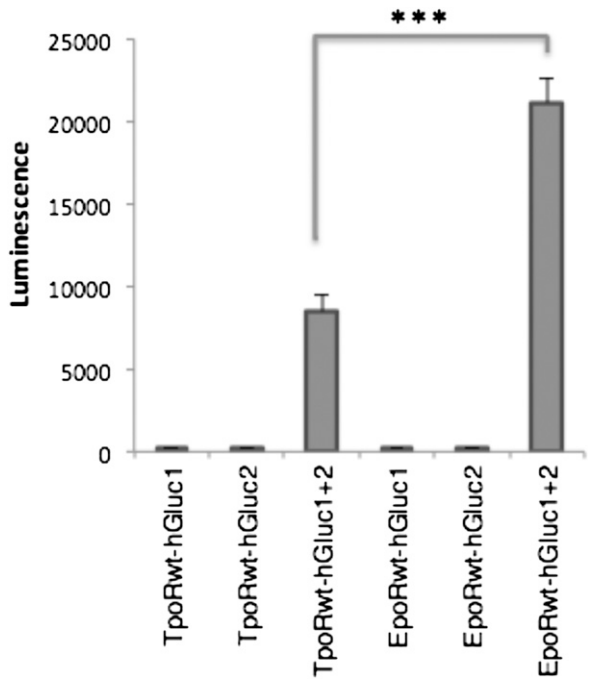

Fig. S5. *Gaussia princeps* luciferase protein fragment complementation assay on HEK293 cells expressing hGluc1 and hGluc2-tagged TpoR and EpoR. Average luminescence of triplicate values ± SD normalized to expression levels of Gluc-tagged proteins by Western blot is shown for one representative experiment out of at least three. ***$P < 0.0001$, ANOVA.

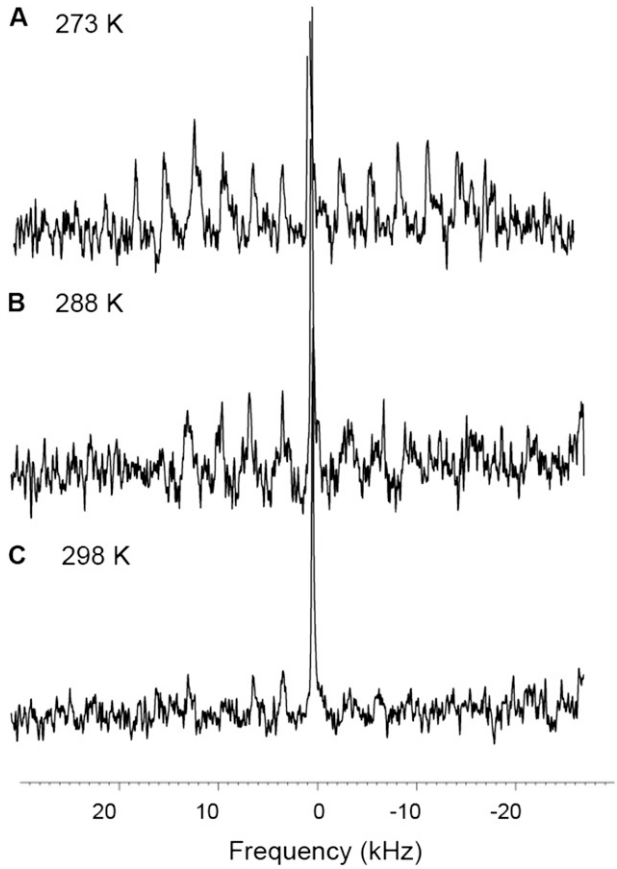

Fig. S6. Temperature dependence of ^{2}H side band pattern in the TpoR TM peptides labeled at Leu512. As the temperature is raised, there is loss of intensity of the deuterium MAS side bands due to increased rotational diffusion of the TM domain of the TpoR. The temperature dependence of the signal intensity is consistent with the suggestion that the frequency of the molecular motion interferes with averaging by MAS. For the W515 mutant peptides, dimerization decreases rotational diffusion, which results in an increase in the side band intensity.

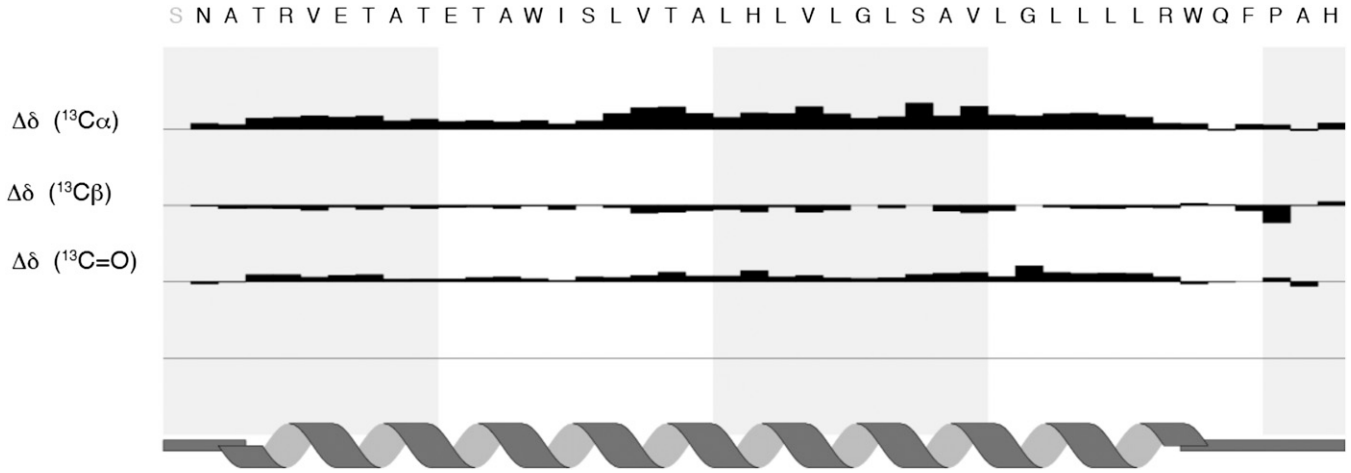

Fig. S7. The TM helix of TpoR TM peptides (residues 481–520) extends through Arg514-Trp515. Solution NMR chemical shift changes characteristic of protein secondary structure are plotted for the TM and JM (RWQFP) region of the TpoR. The output is from the program CCPN (1), which uses the program DANGLE (2) to predict dihedral angle values (and thus, local secondary structure) from the chemical shifts of backbone atoms. The Δδ values for the secondary chemical shifts of the Cα, Cβ, and C′ atoms are measured relative to random coil chemical shifts. Chemical shift deviations from the random coil values provide insight into secondary structure adopted by the peptide, as described by Wishart et al. (3). These deviations from random coil values support the DANGLE-derived data indicating that the residues Arg514-Trp515 are in an α-helical conformation.

1. Vranken WF, et al. (2005) The CCPN data model for NMR spectroscopy: Development of a software pipeline. *Proteins* 59(4):687–696.
2. Cheung MS, Maguire ML, Stevens TJ, Broadhurst RW (2010) DANGLE: A Bayesian inferential method for predicting protein backbone dihedral angles and secondary structure. *J Magn Reson* 202(2):223–233.
3. Wishart DS, Sykes BD (1994) The 13C chemical-shift index: A simple method for the identification of protein secondary structure using 13C chemical-shift data. *J Biomol NMR* 4(2): 171–180.