

## His499 Regulates Dimerization and Prevents Oncogenic Activation by Asparagine Mutations of the Human Thrombopoietin Receptor\*

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Running Title: Tpo Receptor Dimerization and Activation

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**ABSTRACT**

Ligand binding to the extracellular domain of the thrombopoietin receptor (TpoR) imparts a specific orientation on the receptors' transmembrane (TM) and intracellular domains that is required for physiologic activation via receptor dimerization. To map the inactive and active dimeric orientations of the TM helices, we performed asparagine (Asn) scanning mutagenesis of the TM domains of the murine and human TpoR. Substitution of Asn at only one position (S505N) activated the human receptor, while Asn substitutions at several positions activated the murine receptor. Second-site mutational studies indicate that His499 near the N-terminus of the TM domain is responsible for protecting the human receptor from activation by Asn mutations. Structural studies reveal that the sequence preceding His499 is helical in the murine receptor, but non-helical in peptides corresponding to the TM domain of the inactive human receptor. The activating S505N mutation and the small molecule agonist eltrombopag both induce helix in this region of the TM domain, and are associated with dimerization and activation of the human receptor. Thus, His499 regulates the activation of human TpoR and provides additional protection against

activating mutations, such as oncogenic Asn mutations in the TM domain.

The thrombopoietin (Tpo)<sup>2</sup> or Mpl receptor regulates megakaryocyte development and supports hematopoietic stem cell survival (1). The Tpo receptor (TpoR) shares general homology with other members of the cytokine receptor family, including the erythropoietin (Epo) and granulocyte colony stimulating factor (GCSF) receptors (2). These receptors contain a large extracellular domain that binds signaling hormones in a 2:1 ratio of receptor to hormone (3-5) along with a single membrane spanning helix and an intracellular domain that associates non-covalently with a tyrosine kinase.

The minimal functional region of the intracellular domain stretches from the boundary of transmembrane (TM) domain and includes three regions with limited sequence homology: a conserved switch region, Box 1 and Box 2. The switch region contains a hydrophobic motif whose orientation is required for signaling (6,7). Box 1 is characterized by a proline rich motif (PXXPP) that is crucial for cytokine receptors to bind and activate their cognate Janus kinases (JAKs) (8-10). Box 2 is loosely defined as a motif with acidic and hydrophobic residues and is required for maximal

cell growth in response to cytokines.

The TpoR has several unique features. First, the extracellular domain is duplicated, and deletion of the membrane distal domain causes constitutive activation (11). Second, there are 5 amino acids (RWQFP) inserted directly C-terminal to the TM domain whose deletion or mutation causes ligand-independent receptor activation (12). Finally, clinically relevant mutations have been identified that induce constitutive receptor activity in the extracellular juxtamembrane (JM) region (T487A, (13)), the TM domain (S505N, (14,15)) and in the intracellular RWQFP motif (W515K/L/A, (12,16-19)). The fact that these are clustered in and around the TM domain reflects the importance of this region in the regulation of receptor activation.

It is generally accepted that the active signaling form of cytokine receptors is a dimer, but whether the inactive state is dimeric or monomeric is unknown. Several lines of evidence exist to suggest that the Epo receptor (EpoR), one of the most extensively studied cytokine receptors, exists as a preformed dimer and activation is induced by structural reorganization of the dimer after hormone binding. Crystallographic studies of the EpoR extracellular domain in the presence and absence of ligand suggest that the full-length inactive receptor is dimeric and hormone binding causes subunit rotation for activation (4,20). A similar model has been proposed for the structurally related growth hormone receptor (21-23). The first evidence that the TM domain contributes to dimerization of the inactive EpoR came from co-patching measurements using chimaeras of the prolactin and Epo receptors in HEK293-derived BOSC cells (24). These studies found the TM domain of the EpoR to be both necessary and sufficient for receptor dimerization in the context of the full-length receptor. This conclusion is supported by biophysical measurements showing that the isolated TM domain of the murine EpoR associates as a dimer (25). However, unlike the TpoR, the TM domain of the EpoR is not a hot-spot for activating mutations.

Much less is known about whether the TpoR forms an inactive dimer that changes orientation upon ligand binding. Studies using coiled-coil fusions to the TM-cytosolic domain of the murine TpoR showed that one dimeric interface is inactive, one interface mimics the physiologic functions of TpoR and several orientations induce partial levels of activation *in*

*vitro* and in bone marrow transplantation experiments *in vivo* in mice (26). However, these studies use an artificial receptor where the large, native extracellular domain dimer (preformed or ligand-bound) is replaced with a short, tightly associated coiled coil that forces symmetric dimerization. Biophysical studies on the association of the human TpoR TM domain using cysteine mutagenesis and the TOXCAT reporter assay indicate that the wild type TM sequence (residues 491-515) associates as a dimer (27). However, we have recently shown that the five amino acid (RWQFP) insert at the intracellular TM-JM boundary of the TpoR impairs dimerization of the upstream TpoR TM helix (28). We further showed that mutation of Trp515 within this motif leads to dimerization of the TM-JM region of TpoR by changing the orientation of the TM helix relative to the membrane bilayer normal (28). While the studies on isolated TM-JM regions of TpoR reveal that they have a propensity to dimerize, it remains an open question as to whether the full-length receptor is primarily dimeric, monomeric or exists in a monomer-dimer equilibrium.

In this study, asparagine-scanning mutagenesis of the murine TpoR (mTpoR) and human TpoR (hTpoR) was used to modulate the orientation and dimerization of the TM domain of the receptor. The asparagine mutations provide an alternative to the coiled-coil dimerization sequences we have previously used to engineer TpoR dimers (26). Asparagine is able to mediate dimerization of TM helices through strong hydrogen bonding interactions, and often does so in a sequence-independent fashion (29-31). Moreover, asparagine-scanning mutagenesis addresses the question of whether the clinically observed S505N mutation in humans is unique or just one of many mutations that lead to constitutive activation. We make use of full-length receptors, truncated receptors, and peptides corresponding to only the TM-JM domain for comparative studies between the murine and human receptors. Together, our results provide the structural basis for why the hTpoR cannot be activated by asparagine mutations that readily activate the mTpoR, suggesting an evolutionary mechanism that results in resistance to oncogenic activation of hTpoR.

## EXPERIMENTAL PROCEDURES

*cDNA Constructs and Cells*—Mouse and human TpoR cDNAs cloned in the pMX-IRES-

GFP bicistronic vector contained a hemagglutinin (HA) tag at the NH<sub>2</sub>-terminus. Site-directed mutagenesis was achieved by the overlapping-extension PCR amplification method. The PCR reaction was performed using 1 µl of pfuTurbo DNA polymerase (2.5 U/µl) following the protocol in the QuickChange XL site-directed mutagenesis kit (Stratagene, Santa Clara, CA), as described (28). Sanger sequencing of the entire coding region was used for each construct.

**Cell Proliferation Assay**—Pro-B interleukin 3 (IL-3) dependent Ba/F3 cells were maintained in RPMI 1640 and 10% fetal calf serum using WEHI cell supernatant as a source of IL-3. TpoR Asn variants subcloned in the pMX-IRES-GFP were used for packaging ectopic retrovirus in BOSC cells. Cells were infected in medium containing polybrene with packaged retrovirus and cell pools expressing similar levels (top 15%) of green fluorescence protein (GFP) coded by the bicistronic retroviral vectors were isolated using flow cytometry cell sorting. Cell-surface TpoR was assessed by staining with anti-HA antibodies (Covance, Princeton, NJ; 10 µg/ml) and phycoerythrin conjugated donkey anti-mouse IgG secondary antibodies (28). The levels of TpoR total protein expression were determined by Western blotting using anti-HA antibodies (Roche Pharmaceuticals). For determining the maturation state of the N-glycosylated TpoR mutants we used digestion with Endoglycosidase H (Endo-H) for 4 h followed by Western blotting with anti-HA antibodies, as described (6). Cell proliferation in the presence or absence of cytokines was measured using the CellTiter-Glo Luminescent cell viability assay (Promega, Madison, WI).

**Dual-luciferase Transcriptional Assay of STAT Activation**—Transcriptional activation of STAT3 and STAT5 downstream TpoR variants was assessed in γ-2A cells (JAK2-deficient cells reconstituted with JAK2) using the dual-luciferase assay kit (Promega, Madison, WI). The assay measures luciferase luminescence after transient transfection of the pGRR5 reporter containing the firefly luciferase and STAT3/STAT5 binding sites and a control reporter (pRL-TK) containing *Renilla* luciferase. The ratio luminescence of these two luciferases provides a measure of STAT activation (26,32). Luminescence was detected in cell lysates with a Perkin-Elmer Victor-X light analyzer.

**Split-Luciferase Complementation Assay of Receptor Dimerization**—TpoR dimerization was

measured using a complementation assay in which two halves of *Gaussia princeps* luciferase were attached separately to the C-terminus of TpoR. The cDNA of TpoR was inserted between the NotI and ClaI restriction sites of the pcDNA3.1/Zeo vector upstream of either the hGluc1 fragment coding amino acids 1–93 (hGluc1) or the fragment coding amino acids 94–169 (hGluc2) of *Gaussia princeps* luciferase (33). BOSC cells plated on 24-well plates were co-transfected with the plasmids (coding hGluc1 and hGluc2) using Lipofectamine (Invitrogen). Twenty-four hours after transfection cells were trypsinized and resuspended in 150 µL of DMEM/F-12 without phenol red (Gibco). Cell samples were flash-frozen on dry ice for 10 min and thawed in a water bath at 37 °C for 10 min (cycle was repeated twice). After centrifugation (10,000 g, 5 min) the supernatants were collected and aliquoted (100 µL per well) in 96-well white plates (Nunc, Thermo Fisher Scientific) to measure the luciferase activity. Native coelenterazine (NanoLight Technology) was reconstituted as a stock solution (1 mg/mL in methanol) and diluted to a final concentration of 20 µM in DMEM/F12 at room temperature for injection (100 µL). Signal intensities (integrated over 10 s, with an injection delay of 2 s) were read on a Perkin-Elmer Victor X light analyzer. Aliquots of the supernatants were also used to detect the proteins on Western blots (33).

**Membrane Reconstitution of TM-JM peptides for Biophysical Studies**—Peptides (40 residues in length) corresponding to the TM domain and JM regions of hTpoR (TRVETATETAWISLVLTALHLVLGLSAVLGLLLLRWQFPAH) and mTpoR (ARVSTGSETAWITLVTALLLVLSLSALLGLLLKWQFPAH) were synthesized using solid-phase methods. Deuterated (5,5,5-D<sub>3</sub>) leucine and deuterium-free water were purchased from Cambridge Isotope Laboratories (Andover, MA). Other amino acids and octyl-β-glucoside were obtained from Sigma Chemical (St. Louis, MO). The crude peptide (5–15 mg) was purified by reverse-phase HPLC on a C4 column using gradient elution. Purified TpoR peptides were reconstituted by detergent dialysis using DMPC and DMPG (10:3) and a ~1:60 peptide-to-lipid ratio. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL) as lyophilized powders and used without further purification. The lipid, detergent and peptide were dissolved in organic solvent (e.g. hexafluoro-2-propanol or trifluoroethanol). The mixture of lipid, detergent

and peptide in organic solvent was incubated at 37 °C for over 2 h, and the solvent was removed by evaporation using a stream of argon gas and then the sample was placed under vacuum. The dry mixture was rehydrated with phosphate buffer (10 mM phosphate and 50 mM NaCl, pH 7.4) such that the final concentration of octyl- $\beta$ -glucoside was 5-10% (w/v). The rehydrated sample was then stirred slowly for at least 6 h, and the octyl- $\beta$ -glucoside was removed by dialysis using Spectra-Por dialysis tubing (3500 MW cutoff) for 24 h against phosphate buffer at 37 °C. To confirm that the membrane-reconstituted peptides did not aggregate during dialysis, the samples were loaded onto 10-40% (w/v) sucrose gradients and purified. Samples for IR spectroscopy were dialyzed a second time to remove sucrose. The dichroic ratios were compared before and after dialysis. Samples for NMR spectroscopy were pelleted by ultracentrifugation and, if needed, washed twice to remove sucrose. The pelleted samples were lyophilized, rehydrated with deuterium-depleted water ( $50 \pm 5\%$  weight) and incubated at 37 °C for 24 h before loading into NMR rotors.

**Polarized IR Spectroscopy**—Polarized attenuated total reflection (ATR) Fourier transform infrared (FTIR) spectra were obtained on a Bruker IFS 66V/S spectrometer. Reconstituted hTpoR and mTpoR TM-JM peptides (50-100  $\mu$ g) were layered onto a germanium internal reflection element using a slow flow of air or argon gas directed at an oblique angle to the ATR plate to form an oriented multilamellar lipid-peptide film. IR spectra were obtained using light having either parallel or perpendicular polarization relative to the surface of the germanium plate. The dichroic ratio ( $R_{ATR}$ ) is defined as the ratio between the absorption of parallel ( $A_{||}$ ) and perpendicular ( $A_{\perp}$ ) polarized light. The frequency of the amide I vibration is sensitive to the global secondary structure of protein and the dichroic ratio of the amide I band provides information on the orientation of TM helices relative to the plane of the membrane (34). A detailed analysis has been described previously (34). 1000 scans were acquired and averaged for each sample at a resolution of 4  $\text{cm}^{-1}$ . The calculation of the helix tilt angles from the observed dichroic ratios was based on a value of the transition moment angle ( $\alpha$ ) of 39.5° and a maximum membrane order parameter ( $S_{\text{mem}}$ ) of 0.85 (34). The use of the values of  $\alpha$  and  $S_{\text{mem}}$  are based on measurements of bacteriorhodopsin, an integral membrane protein with 7 transmembrane

helices of known structure and which forms well-ordered, two-dimensional membrane arrays. We do not independently know the disorder of the reconstituted TM helices in our samples. Consequently, we assume that the maximum order obtained is equal to that of bacteriorhodopsin obtained from diffraction measurements (35). This results in a lower estimate for the helix tilt angles reported in Table 1 (i.e. greater disorder in our membrane reconstitutions than that found for bacteriorhodopsin would correspond to lower actual tilt angles for an ideal sample with no disorder).

**Solid-state NMR Spectroscopy**—Deuterium NMR spectra were obtained at a  $^2\text{H}$  frequency of 55.2 MHz on a Bruker Avance NMR spectrometer using a 4 mm MAS probe. A MAS frequency of 3 kHz was used to increase the number of spinning side bands. Single pulse excitation was employed using a 4 - 7.5  $\mu\text{sec}$  90° pulse, followed by a 4.5  $\mu\text{sec}$  delay before data acquisition. The repetition delay was 0.25 sec. A total of 600,000 - 1,500,000 transients were averaged for each spectrum and processed using a 100 Hz exponential line broadening function. Spectra were obtained at 25 °C.

2D dipolar assisted rotational resonance (DARR) NMR experiments (36) were performed at a  $^{13}\text{C}$  frequency of 125 MHz on a Bruker AVANCE spectrometer. The MAS spinning rate was set in the range of 9-11 KHz ( $\pm 5$  Hz). The ramped amplitude cross polarization contact time was 2 ms. Two-pulse phase-modulated decoupling was used during the evolution and acquisition periods with a radiofrequency field strength of 80 kHz. Internuclear  $^{13}\text{C}\cdots^{13}\text{C}$  distance constraints were obtained from using a mixing time of 600 ms. The sample temperature was maintained at -50 °C ( $\pm 2$  °C). All  $^{13}\text{C}$  solid-state MAS NMR spectra were externally referenced to the  $^{13}\text{C}$  resonance of neat TMS at 0 ppm at room temperature. Using TMS as the external reference, we calibrated the carbonyl resonance of solid glycine at 176.46 ppm. The chemical shift difference between  $^{13}\text{C}$  of DSS in  $\text{D}_2\text{O}$  relative to neat TMS is 2.01 ppm.

## RESULTS

**Asparagine Mutations in the Transmembrane Domain of the Human and Murine TpoR Induce Different Effects**—The TM sequences of human and murine receptors are shown in Fig. 1A along with a consensus sequence for mammalian TpoRs. Across a wide range of species, the TM domain is relatively well

conserved. Ser505 and Trp515 both have a sequence identity of ~96%. The S505N is associated with familial forms of thrombocytosis, while the W515K,L,A mutations are associated with essential thrombocythemia and primary myelofibrosis. There are only a few residues with less than 90% identity (shown in red). There are four differences between human and mouse in the TM-JM sequence shown. Based on sequence comparison between TpoRs from 50 species, leucine at positions 492 and 500 in the mouse, and glycine and arginine at positions 503 and 514, respectively, in the human are the consensus residues.

Asparagine, the residue that leads to constitutive activation upon mutation at Ser505 (human), is able to mediate dimerization of TM helices as left-handed coiled coils. The early studies of leucine zippers involved Asn residues at the “a” position in the heptad repeat characteristic of a left-handed coiled coil (37,38). The crystal structure of the GCN4 transcription factor shows that the amide side chain of Asn forms an asymmetric hydrogen bond across the dimer interface (38) and dimerization studies showed that this contributes about -2 kcal/mol of binding energy (39).

Helical wheel diagrams illustrate the possible dimer interfaces in hTpoR and mTpoR (Fig. 1A,B). Such dimeric interfaces are imposed when the coiled-coil domain of Put3 is fused at consecutive positions to the TM domain of mTpoR, thus replacing the extracellular domain (26). There are seven possible symmetric interfaces that might mediate dimerization in the native receptor and that were created by progressive deletion of residues from the N-terminal end of the TM domain after fusion with the Put3 coiled coil (26). These are designated *cc-TpoR-0* through *cc-TpoR-VI*. The *cc-TpoR-II* interface was found to be inactive, whereas the other interfaces exhibited various degrees of activity.

To investigate whether the full-length TpoR can be activated by asparagine mutations on different TM helix interfaces, TM residues 503-510 were individually substituted with asparagine in the hTpoR and the receptor activity was monitored using a luciferase assay that reports on STAT5 transcriptional activation (see Methods). Substitution with Asn only at a single position (S505N) leads to ligand independent activation of the hTpoR (Fig. 1C). This position is the same as that detected in clinical studies showing only a

single site (Ser505) where an Asn mutation leads to disease. The results on STAT5 transcriptional activity were confirmed with cell proliferation assays in stable Ba/F3 transduced cells (Fig. 1D). For these measurements, each receptor mutant was retrovirally transduced into Ba/F3 cells and sorted using flow cytometry for similar levels of expression. Only the S505N mutant led to cytokine independent cell proliferation. Importantly, several substitutions (L508N, G509N, L510N) led to receptors that were unable to traffic to the cell surface and were, as a consequence, inactive when Tpo was added.

Asparagine scanning measurements of STAT5 transcriptional activation of the mTpoR reveal a very different picture (Fig. 2A). In contrast to hTpoR, asparagine substitutions at positions 494, 498, 500, 501 and 502 in mTpoR stabilize active receptors in the absence of ligand. These results were confirmed by cell proliferation assays in Ba/F3 cells for positions 494, 498, 500 and 502 (Fig. 2B). Asn substitutions at 4 different positions of the mTpoR clearly lead to activation, while only S505N (equivalent to S498N in the mouse) activates the hTpoR.

*Mutation of His499 to leucine leads to increased cell-surface localization and signaling by the hTpoR G509N mutant-* One of the largest sequence differences in the TM and JM regions between the murine and human receptors is at position 499 where histidine in human (His499) is replaced by leucine in mouse (Leu492). This TM residue is unique to TpoR and GCSFR within the family of type-I cytokine receptors and only appeared later in species evolution. The histidine in the human receptor has previously been shown to bind eltrombopag, a small molecule agonist of hTpoR, leading to receptor activation (40). In this section, we explore the influence on activation of the H499L and L492H mutants in the context of the human and murine receptors, respectively, and show how these mutations influence the ability of Asn substitutions within the TM domain to activate TpoR. STAT5 activation and cell proliferation were measured using the dual-luciferase and the cell viability assays, respectively, on the murine (A) and human (B) receptors with swapped residues (i.e. H499L and L492H) (Fig. 3). Several conclusions can be drawn.

First, mTpoR G502N (the most strongly activating single Asn mutant) is not active in the context of the mTpoR G502N/L492H double mutant. Indeed, placement of histidine at position

492 in the mTpoR G502N mutant abolished the activity of the mutant in the absence and the presence of ligand, yielding the same phenotype as the human receptor with the corresponding G509N substitution.

Second, the mTpoR is not sensitive to eltrombopag, while substitution of L492H in the mTpoR imparts sensitivity to eltrombopag as previously observed (40). This confirms that eltrombopag binding requires the presence of histidine in the TM sequence. In contrast, removal of histidine at position 499 in the hTpoR-G509N mutant could restore the activity of this mutant in the presence and in the absence of ligand. As a control, the human receptor lacking His499 could no longer respond to eltrombopag.

Third, the hTpoR-G509N mutant (equivalent to mTpoR G502N) does not traffic to the plasma membrane. The hTpoR-G509N mutant is retained intracellularly in an endoglycosidase H (EndoH)-sensitive form (Fig. 4), indicating it contains high mannose N-glycosylation, characteristic of immature receptor retained in the endoplasmic reticulum (ER), or possibly a receptor that cannot progress past the *cis*-Golgi. In contrast, the double mutation (H499L, G509N) allows the receptor to traffic to the plasma membrane (Fig. 4), and to induce STAT5 activation and autonomous proliferation in Ba/F3 cells (Fig. 3C). H499L appears to also rescue activity for additional Asn mutants in the hTpoR. For example, V507N is partially rescued by the H499L mutation and exhibits weak activity (data not shown). Rescued receptors become EndoH-resistant and are localized at the cell-surface (Fig. 4). In the background of the mouse TpoR, the L492H mutation inactivates the Asn mutants and impairs their ability to traffic to the cell-surface (data not shown).

*Dimerization of hTpoR Causes Straightening of the TM Helix near His499*—The structural basis underlying the mechanism by which His499 influences receptor activation and cell surface expression is a challenge to study in full-length Tpo receptors. However, the original studies describing the influence of Asn mutations on TM helix dimerization were undertaken on isolated TM domains that independently fold into helical secondary structure when reconstituted into membrane bilayers (29–31). Indeed, we have previously shown that the activating S505N and W515K mutations in the context of TM-JM peptides of the human TpoR lead to a change of the tilt angle of the TM helix and induce

dimerization, consistent with measurements on the full-length receptor using the split-luciferase assay (28). Here, we extend these studies to the G509N and H499L mutants in hTpoR and to the G502N and L492H mutants in mTpoR (Table 1).

Polarized FTIR spectroscopy provides information on the global secondary structure and orientation of membrane-reconstituted TM-JM peptides. Typically these peptides fold into  $\alpha$ -helical secondary structure that gives rise to an amide I vibrational band at  $\sim 1655\text{ cm}^{-1}$  in the IR spectrum. The orientation of the TM helix (i.e. the helix tilt angle relative to the bilayer normal, see Methods) is probed in polarized IR spectra by measurements of the dichroic ratio ( $R_{ATR}$ ). Hydrophobic peptides (20–24 residues in length) typically exhibit dichroic ratios of  $>3.0$ , corresponding to a maximum helix tilt of  $\sim 28^\circ$ . Lower dichroic ratios indicate something unusual in the TM sequence (or simply reflect poor membrane reconstitutions). Our previous FTIR studies on hTpoR TM-JM peptides revealed dichroic ratios of  $\leq 2.8$  (28). In Fig. 5A,B we show using parallel reconstitutions that mTpoR-WT peptides exhibit a striking increase in the dichroic ratio compared to hTpoR-WT, corresponding to a decrease in the helix tilt angle from  $35^\circ$  to  $20^\circ$ . This change in orientation can be mimicked in the hTpoR by the H499L mutation, which exhibits the same dichroic ratio (3.2) within error as the mTpoR (Table 1).

A second mechanism for increasing the dichroic ratio of the hTpoR-WT TM helix is incorporation of the S505N mutation. The hTpoR-S505N dichroic ratio (3.4) is closer to mTpoR-WT (3.3) than to hTpoR-WT (2.7). We attribute this increase to a shift in the monomer-dimer equilibrium toward dimer in the S505N mutant (28). The mTpoR-S498N and hTpoR-S505N peptides exhibit similar dichroic ratios (3.3 versus 3.4), suggesting they adopt similar active orientations.

We also compared the homologous hTpoR-G509N and mTpoR-G502N mutants. The mTpoR-G502N peptide has a much higher dichroic ratio (3.2) than hTpoR-G509N (2.5) consistent with the higher activity of the full length mTpoR-G502N mutant. The dichroic ratio of hTpoR-G509N is slightly lower than hTpoR-WT (2.7). The presence of two polar residues (His499 and Asn509) appears to alter the orientation of the TM helix in the membrane and may be associated with the poor ability of the G509N mutant to traffic. Similarly, introducing a

histidine (at the equivalent His499 position) in the murine coiled-coil constructs induces TpoR protein instability as observed by loss of activity and protein expression at the cell surface (data not shown).

Previous NMR studies on the structure and orientation of the TM helix of hTpoR found that there was a break in helical secondary structure near Ala497, one turn before His499 (41). The low dichroic ratio of the amide I band observed in FTIR spectra of wild-type hTpoR would be consistent with this observation. To directly probe the helical secondary structure in this region of the peptide in mTpoR and hTpoR, we made use of intramolecular distance measurements using two-dimensional (2D)  $^{13}\text{C}$  NMR spectroscopy with magic angle spinning (MAS) (36).

2D  $^{13}\text{C}$  NMR spectra were obtained on the hTpoR and mTpoR TM sequences isotopically labeled with 1- $^{13}\text{C}$ -Leu494/487, 1- $^{13}\text{C}$ -Val495/488, and  $^{13}\text{C}\beta$ -Ala497/490 (human/mouse TpoR numbering). These positions just precede His499 or Leu492 in the hTpoR and mTpoR sequences, respectively. In the 2D NMR spectra, intramolecular cross peaks are observed between  $^{13}\text{C}$  sites with a through-space separation of  $\leq 6 \text{ \AA}$ . For example, in mTpoR the  $^{13}\text{C}\beta$ -carbon of Ala490 is in close proximity to the  $^{13}\text{C}=\text{O}$  groups of Leu487 (4.3  $\text{\AA}$ ) and Val488 (5.4  $\text{\AA}$ ) in  $\alpha$ -helical secondary structure, and cross peaks between these  $^{13}\text{C}$ -resonances are observed in the 2D NMR spectrum. Fig. 5C shows the full 2D DARR spectrum of mTpoR-WT where a distinct cross peak is observed between  $^{13}\text{C}\beta$  Ala490 and either  $^{13}\text{C}=\text{O}$  Leu487 and/or Val488, whose resonances are not resolved. The cross peak (highlighted in Fig. 5E) confirms this region of mTpoR-WT is  $\alpha$ -helical. In contrast, this cross peak is absent in hTpoR (Fig. 5D) revealing that this region does not adopt helical secondary structure.

In addition to the observation of intra-helical cross peaks in mTpoR, there are distinct changes in chemical shifts of the  $^{13}\text{C}=\text{O}$  resonances between the hTpoR and mTpoR sequences (Table 1). The  $^{13}\text{C}=\text{O}$  chemical shifts above  $\sim 174 \text{ ppm}$  in the mTpoR-WT peptide are consistent with a helical geometry, whereas chemical shifts below  $\sim 174 \text{ ppm}$  are consistent with unraveling of the helix just N-terminal to His499.

Table 1 summarizes the polarized IR and NMR measurements on hTpoR-WT, mTpoR-WT and several mutants. There is a general correlation

between the dichroic ratio and the presence of helical secondary structure above the Leu492 (mTpoR) and His499 (hTpoR). That is, the TM helices adopt a more parallel orientation (low tilt) when helical structure is observed around Leu492 or His499. In the section below, we show that these changes are associated with the ability of the TM helix to dimerize. The comparisons suggest that hTpoR-H499L is structurally similar to mTpoR-WT, and mTpoR-L492H is structurally similar to hTpoR-WT.

*The TM Domain of mTpoR has a Stronger Propensity to Dimerize than hTpoR*—In the wild-type and mutant TpoR peptides, dimerization can be estimated from deuterium MAS NMR spectra (28). In these experiments, the peptides are deuterated at Leu512 (hTpoR) or Leu505 (mTpoR). Comparison of the deuterium MAS NMR spectra of hTpoR-WT and mTpoR-WT reveals a large difference in the MAS spectra (Fig. 6A,B). We have previously shown that fast rotational diffusion of the hTpoR monomer results in the loss of the side band pattern and that mutations in the TM and JM regions that lead to dimerization restore the MAS rotational side bands (28). On the basis of the observed sideband pattern, we conclude that the monomer-dimer equilibrium in mTpoR-WT is shifted toward dimer compared to hTpoR-WT. The deuterium side band pattern characteristic of dimerization is observed for the activating mutants of hTpoR (S505N, G509N) (Table 1). TM dimerization is also observed for the activating mutants of mTpoR (S498N, G502N and W508K).

The *Gaussia princeps* luciferase complementation assay (28,33) was used to assess the dimerization status of the full length, as well as of truncated mouse and human TpoRs containing transmembrane/intracellular (TM-IC) domains. In this assay, two proteins of interest are fused to complementary fragments of *Gaussia princeps* luciferase, denoted hGluc1 and hGluc2. If the proteins interact, luciferase fragments are brought together, fold into their native structure and the reconstituted luciferase enzyme catalyzes the oxidation of the substrate coelenterazine, which can be detected and quantified using luminescence readers.

The carboxyl terminus of murine or human TpoR was fused to complementary fragments of *Gaussia princeps* luciferase (hGluc1 and hGluc2) and transiently co-expressed in BOSC cells where their expression levels were comparable and could be assessed by Western blot

(data not shown). The expression of these constructs individually, as expected did not result in reconstitution of luciferase activity (28). The complementation of hGlu-tagged murine and human full-length TpoR was relatively low and appeared to be at similar levels (Fig. 6C, condition 1 and 2), indicating that the level of preformed dimers detected by proximity of cytosolic domains is low. Importantly, removal of the extracellular domain resulted in significantly higher dimerization of the mTpoR (condition 4), but not of the hTpoR (condition 3) whose dimerization remained the same compared to the full-length receptor. Thus, the hTpoR TM-IC segment possesses an additional mechanism to prevent its self-dimerization that could be ascribed to His499. Indeed, substitution of His499 to leucine allows dimerization of the TM-IC form of hTpoR (condition 5), while substitution of Leu492 to histidine in the TM-IC form of the mTpoR dramatically decreased dimerization, as assayed by luciferase complementation (condition 6).

The results in Fig. 6C for the TM-IC constructs are consistent with the NMR analysis of the TM-JM peptides, namely that His499 prevents dimerization of the TM domain of hTpoR in the context of a truncated TpoR lacking the extracellular domain. Using the dual-luciferase assay to monitor receptor activity, we show in Fig. 6D that the TM-IC constructs of mTpoR are inactive despite increased dimerization. In the hTpoR constructs, truncation of the extracellular domain abolishes Tpo-dependent activity, yet still allows activation upon the addition of eltrombopag. This activation is not detected in the H499L mutant.

*Eltrombopag Induces Dimerization of hTpoR TM Helix Peptides*—Comparison of the hTpoR and mTpoR TM-JM peptides shows that the monomer-dimer equilibrium is shifted toward monomer in hTpoR-WT compared to mTpoR-WT (Fig. 6A,B). Binding of the small molecule agonist eltrombopag induces receptor activation and requires the presence of His499 (40). In the absence of activation, His499 was previously shown to cause a kink in the TM helix (41). We asked whether this structure changes in the presence of eltrombopag, and whether eltrombopag influences dimerization of the hTpoR TM domains. Comparison of hTpoR WT peptides without (Fig. 6A) and with eltrombopag (Fig. 7B) shows a slight increase in the intensity of the deuterium MAS side bands, consistent with a shift toward the peptide dimer. Furthermore, the

secondary structure around His499 becomes helical in the hTpoR-WT peptide in the presence of eltrombopag (Table 1). Increased dimerization agrees with the studies on the TM-IC constructs containing the full IC domain, which exhibit an increase in activity upon eltrombopag binding (Fig. 6D). We also asked whether substituting His499 with leucine, the corresponding residue in mTpoR would influence dimerization. Indeed, hTpoR H499L also becomes more dimeric compared to hTpoR-WT (Fig. 7C), consistent with an observed increase in the dichroic ratio from polarized IR measurements (Table 1), while the mTpoR L492H peptide becomes more monomeric compared to mTpoR-WT (Fig. 7A). The dimeric interfaces in Fig. 7B and C are predicted to be different, between the inactive dimeric and active dimeric states of hTpoR (see below).

*Rotational Orientation of TM Helices in Inactive and Active Dimers*—Interhelical NMR measurements of specifically labeled 41 residue mTpoR peptides (Glu481 to Ser524) allow us to probe the interface that mediates dimerization. In these experiments, two mTpoR peptides with complementary  $^{13}\text{C}$  labeling were reconstituted together into DMPC:DMPG bilayers at a peptide:lipid molar ratio of 1:60. The positions of the labels were selected on the basis of our previous engineered Put3 constructs that defined the inactive and active interfaces.

To probe the inactive dimer interface, 2D  $^{13}\text{C}$  NMR spectra were obtained on mTpoR-WT peptides containing  $^{13}\text{C}$ -labeled Ala499 and Gly502. One peptide was labeled with  $^{13}\text{C}\beta$ -Ala499 and  $^{13}\text{C}=\text{O}$  Gly502, and the other was labeled with  $^{13}\text{C}=\text{O}$  Ala499 and  $^{13}\text{C}\alpha$  Gly502. Ala499 is predicted to be in the inactive interface, while Gly502 is at the boundary between inactive and active interfaces. We observe a cross peak between the diagonal  $^{13}\text{C}\beta$  and  $^{13}\text{C}=\text{O}$  resonances of Ala499 (Fig. 8A) and between the diagonal  $^{13}\text{C}\alpha$  and  $^{13}\text{C}=\text{O}$  resonances of Gly502 (Fig. 8B). These  $^{13}\text{C}$ -labeled carbons are on different peptides, and the cross peak intensities are consistent with an interhelical Ala499  $\text{C}\beta$  to  $\text{C}=\text{O}$  distance of  $\sim 5.4$  Å, and an interhelical Gly502  $\text{C}\alpha$  to  $\text{C}=\text{O}$  distance of  $\sim 5.2$  Å. An intrahelical cross peak is also observed between the Gly502  $^{13}\text{C}\alpha$  resonance and the Ala499  $^{13}\text{C}=\text{O}$  resonance. These  $^{13}\text{C}$  labels are on the same peptide, and are estimated to be  $\sim 4.4$  Å apart in an  $\alpha$ -helical geometry. An intrahelical cross peak between the Ala499  $^{13}\text{C}\beta$  and Gly502  $^{13}\text{C}=\text{O}$  resonances was

not observed as the distance between  $^{13}\text{C}$  sites is  $>6 \text{ \AA}$  in an  $\alpha$ -helix.

To probe the potential active interface(s), parallel 2D NMR experiments were undertaken on mTpoR-S498N (Fig. 8C,D). In contrast to the results above, we do not observe interhelical Ala499-Ala499 or Gly502-Gly502 cross peaks in peptides containing this activating mutation confirming that we lose the inactive interface. The absence of contacts between these residues is consistent with a shift in the dimer interface to either the active *cc*-TpoR-I or *cc*-TpoR-V interfaces defined by engineered Put3 dimers, both of which contain Ser498 in the interface.

Models of the TM domain of the hTpoR in inactive and active orientations are shown in Fig. 9A. The inactive receptor exists in a monomer-dimer equilibrium. The split luciferase measurements of the full-length receptor along with deuterium NMR measurements of the hTpoR-WT TM-JM peptides indicate that the equilibrium is shifted toward monomer. His499 induces a break in the TM helix. The side chain of His499 is predicted to reside within the headgroup region of phospholipids on the extracellular side of the bilayer. Given the bilayer thickness, this requires the RWQFP motif to partition into headgroup region of the phospholipids on the intracellular side of the bilayer. The active orientation is modeled as a dimer (Fig. 9A, right). In the wild-type full-length receptor, ligand binding drives a shift of the receptor dimer to an active orientation with Ser505 in the interface. Dimerization is also mediated by hydrogen bonding interactions involving the Asn side chains in the S505N mutant (Fig. 9A, right). In the TM-JM peptides, dimerization induces helical secondary structure in the region just N-terminal to His499 and reduces the overall dichroic ratio (helix tilt angle).

The inactive mTpoR also exists in a monomer-dimer equilibrium (Fig. 9B). In the absence of the extracellular domain, the equilibrium shifts toward dimer. The interface mediating the inactive dimer in TM-JM peptides involves packing of Ala499 and Gly502. This interface is the same as that found in the engineered Put3 constructs (26). In the wild-type full-length receptor, ligand binding also drives a shift of the receptor dimer to an active orientation with Ser498 in the interface. Hydrogen bonding interactions involving Asn side chains stabilizes this orientation in the S498N mutant (Fig. 9B, right).

## DISCUSSION

We show that several asparagine mutations in the TM domain of the mTpoR activate signaling, while only one asparagine substitution (S505N) does so for the hTpoR. Thus, human TpoR appears to be largely protected from oncogenic activation by Asn mutations. This major difference can be abolished if His499 in the hTpoR is substituted by leucine, the homologous residue in the mTpoR. We further show that His499 locally influences the structure and orientation of the TM helix and this change influences the ability of receptors with specific TM asparagine mutations to traffic to the cell surface.

*Receptor Dimerization in TpoR Signaling*—Dimerization of the cytokine receptors is required for activation. The EpoR (24), PIR (42) and GHR (22,23) have been found to associate as pre-formed (inactive) dimers mediated by TM domain interactions. The implication is that there is a change in TM helix interfaces upon ligand binding and that this change is relayed to the intracellular domain to allow receptor activation.

Our previous studies indicated that, upon ligand binding, or in the constitutively active S505N and W515K mutants, the monomer-dimer equilibrium shifts toward the active dimer (28). We extend these results here using the split luciferase assay to show that both the full-length hTpoR and mTpoR exist at a low level as preformed dimers, with the majority of chains detected as monomers. Interestingly, removal of the extracellular domain induces receptor dimerization in the mTpoR, but not the hTpoR.

The model that emerges is one where ligand binding triggers a conformational change in the extracellular domain that allows receptor dimerization in an active conformation. Using structural approaches, we are able to mimic the dimerization behavior of the murine and human receptors when the extracellular domain is removed (i.e. the TM-JM peptides and the TM-IC constructs are largely monomeric for hTpoR and dimeric for mTpoR). This observation indicates that the extracellular domain blocks the ability of the TM helices to directly interact in the mTpoR. In the hTpoR, there is an additional mechanism that blocks helix association, namely the change in the helix conformation induced by His499. The H499L mutation induces constitutive dimerization of hTpoR in the absence of the extracellular domain in both the TM-IC constructs and the TM-

JM peptides. In both the wild-type hTpoR receptor constructs and in the TM-JM peptides, the ability of the activating Asn mutations and eltrombopag (for hTpoR) to induce dimerization strongly suggests that helix association is a key element of the activation mechanism.

The comparative studies on dimerization using the split luciferase assay between the full length WT TpoRs and the corresponding truncated TM-IC constructs show large differences in receptor dimerization. We have previously compared dimerization for hTpoR-WT and mEpoR-WT using the split luciferase assay and observed a two-fold difference in bioluminescence indicating that both the hTpoR and mEpoR receptors exist in a monomer-dimer equilibrium with mEpoR shifted more toward the receptor dimer (28). The subpopulation of pre-formed dimers may be the relevant form for rapid signaling by TpoR. While our studies reveal clear differences in dimerization in comparisons of (for e.g.) the full-length hTpoR and hTpoR TM-IC H499L, they open up the question as to how different cell types or membrane environments influence the monomer-dimer equilibrium. For example, there may be differences in the monomer-dimer equilibrium (and hence the level of pre-formed dimers) between TpoR-expressing cells, namely hematopoietic stem cells and megakaryocyte progenitors or there may be a shift in equilibrium if the membrane environment changes, such as incorporation of receptors into lipid rafts. The ability of the cell to modulate the monomer-dimer equilibrium would provide an additional control mechanism for receptor activation.

**TM Helix Orientation in TpoR Signaling—**The orientation of the TM and intracellular domains within dimers of single TM receptors is generally important for activation. In both the EpoR (32) and TpoR (26) we have shown using Put3 coiled-coil constructs that receptor activity is dependent on the orientation of the TM helix. For mTpoR, only one dimeric interface (*cc*-TpoR-*II*) is inactive. The *cc*-TpoR-*II* interface is lined with leucines, a AxxG motif, and ends with glutamine 509 within the K/RWQFP motif, corresponding to Gln516 in the hTpoR. The AxxG sequence has similarities with the GxxxG sequences known to mediate TM dimerization (43,44). The close packing of the TM helices due to these small side chains places the mTpoR glutamine 509 into a position where it can mediate interhelical hydrogen bonding and stabilize dimerization. We

ascribe this inactive interface to the same interface that mediates dimerization of the inactive TM-IC constructs of mTpoR. Moreover, we show with NMR (Fig. 8) that the TM-JM peptides (also lacking a large extracellular domain and hence similar to the TM-IC constructs) dimerize via the same interface as in the inactive *cc*-TpoR-*II* construct.

In contrast, several interfaces in the Put3 coiled-coil constructs have previously been shown to exhibit various levels of activity (26). *cc*-TpoR-*I* induced high numbers of platelets, without other cytological features of myeloproliferation in bone marrow, and *cc*-TpoR-*V* induced a myeloproliferative phenotype with small red blood cells and variable levels of thrombocytosis (26). Thus, the active helix interfaces identified in the Put3 experiments are consistent with the Asn substitutions reported here, at least for the mTpoR. That is, the *cc*-TpoR-*I* and *cc*-TpoR-*V* interfaces involve the residues where asparagine mutations lead to the strongest activity (V494N, S498N, L501N and G502N). In *cc*-TpoR-*V*, the dimer interface is mediated by an SxxxG sequence, which again allows close helix packing. In our NMR studies, the S498N mutation induces a change in the conformation of the mTpoR peptide dimer and we lose the contacts between Ala499 and Gly502 characteristic of the inactive dimer (Fig. 8). The low energy structure shown in Fig. 9B corresponds to *cc*-TpoR-*I*, in which Asn498 forms interhelical hydrogen bonds.

**Eltrombopag Drives Dimerization of hTpoR-WT—**Eltrombopag is an FDA-approved drug used for the treatment of thrombocytopenia (low platelet numbers). Previous studies have found that eltrombopag can activate hTpoR, but not mTpoR (40). Eltrombopag and related small molecule agonists have been found to interact with His499 (41). We find that this transmembrane-acting compound increases the dichroic ratio in FTIR measurements, induces helix in the Leu494-Ala497 sequence in the TM domain and leads to receptor dimerization as assayed by deuterium NMR line shapes. The changes in dimerization induced by eltrombopag (Fig. 7B) are weaker than those observed for the H499L mutant (Fig. 7C). Nevertheless, eltrombopag appears to induce receptor activation in a similar manner as the S505N mutation, namely, both overcome the ability of His499 to inhibit TM dimerization.

**Multiple Levels of Regulation Involving the TM and JM Regions of TpoR—**The wild-type hTpoR has several mechanisms for regulating

dimerization of the TM region of the receptor. These involve the extracellular domain, the TM His499 residue and the intracellular RWQFP insert. Removal of the extracellular domain or the H499L mutation alone does not induce dimerization. Both are required in the same construct. Ligand binding within the extracellular domain or mutations of Trp515 within the RWQFP insert can both overcome these mechanisms leading to an active receptor conformation. In the mTpoR, removal of the extracellular domain induces dimerization in an inactive conformation. Dual-luciferase measurements of receptor activation show that the TM-IC constructs of mTpoR are inactive. Thus, dimerization *per se* does not suffice for activation. Interestingly, the TM-IC constructs differ from the viral Mpl protein of the myeloproliferative leukemia virus by the presence of an extracellular domain of 109 amino acids comprised of 43 from the original murine sequence and 66 from the Friend murine leukemia virus. This suggests that the extracellular amino acids in the v-Mpl are able to stabilize the TM-IC portion of the receptor in an active orientation.

Our results reveal that the TM helices in TpoR are not passive elements in ligand-induced dimerization. The TM helices have a stronger propensity to form dimers or higher order

oligomers than to remain in a monomeric state. The extracellular, juxtamembrane and intracellular domains all contribute to modulating TM association. This observation may generally hold for ligand-induced activation of single TM receptors. For example, the TM helices in the *ErbB* family of receptor tyrosine kinases all have a strong propensity to dimerize (45) and removal of the extracellular domain can lead to active dimers (46,47) or can trigger intramembrane proteolysis (48,49). In these receptors, the juxtamembrane sequences play an influential role in dimerization and activation (50). Mutation of Val664 to Gln in the TM sequence of the *ErbB2-Neu* receptor can induce dimerization and constitutive receptor activation in a manner similar to the S505N mutation in hTpoR (51).

The additional layer of activity control exerted by His499 in hTpoR may reflect the central role that this receptor plays in hematopoiesis. TpoR not only regulates megakaryocyte/platelet development, but also contributes to the survival of hematopoietic stem cells, which will give rise to all other blood cells. The acquisition of histidine at position 499 during evolution protects the hTpoR from the transforming effect of several Asn TM mutations that are activating in the mTpoR.

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### Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

### Author Contributions

SOS and SNC conceived and designed the study, interpreted the data and wrote the paper. EL and JPD designed and performed experiments ( $\gamma$ 2A luciferase assays, Ba/F3 cell retroviral transduction, sorting, cell surface labeling and proliferation) and analyzed the data, prepared the figures and wrote the paper. JS designed and performed experiments ( $\gamma$ 2A luciferase assays, Ba/F3 cell retroviral transduction, sorting, cell surface labeling and proliferation). SMM performed cloning and expression of TpoR constructs. EL performed intracellular TpoR metabolism studies. TS synthesized and purified the TM peptides. SD carried out membrane reconstitutions and FTIR studies. VG performed the *Gaussia* luciferase experiments.

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## FOOTNOTES

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<sup>1</sup> Both authors contributed equally to this work.

<sup>2</sup> The abbreviations used are: Asn, asparagine; ATR, attenuated total reflection; DARR, dipolar assisted rotational resonance; Epo, erythropoietin; ETPG, eltrombopag; FTIR, Fourier transform infrared; GFP, green fluorescence protein; GCSF, granulocyte colony stimulating factor; HA, hemagglutinin, hTpoR, human thrombopoietin receptor; JAK, Janus kinase; JM, juxtamembrane; mTpoR, murine thrombopoietin receptor; Mpl, myeloproliferative leukemia; Tpo, thrombopoietin; TM, transmembrane.

## FIGURE LEGENDS

**FIGURE 1.** *Asparagine substitutions in the transmembrane domain of the human TpoR lead to activation of only one dimeric interface.* *A*, TM sequences of hTpoR (upper) and mTpoR (middle) showing the position of His499 and the intracellular R/KWQFP motif. A consensus sequence (bottom) of 50 different mammalian TpoRs highlights (red) the positions with sequence identity of <90%. Four of these residues bracket Ser498 (murine) and Ser505 (human), which is highly conserved. *B*, Helical wheel diagrams of hTpoR (left) and mTpoR (right) showing the dimer interfaces that would be predicted for interacting helices in a left-handed coiled-coil geometry. *C*, Transcriptional activation of hTpoR after substitution of Gly503 to Leu510 with asparagine.  $\gamma$ 2A cells were transiently transfected with JAK2 and the wild type TpoR or the different TpoR mutants and the activity of these receptors was assessed by measuring luciferase luminescence (in relative luciferase units, rlu) in the absence or in the presence of 50 ng/ml of Tpo as indicated. Only the S505N mutation triggers ligand-independent dimerization and signaling. *D*, BaF3 stably expressing the hTpoR variants were grown in the absence of cytokine or in the presence of different concentration of Tpo as indicated. Only TpoR S505N induced BaF3 autonomous growth. The presented values are averages of triplicates  $\pm$  standard deviation (SD) for one representative experiment out of at least three. \* $P$ <0.01, \*\* $P$ <0.001, one way ANOVA.

**FIGURE 2.** *Asparagine substitutions in the transmembrane domain of the murine TpoR lead to activation of several dimeric interfaces.* Leu492 to Gly502 were substituted with asparagine and these mutant receptors were assessed for their ability to signal in the absence of cytokine. *A*,  $\gamma$ 2A cells were transiently transfected with the constructs and the transcriptional activation of STAT5 was measured by luciferase luminescence (in relative luciferase units, rlu). V494N, S498N, L500N, L501N and G502N triggered signaling in the absence of stimulation. *B*, BaF3 cells stably expressing the different constructs were grown and cell viability was measured in the absence of the presence of the different concentrations of Tpo ligand as indicated. V494N, S498N, L500N and G502N allow proliferation of BaF3 in the absence of cytokine. The values presented are averages of triplicates  $\pm$  standard deviation (SD) for one representative experiment out of at least three. \* $P$ <0.01, \*\* $P$ <0.001, one way ANOVA between the wild-type vs. each mutant in the absence of cytokine.

**FIGURE 3.** *Addition or removal of the TM histidine modifies the activity of the Asn mutants.* *A*, Introduction of His instead of Leu at the homologous position in the murine receptor abrogated the activity of the G502N mutant as indicated on the dual luciferase assay performed in transiently

transfected  $\gamma$ 2A cells. As control, eltrombopag (ETPG) was added and was able to activate the L492H receptor. *B*, Substitution of His499 by leucine restored the activity of the G509N mutant. Eltrombopag was no longer able to activate the receptor lacking His499. *C*, BaF3 cells stably expressing the TpoR variants were assessed for their ability to grow in the absence of cytokine (red) or in the presence of different concentrations of Tpo (gradient of blue, 1 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml) and eltrombopag (green, 0.7  $\mu$ g/ml). Cells were all able to grow in the presence of IL3 (white). The human double mutant G509N/H499L and its murine homolog G502N were able to induce BaF3 autonomous growth in the absence of cytokine. Histograms are averages of triplicates  $\pm$  standard deviation (SD) for one representative experiment out of at least three

**FIGURE 4.** *TpoR G509N is not expressed at the cell surface.* *A*, Cell surface expression of HA-tagged TpoR was assessed by FACS using an anti HA.11 antibody. *B*, BaF3 cells were sorted for the same level of GFP. *C*, The total level of TpoR protein was assessed by Western blot with Np40 cells extracts using an anti-HA antibody. *D*, Endoglycosidase H (EndoH) and peptide-N-glycosidase F (PNGase) treatment of stable BaF3 cell lines. Sorted BaF3 stably expressing the TpoR asparagine variants have been treated with endoH (E) or PNGase treatment (P) or were not treated (-) and the total level of TpoR protein was assessed using an anti HA antibody by Western blot. All the receptors, except G509N mutant, were resistant to EndoH treatment revealing that the proteins are mature. The G509N mutant, which was fully degraded by EndoH is likely immature and does not process to the cell surface because of some misfolding that blocks it at the RER-Golgi transport system. All the receptors depend on N-glycosylation for processing as revealed by the PNGase treatment.

**FIGURE 5.** *Comparison reveals differences in the secondary structure and orientation of hTpoR and mTpoR TM peptides.* *A* and *B*, Polarized IR spectra of hTpoR-WT and mTpoR-WT. *C*, 2D DARR NMR of mTpoR-WT using TM peptides isotopically labeled at 1- $^{13}$ C Leu487, 1- $^{13}$ C Val488, and  $^{13}$ C $\beta$  Ala490. The cross peak (boxed) between the  $^{13}$ C $\beta$  Ala490 and the 1- $^{13}$ C resonances of 1- $^{13}$ C-Leu487 and/or 1- $^{13}$ C Val488 is consistent with  $\alpha$ -helical secondary structure in this region of the TM-JM peptide. *D*, Row through the  $^{13}$ C $\beta$  Ala497 resonance of hTpoR showing the absence of a cross peak to either Leu494 or Val495, which is consistent with a kink or break in helical secondary structure in this region of the TM-JM peptide. *E* Row through the  $^{13}$ C $\beta$  Ala490 resonance of mTpoR showing the strong cross peak to Leu487 and/or Val488.

**FIGURE 6.** *Comparison reveals differences in dimerization of the hTpoR and mTpoR TM peptides.* Deuterium MAS NMR spectra of hTpoR-WT (*A*) and mTpoR-WT (*B*). The peptides contained deuterated leucine at positions 512 (hTpoR) or 505 (mTpoR). *C*, Dimerization of full-length hTpoR and mTpoR. Dimerization was detected using the split-luciferase assay in cell lysates. hGluc-tagged full-length or transmembrane/intracellular (TM-IC) forms of TpoR as well as mutant hTpoR(TM-IC) H499L were co-expressed in BOSC cells and luciferase activity was measured after cell lysis. *D*, STAT5 transcriptional activity as measured by the split-luciferase assay. Histograms are mean  $\pm$  standard error of the mean of 4 independent experiments. \* $P < 0.01$ , \*\* $P < 0.001$ , one way ANOVA.

**FIGURE 7.** *Influence of His499 and eltrombopag on TM dimerization.* Deuterium NMR spectra are shown of mTpoR-L492H (*A*), hTpoR-WT with eltrombopag (*B*) and hTpoR-H499L (*C*). The absence of side bands in the MAS spectra of mTpoR-L492H reflects rapid axial rotation of the peptide in the membrane bilayers. Dimerization induced in the hTpoR-WT peptide by eltrombopag and by the H499L mutation reduces axial diffusion rate and leads to increased intensity in the MAS sidebands.

**FIGURE 8.** *Interhelical NMR measurements of mTpoR peptides (Glu481 to Ser524).* *A*, Row through the diagonal resonance of the Ala499 C $\beta$  carbon reveals a cross peak with the Ala499 C=O resonance. In an inactive dimer the Ala499 C $\beta$  to C=O distance is predicted to be  $\sim 5.4$  Å. *B*, Row through the diagonal resonance of the Gly502  $^{13}$ C $\alpha$  diagonal resonance reveals a cross peak with the Gly502  $^{13}$ C=O resonance, where the predicted interhelical distance is  $\sim 5.2$  Å. In this sample, an intrahelical cross peak is also observed between the Gly502 C $\alpha$  resonance and the Ala499 C=O resonance. *C* and *D*, DARR NMR

experiments were undertaken on mTpoR-S498N using the same labeling strategy as in (A,B). We do not observe Ala499-Ala499 or Gly502-Gly502 cross peaks in peptides containing this activating mutation.

**FIGURE 9.** *Models of the monomeric and dimeric TM domains of the hTpoR and mTpoR.* *A*, Human TpoR. FTIR and NMR measurements on the TM-JM domain of hTpoR show that the wild-type peptide is primarily monomer (left) with a break in helical secondary structure in the region N-terminal to His499. In the S505N mutant of the hTpoR TM-JM domain (right), an increase in the dichroic ratio of the amide I vibration indicates that the TM helix is oriented closer to the bilayer normal, while the NMR measurements indicate that helix is induced in the region N-terminal to His499. In the full-length receptor, we propose that the monomer and inactive dimer (middle) are in equilibrium, and that either ligand binding or the S505N mutation shifts the equilibrium to the active dimer with either Ser505 (WT) or Asn505 (mutant) in the dimer interface. *B*, Murine TpoR. FTIR and NMR measurements on the TM-JM domain of mTpoR show that the wild-type peptide is primarily dimer (middle) in which dimerization is mediated by close packing of Ala499 and Gly502. *D*, In the full-length receptor, we propose that the monomer (right) and inactive dimer are in equilibrium, and that either ligand binding or the S498N mutation shifts the equilibrium to the active dimer with either Ser498 (WT) or Asn498 (mutant) in the dimer interface. The dimer structures of hTpoR-S505N, mTpoR-WT and mTpoR-S505N were taken from computational searches using the program CHI (52,53) as previously described (26). These low energy structures correspond to the average of several structures in the searches that form clusters and best reproduce the experimental data presented here.

**Table 1. Orientation, secondary structure and association of hTpoR-WT, mTpoR-WT and their mutants.**

| Sequence               | Dichroic ratio <sup>a</sup> | Helix tilt angle | Leu494-Ala497 Structure | Leu494, Val495 <sup>13</sup> C=O Shift (ppm) | Association |
|------------------------|-----------------------------|------------------|-------------------------|----------------------------------------------|-------------|
| hTpoR-WT               | 2.7 ± 0.1                   | 35°              | Extended                | 172.3, 172.3                                 | Monomer     |
| S505N                  | 3.4 ± 0.1                   | 17°              | Helix                   | 175.9, 175.9                                 | Dimer       |
| G509N                  | 2.5 ± 0.2                   | 40°              | Extended                | 172.3, 172.3                                 | Dimer       |
| H499L                  | 3.2 ± 0.1                   | 23°              | Helix                   | 176.1, 176.1                                 | Dimer       |
| hTpoR-WT + eltrombopag | 3.0 ± 0.1                   | 28°              | Helix                   | 175.3, 175.3                                 | Dimer       |
| mTpoR-WT               | 3.3 ± 0.1                   | 20°              | Helix                   | 176.3, 176.3                                 | Dimer       |
| S498N                  | 3.3 ± 0.1                   | 20°              | Helix                   | 177.2, 177.2                                 | Dimer       |
| G502N                  | 3.2 ± 0.1                   | 25°              | Helix                   | 176.6, 176.6                                 | Dimer       |
| L492H                  | 2.6 ± 0.2                   | 38°              | Extended                | 173.1, 173.1                                 | Monomer     |

<sup>a</sup> The error in the dichroic ratio corresponds to the standard deviation in 3 replicate runs.

FIGURE 1

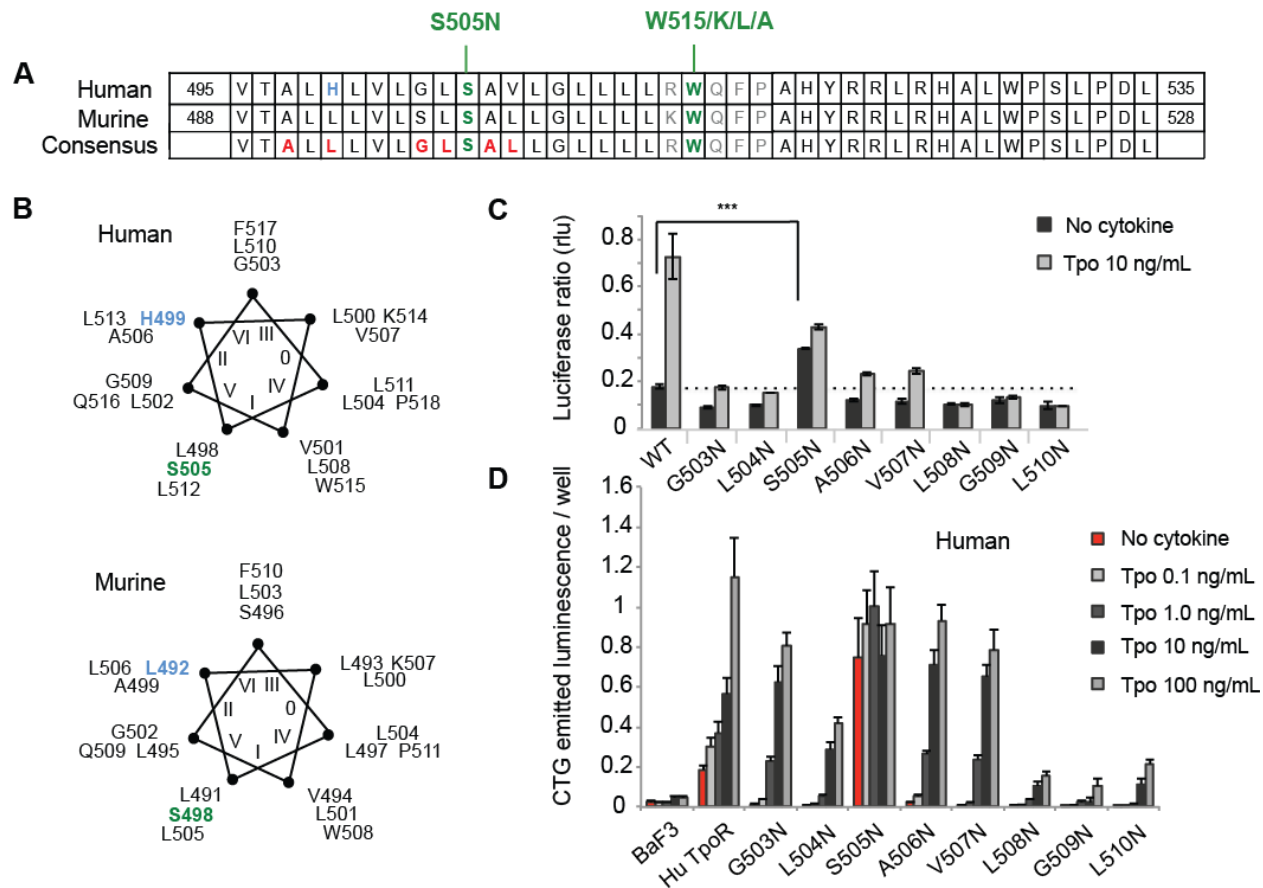


FIGURE 2

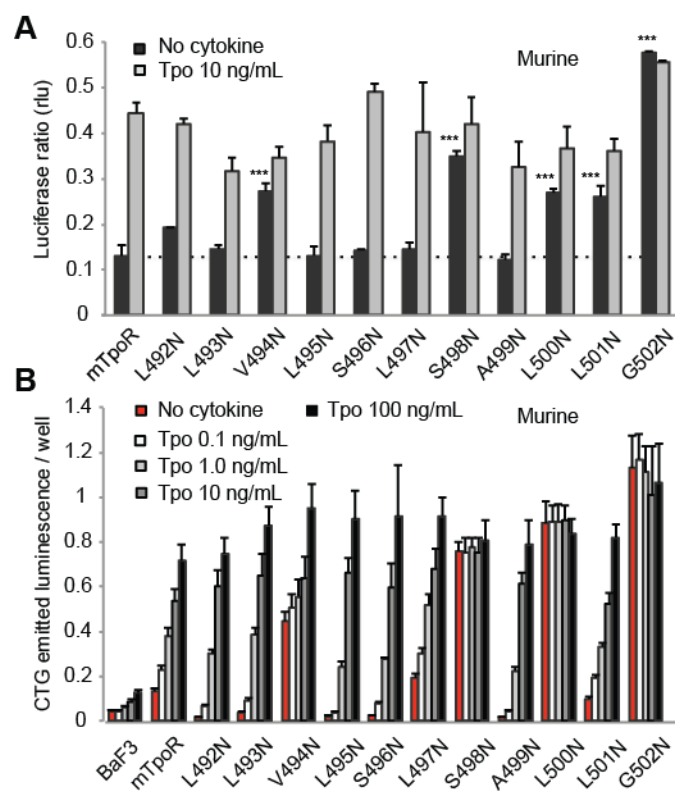
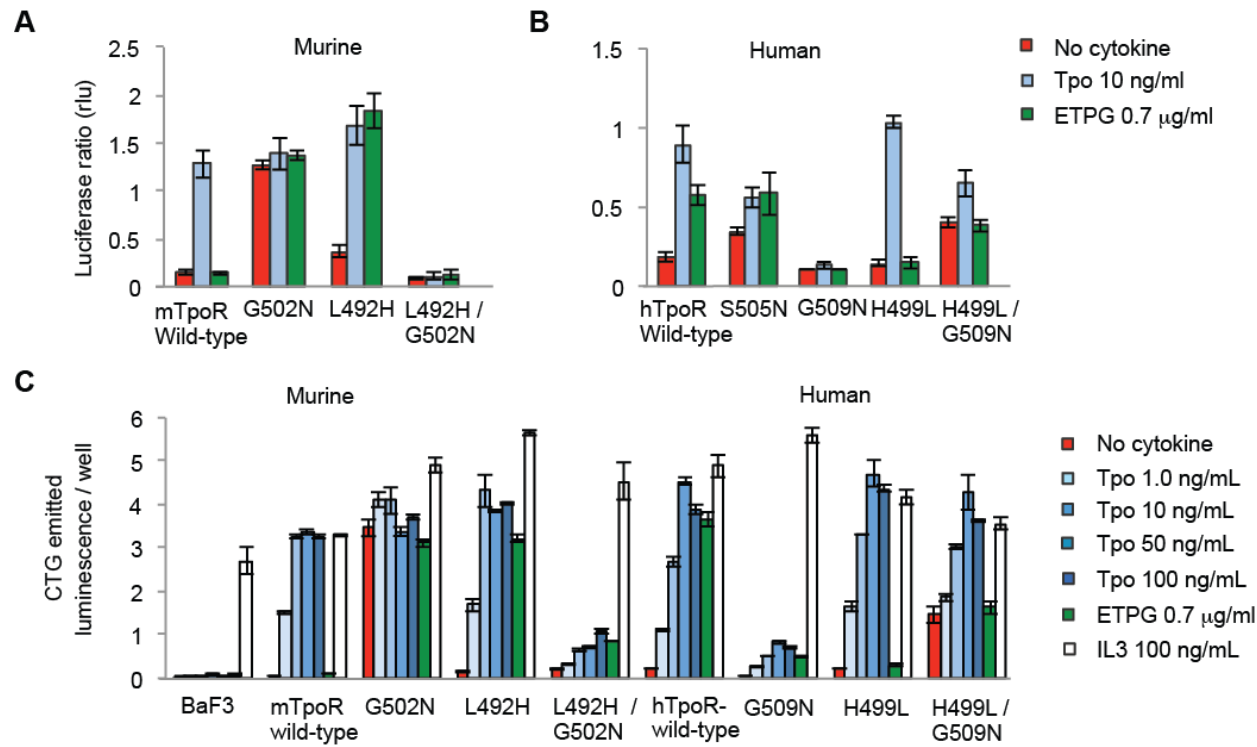


FIGURE 3



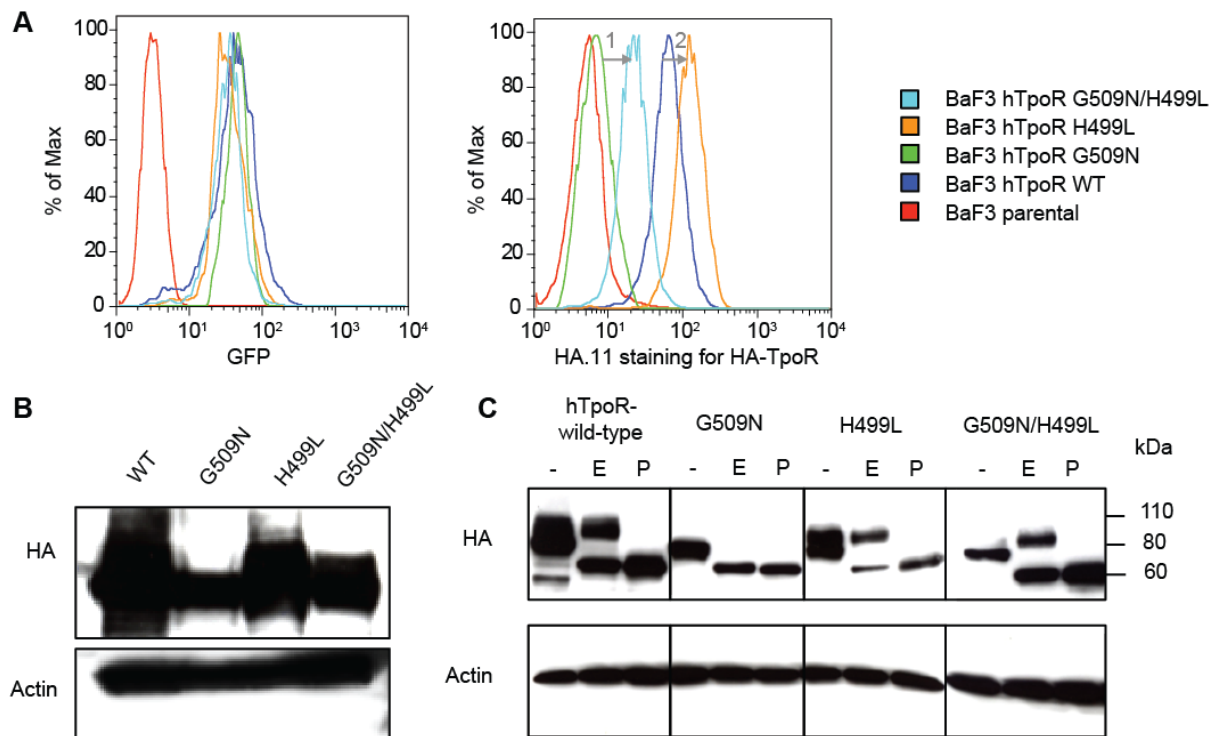
**FIGURE 4**


FIGURE 5

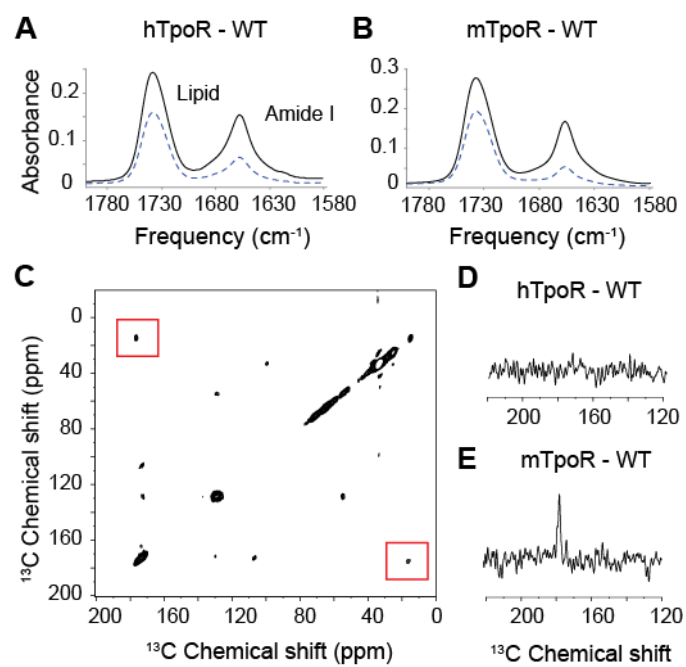
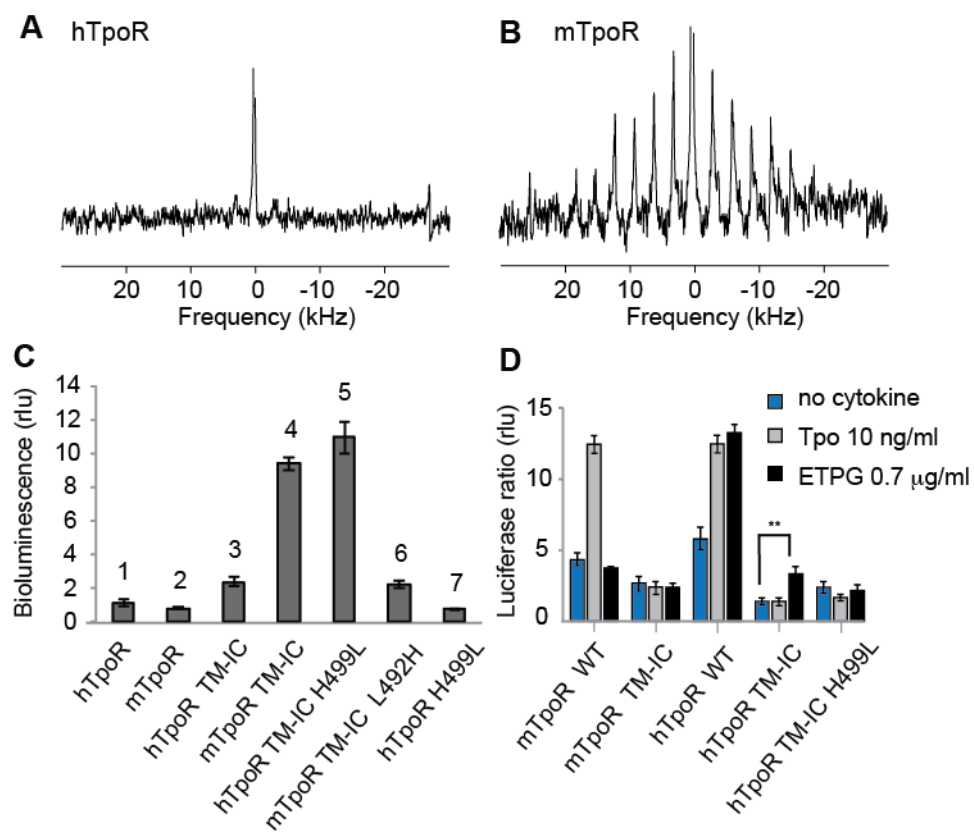
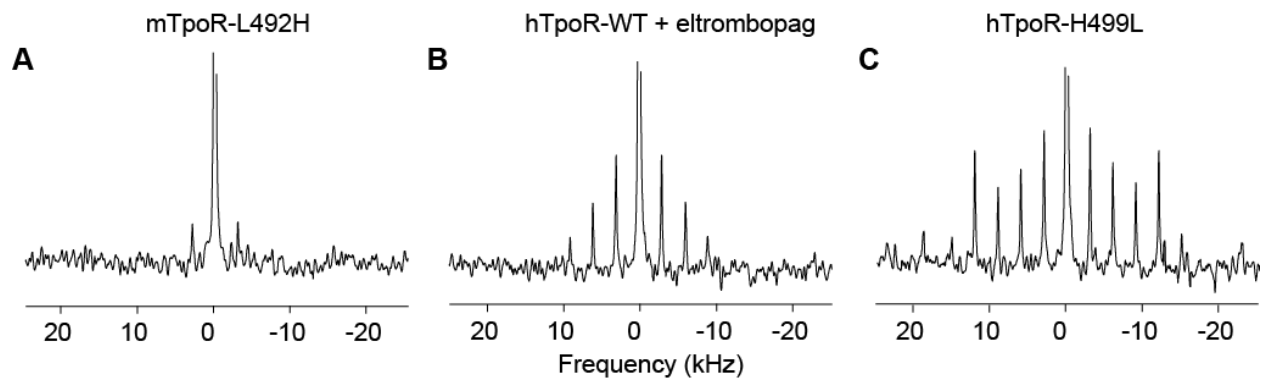


FIGURE 6



**FIGURE 7**



**FIGURE 8**

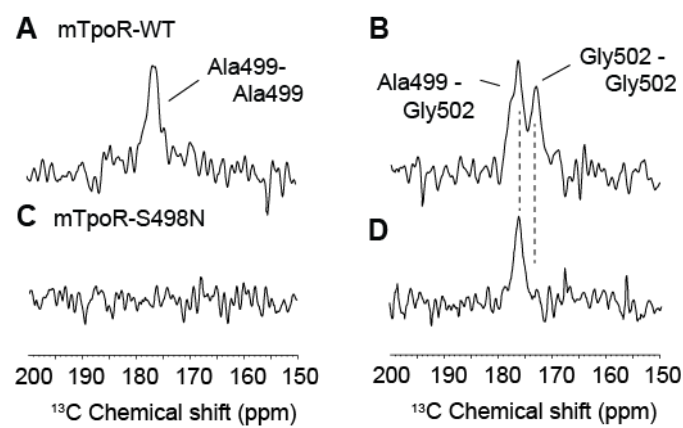
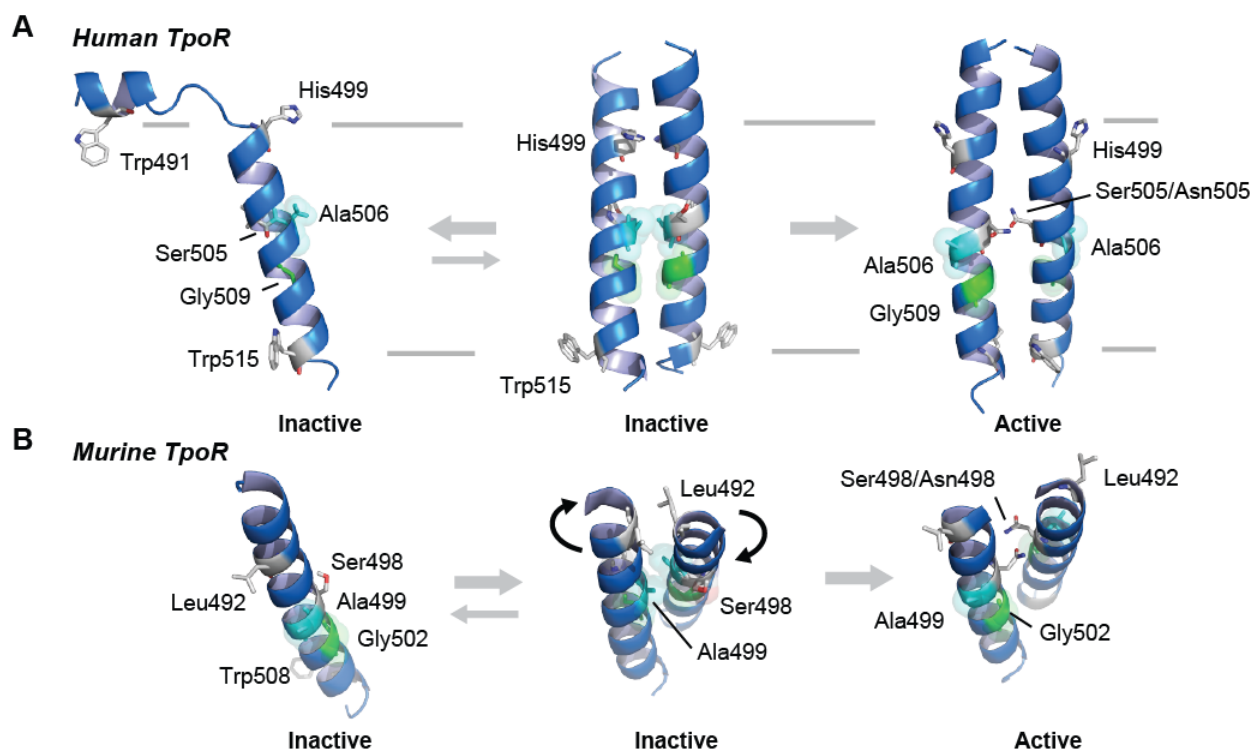


FIGURE 9



**His499 Regulates Dimerization and Prevents Oncogenic Activation by Asparagine Mutations of the Human Thrombopoietin Receptor**

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