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MPL Mutations in Essential Thrombocythemia Uncover A Common Path of Activation with Eltrombopag Dependent on W491

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Abstract:
Mutations in the gene (*MPL*) encoding the human thrombopoietin receptor (TpoR) drive sporadic and familial essential thrombocythemia (ET). We identified two ET patients that harbor double mutations in *cis* in *MPL*, namely L498W-H499C and H499Y-S505N. Using biochemical and signaling assays along with partial saturation mutagenesis we show that L498W is an activating mutation potentiated by H499C, and that H499C/Y enhance the activity of the canonical S505N mutation. L498W and H499C can activate a truncated TpoR mutant, which lacks the extracellular domain, indicating these mutations act on the transmembrane (TM)-cytosolic domain. Using a protein complementation assay we show that L498W and H499C strongly drive dimerization of TpoR. Activation by tryptophan substitution is exquisitely specific for position 498. Using structure-guided mutagenesis we identify upstream amino acid W491 as a key residue required for activation by L498W or canonical activating mutations such as S505N and W515K, as well as by eltrombopag. Structural data point to a common dimerization and activation path for TpoR via its TM domain that is shared between the small molecule agonist eltrombopag and canonical and novel activating TpoR mutations that all depend on W491, a potentially accessible extracellular residue that could become a target for therapeutic intervention.

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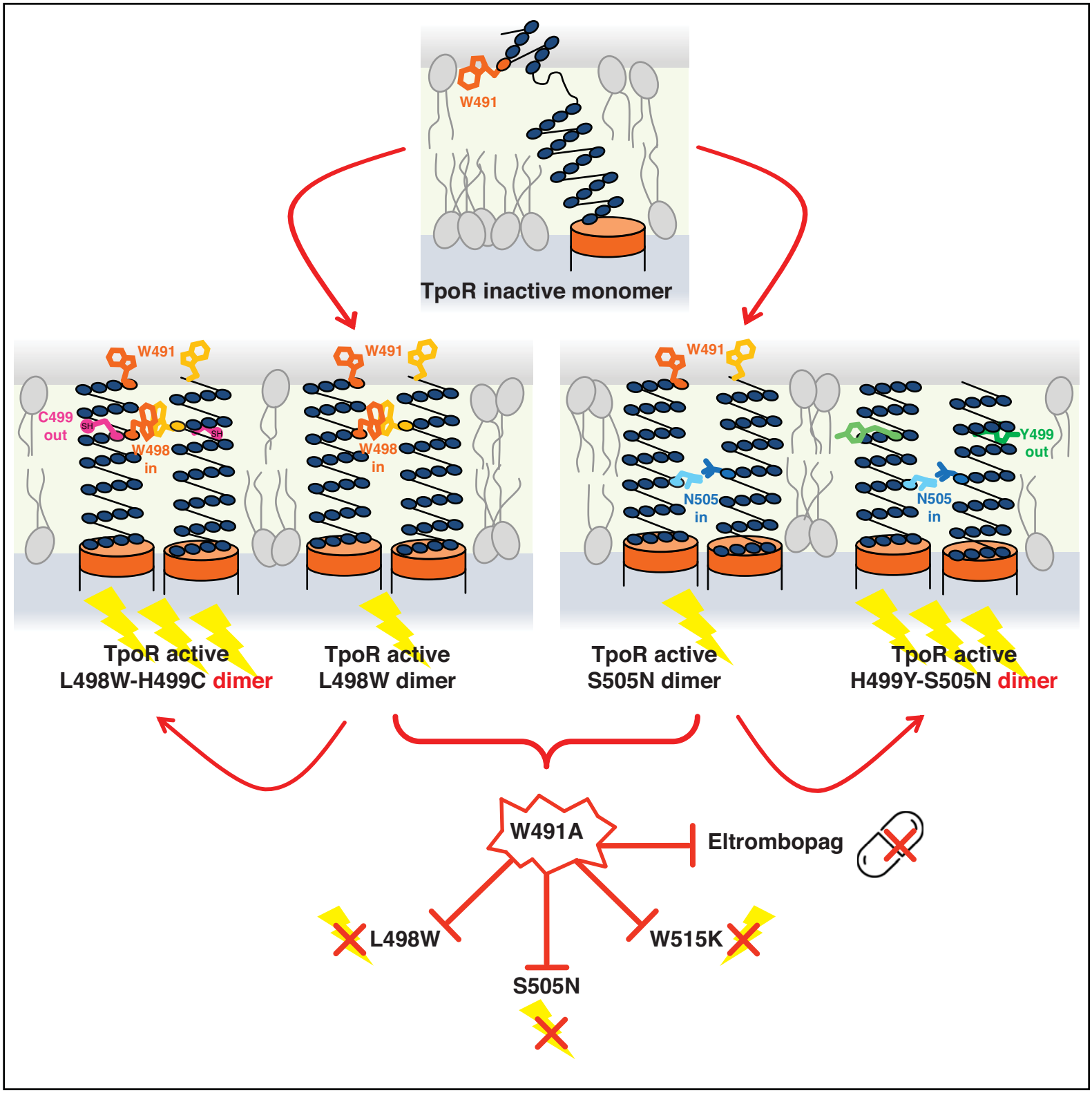
KEY POINTS

1. H499C and H499Y mutations enhance activation of TpoR by a novel L498W and the canonical S505N mutations.
2. Activation of TpoR by eltrombopag and the L498W, S505N and W515K mutants depends on W491, which might be accessible on the cell-surface.

GRAPHICAL ABSTRACT



ABSTRACT

Mutations in the gene (*MPL*) encoding the human thrombopoietin receptor (TpoR) drive sporadic and familial essential thrombocythemia (ET). We identified two ET patients that harbor double mutations in *cis* in *MPL*, namely L498W-H499C and H499Y-S505N. Using biochemical and signaling assays along with partial saturation mutagenesis we show that L498W is an activating mutation potentiated by H499C, and that H499C/Y enhance the activity of the canonical S505N mutation. L498W and H499C can activate a truncated TpoR mutant, which lacks the extracellular domain, indicating these mutations act on the transmembrane (TM)-cytosolic domain. Using a protein complementation assay we show that L498W and H499C strongly drive dimerization of TpoR. Activation by tryptophan substitution is exquisitely specific for position 498. Using structure-guided mutagenesis we identify upstream amino acid W491 as a key residue required for activation by L498W or canonical activating mutations such as S505N and W515K, as well as by eltrombopag. Structural data point to a common dimerization and activation path for TpoR via its TM domain that is shared between the small molecule agonist eltrombopag and canonical and novel activating TpoR mutations that all depend on W491, a potentially accessible extracellular residue that could become a target for therapeutic intervention.

INTRODUCTION

Myeloproliferative neoplasms (MPNs) Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) patients harbor driver mutations in *JAK2*, *CALR* or *MPL*[1]. Mutations in the human thrombopoietin receptor (TpoR) are restricted to ET and PMF, largely W515K/L/A/R substitutions in the amphipathic juxtamembrane (JM) domain[2,3] and more rarely S505N in the transmembrane domain (TMD)[4,5]. These mutations lead to ligand-independent receptor dimerization and resultant activation of signaling via JAK2[6-8]. While 20% of ET and 10-15% PMF patients harbor none of the three driver mutations, some of these "triple-negative" (TN) patients may harbor non-canonical *MPL* mutations[6,9-11].

Here we analyzed two ET patients negative for *JAK2*, *CALR* and *MPL* W515 mutations. We found that each patient harbored double *cis* mutations in *MPL*, namely L498W-H499C and H499Y-S505N. These mutations have never been described in patients and both combinations are active. Using structure-guided mutagenesis, dimerization, biochemical and signaling assays we define a broad mechanism of activation shared by these new mutants, as well as by the small molecule eltrombopag and the canonical S505N and W515 mutants. This mechanism depends

on a potentially accessible region upstream of the juxtamembrane (JM)-transmembrane (TM) region.

METHODS

HA-tagged human TpoR wild-type (WT) and mutant cDNAs, as well as a TM-intracellular TpoR construct lacking the extracellular domain[12], were cloned into the pMX-IRES-GFP bicistronic vector. Transcriptional basal and ligand-dependent activation of STAT5 downstream of TpoR was tested in γ2A and HEK-293T cells in a dual-luciferase reporter assay (DualGlo®, Promega, Madison, WI)[12]. Cytokine-dependency of proliferation in stably transduced Ba/F3 cells was determined as previously described[12]. The levels of TpoR total protein expression were determined by Western blotting using anti-HA antibodies (Roche Applied Science) or anti-TpoR antibodies (EMD-Millipore). Cell-surface localization of TpoR was evaluated by flow cytometry using anti-TpoR(CD110)-PE antibodies (Clone REA250, Miltenyi Biotec, Bergisch Gladbach). TpoR dimerization was assessed in HEK-293T cells using the NanoBiT® complementation assay[13,14].

RESULTS AND DISCUSSION

Activating effect of tryptophan substitutions at the extracellular proximal transmembrane domain of TpoR

The TpoR L498W-H499C variant was identified by targeted sequencing in a TN ET patient. Whole exome sequencing of genomic bone marrow DNA did not identify any other potential driver when assessing a 171-genes myeloid pathology panel. Targeted NGS to assess variant allele frequency in the patient's peripheral whole mononucleated cells (18%) and granulocytes (43%) pointed toward an acquired mutation (Supplemental Table 1). Both single mutations led to ligand-independent STAT5 transcriptional activity, although L498W exerted stronger effects, and the combination more again (Fig. 1A). When stably transduced and at equivalent levels in the IL3-dependent Ba/F3 cell line, autonomous growth was detected in L498W, which was enhanced in L498W-H499C (Fig. 1B). Cell-surface localization of L498W was similar to that of the WT receptor (Fig. 1C).

We and others had previously shown that the human TpoR is mainly a cell-surface monomer that dimerizes either in the presence of Tpo or of the small molecule agonist eltrombopag[12,15], or when mutated (S505N, W515K), via a structural change in the region around H499. We hypothesized that L498W and H499C induce persistent dimerization of TpoR. As detected by protein complementation using the LargeBit and SmallBit fragments of Nano-luciferase[13,14], each mutation induced strong dimerization of the TpoR cytosolic domains (Fig. 1D and Supplemental 1B).

While TpoR H499C did not support proliferation in Ba/F3 cells, its dimerization was comparable to or higher than that of L498W (Fig. 1D). Cell-surface localization and stability of H499C were increased in comparison with WT or L498W (Fig. 1C and F, Supplemental 1E and F). Total expression of the mutants was comparable across cell lines (Supplemental Fig. 2A). These data suggest that H499C, although increasing cell-surface TpoR localization, dimerizes TpoR in a conformation not optimal for signaling. Indeed, different dimeric orientations are compatible with different degrees of signaling[16,17].

To assess whether dimerization and activation involve only the TM-intracellular domains or require the extracellular region as well, we truncated TpoR upstream of position 489 and introduced the mutations into this receptor construct[13]. Both the single and double mutants exhibited significantly more signaling than the WT truncated receptor (Fig. 1E and Supplemental 2C). Thus, the mutations act on the TM-cytosolic domains. In contrast, treatment with eltrombopag activated the TpoR WT but not the mutants, in agreement with a requirement for extra-amino acid residues upstream of H499[15].

We next asked whether other residues can activate at position 498 and whether Trp can activate at other positions between residues 494 and 504. We found that activation was unique to Trp at 498 (Supplemental Fig. 1C, 1D and 2B). Based on previous structural data showing that dimerization induces a helical conformation around H499[7,12], L498W is predicted to stabilize a TM dimer with W498 facing partially towards the dimer interface, as does S505N (Fig. 1G and Supplemental 1A).

H499Y/C mutations increase the activating effect of S505N TpoR mutation

In a second ET patient, we identified the double mutation H499Y-S505N (in *cis*) by targeted sequencing (Supplemental Table 1). While the H499Y mutant was found to be active alone, the

double mutant exhibited significantly enhanced activation of STAT5 over S505N (Fig. 2A). Both mutants were localized on the cell-surface and in the cells at similar or higher levels than the WT receptor (Fig. 2B and Supplemental 2D). Examining whether residues other than H499C/H499Y could activate at position 499, we found that it was not the case for Leu, Phe, Trp or Asn (Fig. 2C and Supplemental 2E), a finding that is corroborated by a recent study[18]. The next question was whether these H499 mutations also enhanced signaling by other TpoR mutants such as L498W or W515K. H499C and H499Y increased activity of both S505N and L498W, but not W515K (Fig. 2D). The enhancement was specific for the H499 mutations, as L498W failed to enhance S505N or W515K signaling (Fig. 2D). That H499 mutations did not alter W515K activity might reflect different active dimeric interfaces adopted by S505N/L498W versus W515K.

In the active S505N dimer, Asn is in the dimer interface of the coiled-coil formed by the two TMDs, while H499 is oriented away from the interface[12] (Supplemental Fig. 1A, lower panel). We had previously found that substitution of H499 to Leu (the WT residue in murine TpoR) in the TM-IC construct of TpoR induced dimerization, but in an inactive orientation[12]. The unique behavior of H499Y and H499C suggests that these substitutions destabilize the inactive orientation of the wild-type receptor (as in H499L) and allow the TM helices to form more stabilizing interhelical contacts in the active dimer.

TpoR activation by eltrombopag or by L498W, S505N and W515K mutations requires the upstream W491 residue

On the basis of structural and mutational data[7,12] we modeled the active TpoR dimeric state using an alpha-helical structure in the region of H499. Another Trp residue (W491) exists upstream on the same helical face as W498 and S505 (both being predicted to occupy an “a” position in a left-handed coiled coil motif (Fig. 1G and 2E, Supplemental 1A), 25 residues from W515 of the RWQFP insert. The W515 position also belongs to the same face but in a “d” position (Supplemental Fig. 1A)[7,19]. We hypothesized that, when rotated inward, the W491 residue might stabilize the active state promoted by other mutations (L498W, S505N and W515K). Given the size of a Trp in the interface, it could push apart helices, which is necessary to allow crossing at S505, leading to activation[12]. Indeed, replacing W491 with A or K prevented TpoR activation by L498W, by S505N and by W515K (Fig. 2F), although not altering cell-surface or global expression (Fig. 2F and Supplemental 2F). Thus, W491 is crucial for the changes in tilt and orientation required for activation[12].

We also tested whether W491 is required, like H499, for activation by eltrombopag. W491 mutations impaired eltrombopag-mediated activation (Fig. 2F) but did not block Tpo-induced activation. We and others showed by polarized IR spectroscopy and NMR studies that eltrombopag induces helix formation around H499 and TpoR dimerization[12,15]. We propose now that eltrombopag activates TpoR by breaking TpoR-membrane interactions via binding to both W491 and H499 and shares the path of activation with the canonical mutants.

Furthermore, our model in Fig. 2E predicts that rotating S505 and W515 inward, and H499 outward will result in activation stabilized by W491. W491 might potentially be accessible on the extracellular JM domain to become a target for small molecules or antibody-mediated inhibition of more than one pathogenic mutant of TpoR. Other such mutants as the one described here are indeed likely to be identified in TN ET patients and they are likely to depend on W491. Interestingly, a recent study using deep mutagenesis and sequencing reported that mutations at positions 490 and 492 also lessened the activity of the S505N mutant[18]. This entire region might thus be of importance for the activation of TpoR.

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Author Contributions

GL, JPD, IC planned paper strategy, performed experiments, interpreted data and wrote the paper; EL, LV planned specific experiments and interpreted data, SC, BP, BC, JMZ provided data on patients, SOS provided structural models and protein structure predictions, SNC planned research, interpreted data and wrote the paper.

Conflict of interest

SNC is co-founder of MyeloPro Research and Diagnostics GmbH, Vienna, Austria.

REFERENCES

1. Barbui T, Thiele J, Gisslinger H, et al. The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. *Blood Cancer J*. 2018;8(2):15.
2. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med*. 2006;3(7):e270.
3. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood*. 2006;108(10):3472-3476.
4. Ding J, Komatsu H, Wakita A, et al. Familial essential thrombocythemia associated with a dominant-positive activating mutation of the c-MPL gene, which encodes for the receptor for thrombopoietin. *Blood*. 2004;103(11):4198-4200.
5. Ding J, Komatsu H, Iida S, et al. 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*. 2009;114(15):3325-3328.
6. Staerk J, Lacout C, Sato T, Smith SO, Vainchenker W, Constantinescu SN. An amphipathic motif at the transmembrane-cytoplasmic junction prevents autonomous activation of the thrombopoietin receptor. *Blood*. 2006;107(5):1864-1871.
7. Defour JP, Itaya M, Gryshkova V, et al. Tryptophan at the transmembrane-cytosolic junction modulates thrombopoietin receptor dimerization and activation. *Proc Natl Acad Sci U S A*. 2013;110(7):2540-2545.
8. Varghese LN, Defour JP, Pecquet C, Constantinescu SN. The Thrombopoietin Receptor: Structural Basis of Traffic and Activation by Ligand, Mutations, Agonists, and Mutated Calreticulin. *Front Endocrinol (Lausanne)*. 2017;8:59.
9. Vainchenker W, Plo I, Marty C, Varghese LN, Constantinescu SN. The role of the thrombopoietin receptor MPL in myeloproliferative neoplasms: recent findings and potential therapeutic applications. *Expert Review of Hematology*. 2019;12(6):437-448.
10. Cabagnols X, Favale F, Pasquier F, et al. Presence of atypical thrombopoietin receptor (MPL) mutations in triple-negative essential thrombocythemia patients. *Blood*. 2016;127(3):333-342.
11. Milosevic Feenstra JD, Nivarthi H, Gisslinger H, et al. Whole-exome sequencing identifies novel MPL and JAK2 mutations in triple-negative myeloproliferative neoplasms. *Blood*. 2016;127(3):325-332.
12. Leroy E, Defour JP, Sato T, et al. His499 Regulates Dimerization and Prevents Oncogenic Activation by Asparagine Mutations of the Human Thrombopoietin Receptor. *J Biol Chem*. 2016;291(6):2974-2987.
13. Leroy E, Balligand T, Pecquet C, et al. Differential effect of inhibitory strategies of the V617 mutant of JAK2 on cytokine receptor signaling. *J Allergy Clin Immunol*. 2019.
14. Dixon AS, Schwinn MK, Hall MP, et al. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chemical Biology*. 2016;11(2):400-408.
15. Kim M-J, Park S, Opella SJ, et al. NMR Structural Studies of Interactions of a Small, Nonpeptidyl Tpo Mimic with the Thrombopoietin Receptor Extracellular Juxtamembrane and Transmembrane Domains. *Journal of Biological Chemistry*. 2007;282(19):14253-14261.
16. Staerk J, Defour JP, Pecquet C, et al. Orientation-specific signalling by thrombopoietin receptor dimers. *EMBO J*. 2011;30(21):4398-4413.
17. Matthews EE, Thévenin D, Rogers JM, et al. Thrombopoietin receptor activation: transmembrane helix dimerization, rotation, and allosteric modulation. *The FASEB Journal*. 2011;25(7):2234-2244.

18. Bridgford JL, Lee S, Lee C, et al. Novel Drivers and Modifiers of MPL-dependent Oncogenic Transformation Identified by Deep Mutational Scanning. *Blood*. 2019;doi: 10.1182/blood.2019002561.
19. Defour JP, Chachoua I, Pecquet C, Constantinescu SN. Oncogenic activation of MPL/thrombopoietin receptor by 17 mutations at W515: implications for myeloproliferative neoplasms. *Leukemia*. 2016;30(5):1214-1216.

FIGURE LEGENDS

Figure 1.

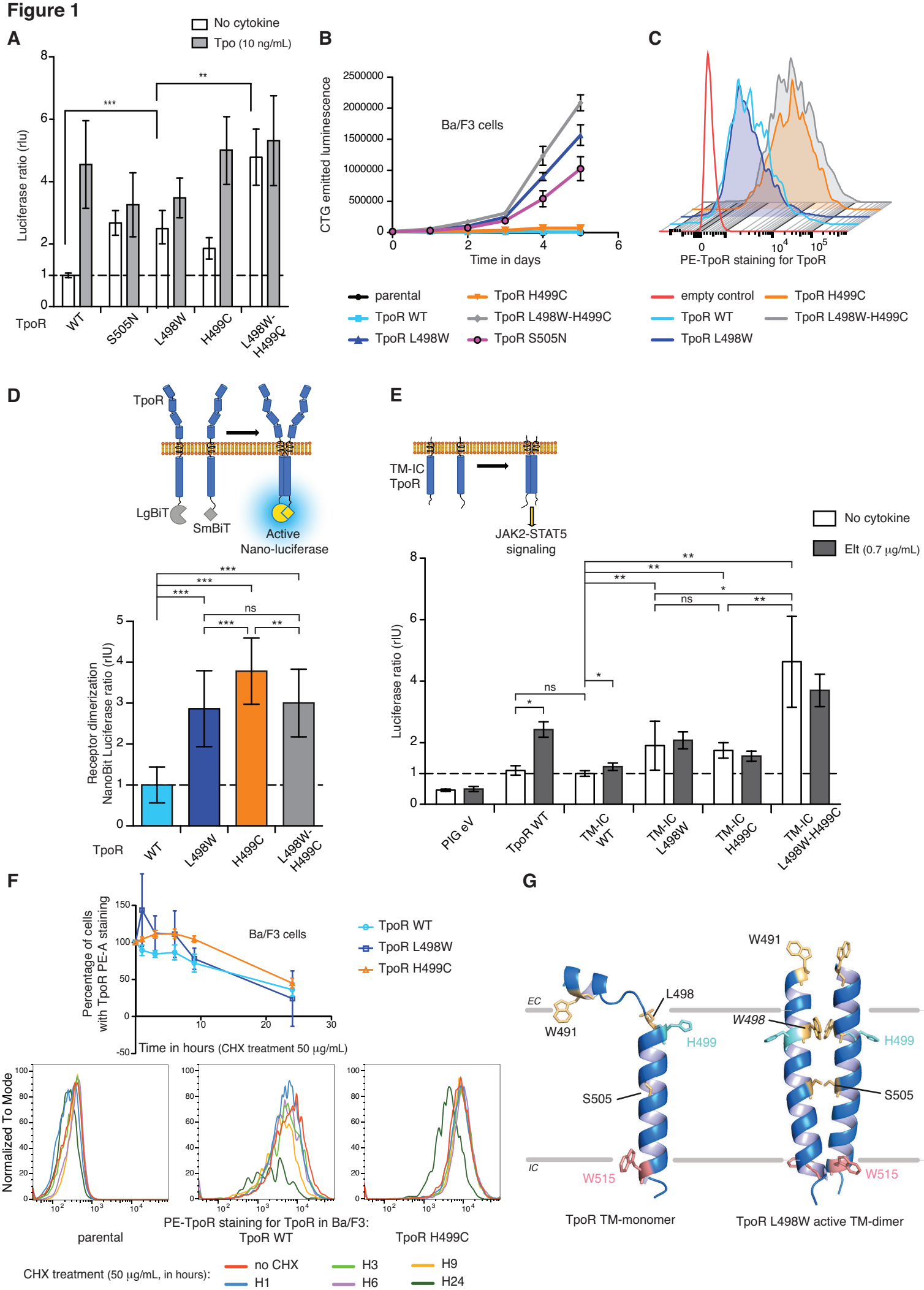
Activation of TpoR signaling by L498W-H499C mutations. A. Effects of H499C and L498W mutations on TpoR signaling. HEK-293T cells were transiently transfected with the indicated HA-TpoR variants, along with cDNAs coding for JAK2, STAT5, SpiLuc *Firefly* luciferase reporter reflecting STAT5 transcriptional activity and normalized with a control reporter (pRL-TK) containing *Renilla* luciferase. Shown are averages of three independent experiments, each performed in triplicate + SD (*error bars*). B. Induction of autonomous proliferation of Ba/F3 cells by L498W and L498W-H499C mutants of TpoR. Ba/F3 cells stably expressing the TpoR mutants of interest were grown in the absence of cytokine. Cell proliferation was assessed by *CellTiter-Glo* (CTG) luminescent cell viability assay. C. H499C increased cell-surface localization of TpoR. HEK-293T cells were transiently transfected with the indicated HA-TpoR variants. TpoR cell-surface localization among FITC-positive cells was assessed by flow cytometry using anti-TpoR antibody. Shown is one representative of two experiments. D. TpoR dimerization in the absence of cytokine. Dimerization of TpoR was determined in HEK-293T using the *NanoBiT* luciferase protein fragment complementation assay using Nano-luciferase fragments fused in-frame with the cytosolic domain of TpoR. E. The extracellular domain of TpoR was not needed for constitutive activation by the L498W-H499C mutant. HEK–293T cells were transiently transfected with the indicated HA-transmembrane-intracellular (TM-IC) TpoR variants, along with cDNAs coding for JAK2, STAT5, SpiLuc *Firefly* luciferase reporter reflecting STAT5 transcriptional activity and normalized with a control reporter (pRL-TK) containing *Renilla* luciferase. Shown are averages of four independent experiments, each performed in triplicate + SD (*error bars*). F. TpoR H499C kept being overexpressed after cycloheximide (CHX) treatment. Ba/F3 cells stably expressing the TpoR mutants of interest were grown in presence of IL-3 and treated with CHX (50 μg/mL). TpoR cell-surface localization among FITC-positive cells was assessed by flow cytometry using anti-TpoR antibody. Upper panel is an average of 3 independent experiments + SD (*error bars*). Lower panel is one representative of three experiments. G. Model of the monomeric WT- and dimeric L498W-transmembrane domains (TMDs) of the TpoR. On the left, the monomeric TMD has a break in the helical secondary structure in the region N-terminal to H499[12]. On the left, in the L498W-active TMD dimer the helix is induced in the region N-terminal to H499. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ns: non-significant; non-parametric multiple comparison Steel test with control. Tpo: human thrombopoietin; TpoR: Tpo receptor; WT: wild-type; Elt:

eltrombopag; rlu: relative luciferase units; LgBit: *Nano-luciferase* large-bit subunit; SmBit: *Nano-luciferase* small-bit subunit; PIG: pMX-IRES-GFP vector; eV: empty vector; CHX: cycloheximide; *EC*: extra-cellular; *IC*: intra-cellular. SD: standard deviation.

Figure 2.

H499 and W491 play opposite roles in regulating activation of TpoR by several activating mutations. A. Effect of TpoR H499Y mutation on constitutive activity of the S505N mutant. B. H499Y and H499Y-S505N increase TpoR cell-surface localizatio. C. Effects of aromatic and aliphatic residues at H499 on TpoR activity. D. Effects of H499Y/C on the constitutive activities of the indicated TpoR mutants (upper panels) and on their cell-surface localization (lower panels). E. Model of the dimeric active S505N- and inactive W491A-transmembrane domains (TMDs) of the TpoR. On the left, in the active S505N mutant, the N505 residues face one-another. On the right, in the inactive W491A mutant, the H499 residues face one another and would therefore be inaccessible to eltrombopag. F. Effects of W491A/K mutations on the indicated TpoR mutants and on stimulations by Tpo or Elt. In A, C, D and F, HEK-293T cells were transiently transfected with the indicated HA-TpoR variants, along with cDNAs coding for JAK2, STAT5, SpiLuc *Firefly* luciferase reporter reflecting STAT5 transcriptional activity and normalized with a control reporter (pRL-TK) containing *Renilla* luciferase. Shown are averages of three to four independent experiments, each performed in triplicate + SD (*error bars*). **: $p<0.01$, ***: $p<0.001$, ns: non-significant, non-parametric multiple comparison Steel test with control. In B and D, HEK-293T cells were transiently transfected with the indicated HA-TpoR variants. TpoR cell-surface localization among FITC-positive cells was assessed by flow cytometry using anti-TpoR antibody. Shown is one representative of two experiments. Tpo: human thrombopoietin; TpoR: Tpo receptor; WT: wild-type; PIG: pMX-IRES-GFP vector; eV: empty vector; Elt: eltrombopag; rlu: relative luciferase units; eV: empty vector; *EC*: extra-cellular; *IC*: intra-cellular.

Figure 1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TM-IC H499C
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F
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Percentage of cells with TpoR PE-A staining
Time in hours (CHX treatment 50 µg/mL)
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TpoR L498W
TpoR H499C
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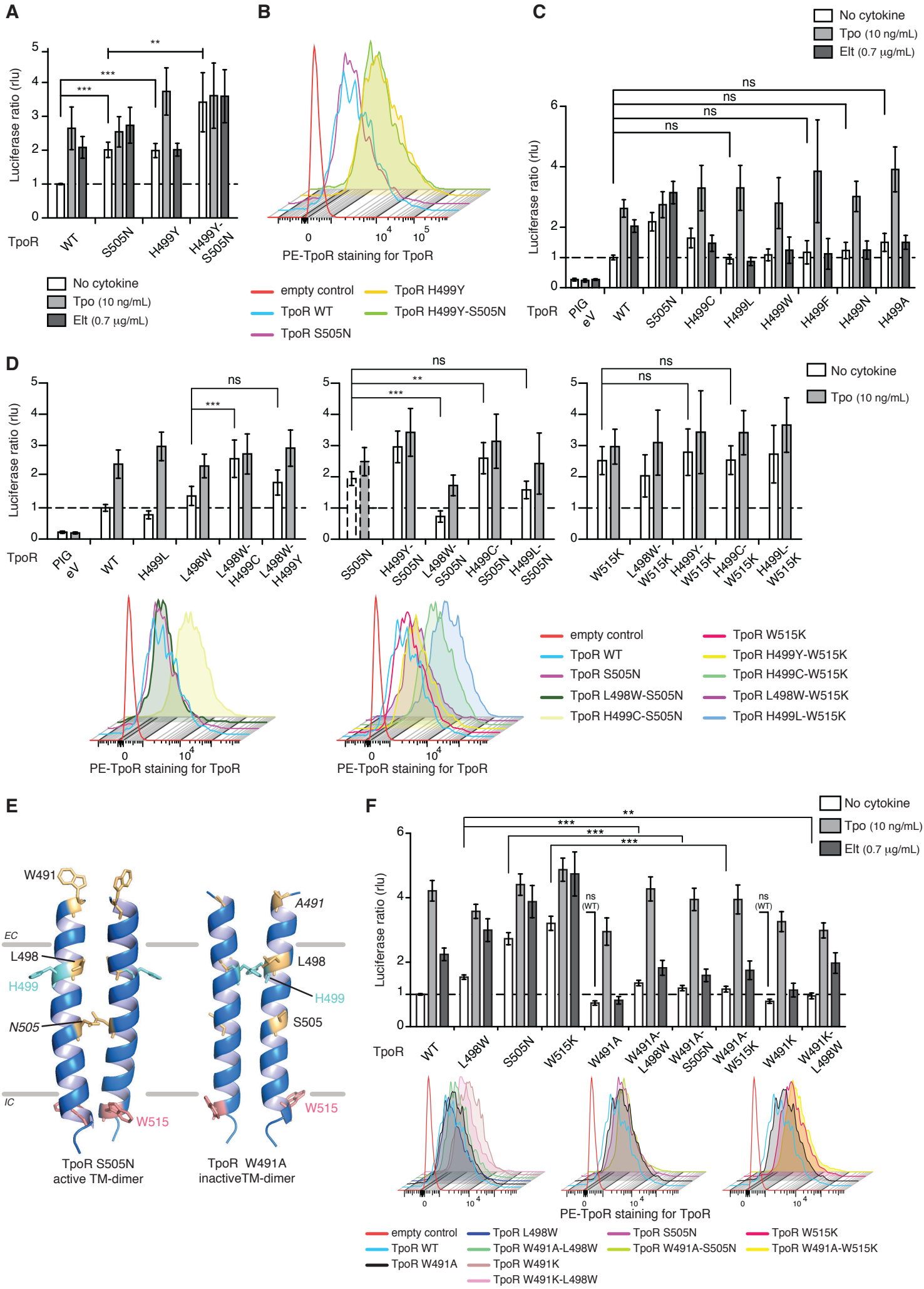
Figure 2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L498W-H499Y
S505N
H499Y-S505N
L498W-S505N
H499C-S505N
H499L-S505N
W515K
L498W-W515K
H499Y-W515K
H499C-W515K
H499L-W515K
PE-TpoR staining for TpoR
PE-TpoR staining for TpoR
empty control
TpoR WT
TpoR S505N
TpoR L498W-S505N
TpoR H499C-S505N
TpoR W515K
TpoR H499Y-W515K
TpoR H499C-W515K
TpoR L498W-W515K
TpoR H499L-W515K
E
W491
EC
L498
H499
N505
IC
W515
A491
L498
H499
S505
W515
TpoR S505N
active TM-dimer
TpoR W491A
inactiveTM-dimer
F
No cytokine
Tpo (10 ng/mL)
Elt (0.7 μg/mL)
Luciferase ratio (rlu)
ns (WT)
TpoR
WT
L498W
S505N
W515K
W491A
W491A-L498W
W491A-S505N
W491A-W515K
W491K
W491K-L498W
PE-TpoR staining for TpoR
empty control
TpoR WT
TpoR W491A
TpoR L498W
TpoR W491A-L498W
TpoR W491K
TpoR W491K-L498W
TpoR S505N
TpoR W491A-S505N
TpoR W515K
TpoR W491A-W515K