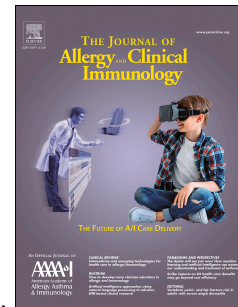


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Functional and structural analysis of cytokine selective *IL6ST* defects that cause recessive hyper-IgE syndrome

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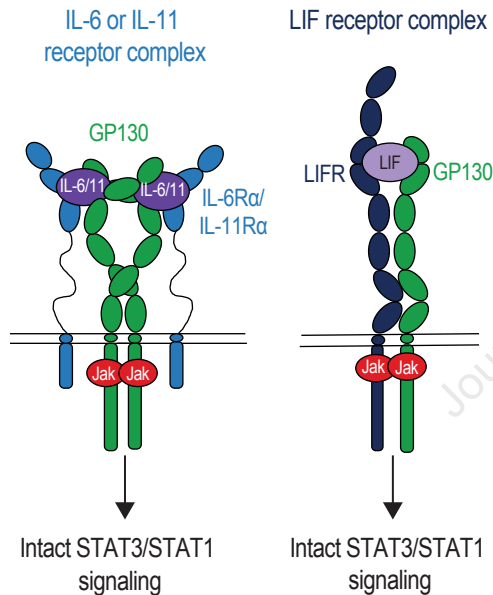
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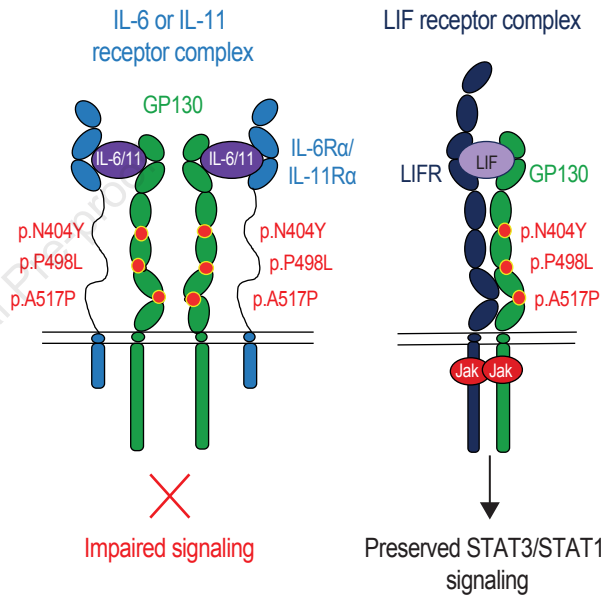
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WT GP130



Pathogenic GP130 variants



Graphical abstract: Representation of three pathogenic GP130 variants, which disrupt the membrane-proximal GP130-GP130 interaction within the hexameric IL-6 and IL-11 receptor complex, but do not destabilize the trimeric LIF receptor complex.

**Functional and structural analysis of cytokine selective *IL6ST* defects
that cause recessive hyper-IgE syndrome**

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CONFLICT OF INTEREST DECLARATION

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ABSTRACT

Background: Biallelic variants in *IL6ST* cause a recessive form of hyper-IgE syndrome (HIES) characterized by high IgE, eosinophilia, defective acute phase response, susceptibility to bacterial infections and skeletal abnormalities due to cytokine selective loss-of-function in GP130 with defective IL-6 and IL-11, variable OSM and IL-27 but sparing LIF signaling.

Objective: To understand the functional and structural impact of recessive HIES-associated *IL6ST* variants.

Methods: We investigated a patient with HIES using exome, genome and RNA sequencing. Functional assays assessed IL-6, IL-11, IL-27, OSM, LIF, CT-1, CLC, and CNTF signaling. Molecular dynamic simulations and structural modeling of GP130 cytokine receptor complexes were performed.

Results: We identify a patient with compound heterozygous novel missense variants in *IL6ST* (p.Ala517Pro, and exon-skipping null_variant p.Gly484_Pro518delinsArg). The p.Ala517Pro variant results in a more profound IL-6 and IL-11 dominated signaling defect compared to the previously identified recessive *IL6ST* variants p.Asn404Tyr, and p.Pro498Leu. Molecular dynamics simulations suggest that the p.Ala517Pro and p.Asn404Tyr variants result in increased flexibility of the extracellular membrane-proximal domains of GP130. We propose a structural model that explains the cytokine selectivity of pathogenic *IL6ST* variants that result in recessive HIES. The variants destabilize the hexameric cytokine receptor complexes whereas the trimeric LIF-GP130-LIFR complex remains stable by an additional membrane-proximal interaction. Deletion of this membrane-proximal interaction site in GP130 consequently causes additional defective LIF signaling and Stüve-Wiedemann syndrome.

Conclusion: Our data provide a structural basis to understand clinical phenotypes in patients with *IL6ST* variants.

Clinical Implication/Key messages: Compound heterozygous variants in *IL6ST* were identified and associated with autosomal recessive hyper-IgE syndrome. Structural analysis show how recessive variants in the extracellular domains of GP130 may differentially affect trimeric- and hexameric complexes and explain pathology by differential cytokine signaling defects.

Capsule summary: We investigate how recessive variants in *IL6ST* cause selective cytokine signaling defects and cause autosomal recessive hyper-IgE syndrome with immunodeficiency and skeletal abnormalities.

Key words: IL-6, IL-11, LIF, *IL6ST*, GP130, STAT3, hyper-IgE syndrome.

Abbreviations

- AD: Autosomal dominant
- AR: Autosomal recessive
- CLC: Cardiotrophin-like cytokine
- CNTF: Ciliary neurotrophic factor
- CT-1: Cardiotrophin-1
- D: Domain
- ER: Endoplasmic reticulum
- EV: Empty vector
- HIES: Hyper-IgE Syndrome
- IL: Interleukin
- JAKs: Janus Kinases
- LIF: Leukemia inhibitory factor
- LIFR: Leukemia inhibitory factor receptor
- MD: molecular dynamics
- ns: Nanoseconds
- OSM: Oncostatin M
- pSTAT3: STAT3 phosphorylation
- STAT3: Signal transducer and activator of transcription 3
- WES: whole exome sequencing
- WT: Wild type

INTRODUCTION

Several cytokine receptors together with the shared signal-transducing β -receptor GP130 enable cellular communication via the interleukin (IL-) 6 cytokine family.^{1, 2} This family includes IL-6, IL-11, IL-27, oncostatin M (OSM), leukemia-inhibitory factor (LIF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), and ciliary neurotrophic factor (CNTF).^{1, 2}

GP130, encoded by *IL6ST*, consists of 6 extracellular domains: an N-terminal Immunoglobulin-like domain (D1); two cytokine binding domains (D2-D3); three membrane-proximal fibronectin type III domains (D4-D6); a transmembrane domain; and an intracellular cytoplasmic domain that contributes to the activation of intracellular signaling cascades.¹ IL-6 interacts with both IL-6R α and GP130, resulting in the formation of a hexameric receptor complex in a 2:2:2 configuration.^{3, 4} By contrast, IL-27/IL-27R, LIF/LIFR and OSM/OSMR form heterotrimeric complexes with GP130, with stoichiometries of 1:1:1.⁵⁻⁷ Signal transduction via GP130 is mediated by Janus Kinases (JAKs) that phosphorylate the intracellular cytoplasmic domain of GP130 resulting in activation of multiple signaling pathways, including the signal transducer and activator of transcription (STAT3) 3 pathway, which regulates immune responses, cell survival and organogenesis.^{1, 2}

Hyper-IgE syndrome (HIES; MIM#147060) is a primary immunodeficiency characterized by elevated IgE levels, recurrent staphylococcal skin abscesses, mucocutaneous candidiasis and recurrent lung infections that can progress to cyst formation and bronchiectasis. The patients experience defective acute-phase response, eosinophilia, eczema, and skeletal abnormalities including retained primary teeth, craniosynostosis and scoliosis.^{8, 9} HIES is typically inherited in an autosomal dominant (AD) manner with dominant-negative *STAT3* mutations accounting

for the majority of AD-HIES.⁸ Autosomal recessive (AR) forms of HIES or HIES-like pathology have been described: these include homozygous mutations of *DOCK8*,¹⁰ *PGM3*,¹¹ and *ZNF341*.^{12, 13} *ZNF341* is a nuclear zinc finger transcription factor that regulates *STAT3* expression by binding to the *STAT3* promoter.¹³ *PGM3* controls GP130 glycosylation and surface expression.¹¹

An additional group of patients with HIES present with genetic variants in *IL6ST*. Whereas complete loss-of-function variants in *IL6ST* that affect all GP130-dependent cytokines cause an extended form of Stüve-Wiedemann syndrome,¹⁴ homozygous missense variants in *IL6ST* with a partial cytokine defect and phenotype of immunodeficiency with elevated IgE and craniosynostosis resembling HIES (MIM#618523) have been described. These variants displayed selective cytokine signaling defects in IL-6, IL-11, IL-27 and OSM signaling while LIF signaling was spared.^{15, 16} In addition to these recessive variants, a total of 12 heterozygous patients from 8 families with AD-HIES due to dominant-negative *IL6ST* variants were-reported.¹⁷ A missense variant in *IL6ST* with a selective IL-11 signaling defect in a patient with craniosynostosis and retained deciduous teeth has also been recently described.¹⁸

The *IL6ST* mutations that gave rise to Stüve-Wiedemann syndrome result in dramatic structural changes that can account for a complete loss of receptor function. By contrast, it is unclear how the non-synonymous single amino acid HIES-causing *IL6ST* variants lead to selective cytokine defects.

Here, we identify a patient who presented with HIES associated with novel compound heterozygous variants in *IL6ST*. We demonstrate that the non-synonymous missense variant

results in selective GP130-dependent cytokine signaling defects in IL-6, IL-11, and to a lesser extent in IL-27 and OSM but allow LIF signaling. Guided by molecular dynamics (MD) simulations and structural data, we propose a model to explain why LIF signaling may be spared explaining the genotype-phenotype associations in these patients.

METHODS

Patient and samples

The study was reviewed by the Stanford Institutional Review Board and the National Human Genome Research Institute Institutional Review Board, with written informed consent obtained from the patient and parent. The patient was enrolled in the Undiagnosed Disease Network study at Stanford University (15-HG_0130). Functional experiments were performed as part of the Oxford GI-biobank project (16/YH/0247) and an approved rare disease subproject.

Whole exome, whole genome and RNA sequencing

Whole exome sequencing (WES) was performed at Baylor Genetics. Transcriptome-wide RNA-seq was performed as previously described.¹⁹

Fibroblast cell culture

Primary skin fibroblasts were established at Stanford University from a skin punch biopsy and maintained in DMEM (Sigma-Aldrich) supplemented with 10% FCS.

GP130 surface expression

Staining of surface GP130 was performed using anti-GP130-biotin (AN-G30; eBioscience) followed by staining with streptavidin-PE CF594 and expression was determined by flow

cytometry. Cells were stained with Fixable Viability Dye eFluor 780 (eBioscience) and dead cells were excluded.

Plasmid expression experiments

A GP130-deficient HEK293 cell line generated using CRISPR/Cas9 was previously described.¹⁵ GP130-deficient HEK293 cells were transiently transfected for 24 hours with plasmids encoding empty vector (EV), WT or GP130 variants p.Ala517Pro, p.Asn404Tyr, p.Pro498Leu or the exon 12 skipping null variant (mutagenesis created by GenScript) (pcDNA3.1(+)) using Lipofectamine 2000 (Thermo Fisher). Cells were co-transfected with a GFP-encoding plasmid for gating on successfully transfected cells.

For live cell confocal imaging or where specified, WT and *IL6ST* variants were cloned into the vector pMM-YPet using the Gateway Cloning system. In brief, point mutations were introduced into *IL6ST* in the entry vector by mismatch primers via mutagenesis PCR. WT and *IL6ST* variants were subsequently transferred from the entry vector to the pMM-YPet destination vector by recombination using the LR Clonase II mix (Thermo Fisher) according to the manufacturer's instructions.

Phosflow STAT3 signaling assay

Phosphorylation of STAT3 (pSTAT3) was determined by Phosflow. Fibroblasts and HEK293 cell lines were plated in 96-well plates and serum starved overnight. Next day, cells were further serum-starved for 2 hours, stained with Fixable Viability Dye eFluor 780 (eBioscience) and stimulated with indicated cytokines (Miltenyi Biotec, R&D, Biolegend) for 15 min. Subsequently, cells were detached with trypsin-EDTA solution and fixed in pre-warmed Cytofix buffer (BD biosciences). After fixation, cells were washed and permeabilized with ice-cold Perm Buffer III (BD biosciences) and stained with anti-STAT3

(pY705)-Alexa Fluor 647 (clone 4/P-STAT3) at room temperature. 1-2 technical replicates were included per independent experiment. Samples were acquired using LSRII and data was analyzed using Flowjo Software (version 10.4.0).

Live cell confocal imaging

GP130-deficient HEK293 cells were seeded in ibidi μ -dishes and transfected with YPet-fused *IL6ST* variants. 48 hours after transfection, cells were incubated for 10 min with CellMaskTM Deep Red Plasma membrane stain and 2 μ M Hoechst 33342 solution (ThermoFisher) before live cell confocal imaging using a Zeiss LSM 980.

Molecular dynamics simulations

MD simulations were performed using NAMD 2.1.3b1²⁰ and the CHARMM22 forcefield^{20, 21} at 310 K. GP130 structures PDB IDs: 3L5I, 3L5H²² and 1I1R²³ were used to construct starting models. Trajectories were analyzed using VMD 1.9.3.²⁴ The mCSM server²⁵ was used to calculate the free energy change as a result of the mutations (see Supplementary Methods for full details).

3D structural modeling

Models of the IL-6/IL-6R α /GP130, IL-11/IL-11R α /GP130, and LIF/LIFR/GP130 complexes were constructed by structural superposition of the complete extracellular domains of GP130 (PDB ID: 3L5H),²² the IL-6 complex (PDB ID: 1P9M),²⁶ IL-11 (PDB ID: 6O4O) and IL11R α (PDB ID: 6O4P),²⁷ the LIF/GP130 complex (PDB ID: 1PVH),²⁸ and the LIF/LIFR complex (PDB ID: 2Q7N)²⁹ (see Supplementary Methods for full details).

Statistical analysis

Results were analyzed using Prism 8 (GraphPad software, Inc.). Significance of differences was determined by nonparametric Mann-Whitney *U* test, one-way ANOVA or Kruskal-Wallis test. *p*-values < 0.05 were considered significant.

RESULTS

Clinical history – Compound heterozygous *IL6ST* variants in a patient with hyper-IgE syndrome.

We investigated an 8-year-old male patient of Native American, Italian and Scottish ancestry, who presented with chronic osteomyelitis, and recurrent septic arthritis of the knees beginning at 4 years of age causing bone and joint destruction. The causative organism for these infections was *Staphylococcus aureus* (**Table 1**). He is the only child from a non-consanguineous family, and no significant family history was reported (**Fig 1, A**). He has a history of chronically elevated IgE (842-1549 IU/mL) and eosinophilia (320-1430 cells/uL) (**Table 1**). Congenital bilateral contractures of the fingers were noted in the newborn period. As a newborn he was additionally diagnosed with pneumothorax and pulmonary hypertension requiring extracorporeal membrane oxygenation. He was hospitalized in the newborn intensive care unit for two months after delivery. An episode of severe pneumonia occurred at 6 months of age with empyema requiring a video-assisted thoracoscopic surgery procedure. Subsequent clinical features included recurrent otitis media, asthma, obstructive sleep apnea with a retruded midface, multiple fractures without major trauma, and multiple food and environmental allergies. He had delayed tooth eruption (primary and secondary teeth) with one retained primary tooth. Additional dysmorphology included a high arched palate, telecanthus, long palpebral fissures and a prominent lower jaw. Pan-craniosynostosis and mid-face retrusion was noted on 3D CT reconstruction (**Fig 1, B**). Plain films show the

effects of destructive arthritis in the patient's left elbow and left knee (**Fig 1, C-E**). In total, clinical features were suspicious for HIES (NIH HIES score of 37).

No cellular defect was identified based on routine immunological assessment. Luminex cytokine measurements from serum samples were compared with all other patients who were investigated for rare immune and non-immune disorders. Cytokines including IL-6, IL-27, LIF, IL-10, IL-17F, IL-22, and IL-23 were measured and no major change in cytokine dysregulations were noted in patient II-1 (see **Fig E1** in this article's Online Repository at www.jacionline.org). The clinical laboratory parameters, infections, and features are summarized in **Table 1** and **Table E4**. A comparison of the clinical phenotypes of all three HIES *IL6ST* mutations are summarized in **Table E4**.

After non-informative STAT3 sequencing, WES identified a missense variant within the *IL6ST* locus (NC_000005.10; Hg19, c.1549G>C, p.Ala517Pro; 5-55248081) (**Table E1** and see **Fig E2, A**, in this article's Online Repository at www.jacionline.org). Upon manual review using Integrative Genomics Viewer, a second *IL6ST* variant was identified in trans (c.1552+3A>C; p.Gly484_Pro518delinsArg; 5-55248075) (see **Fig E2, B**, in this article's Online Repository at www.jacionline.org). Comparison of the patient and maternal WES data indicated that the c.1552+3A>C variant was maternally inherited, which was confirmed by Sanger sequencing (**Fig 1, F**). Whilst DNA of the healthy father was unavailable, inspection of WES and WGS data indicated that these variants are *in trans*, as they never co-occur on the same sequencing read despite their close proximity of 6 base pairs (see **Fig E2, B**, in this article's Online Repository at www.jacionline.org). p.Ala517Pro was predicted to be deleterious by several tools including SIFT (Damaging),³⁰ CADD score (25.1),³¹ and

MutationTaster (Disease-causing)³² (**Table E2**). GnomAD reports an allele count of 2 among 280,866 alleles.

The c.1552+3A>C splice variant was predicted by MaxENTscan to cause a dysfunctional donor site, leading to skipping of exon 12. This variant is not found in gnomAD and not otherwise reported. mRNA sequencing was performed on whole blood from the patient II-1. Despite low expression of *IL6ST* (0.39 TPM), exon 12 skipping was detected along with a normally spliced transcript including the c.1549G>C variant, providing additional evidence for the compound heterozygous inheritance. RNA-seq confirmed the predicted splicing impact of the c.1552+3A>C (p.Gly484_Pro518delinsArg) (**Fig 1, G**) but did not show significant alteration in absolute *IL6ST* transcript abundance (**Table E3**).

Selective loss of IL-6, IL-11, and OSM signaling in patient II-1 primary fibroblasts

We first investigated the functional consequences on cytokine signaling using patient II-1 fibroblasts (**Fig 2**). Using a monoclonal antibody to GP130, we failed to detect GP130 surface expression (**Fig 2, A**). Stimulation with GP130-dependent cytokines (IL-6, IL-11, IL-27, OSM, LIF) in two healthy control fibroblast lines resulted in the expected phosphorylation of STAT3 (pSTAT3) (**Fig 2, B-F**). By contrast, patient fibroblasts showed defective IL-6, IL-11, and to a lesser extent OSM signaling whilst responses to IL-27 and LIF were intact and comparable to control fibroblasts (**Fig. 2, B-F**). The defective IL-6 and IL-11 signaling was most apparent with highest cytokine concentration (100ng/ml) whilst reduced OSM signaling was dose-dependent as the defect was only observed with lower concentrations (1ng/ml). Stimulation with GP130-independent IFN- α , as a control, resulted in similar levels of pSTAT3 (**Fig 2, G**). The selective cytokine defects of the patient fibroblasts with p.Ala517Pro and exon 12 skipping variants were remarkably similar to those fibroblasts that have been previously described in patients carrying homozygous

p.Asn404Tyr, and p.Pro498Leu variants.^{15, 16} The data summarized in a radar chart highlights the comparable responses to LIF (and IL-27) between control and patient fibroblasts whereas profound defects are seen for other GP130-dependent cytokines (**Fig 2, H**).

The c.1552+3A>C exon skipping splice variant is a null allele.

We assessed the functional consequences on the maternally inherited c.1552+3A>C splice variant. The exon 12 skipping variant disrupts the D5 domain of GP130 (see **Fig E3, A**, in this article's Online Repository at www.jacionline.org). A structural analysis predicts defective signal transduction due to disruption of the D5-D6 interdomain interface and a less well-ordered D5. This likely results in a significantly impaired geometry of GP130 that does not bring the two intracellular cytoplasmic domains in close proximity to allow downstream signaling. The c.1552+3A>C splice variant was not detectable by surface staining using an anti-GP130 antibody and showed complete loss of GP130-dependent signaling whereas response to the GP130-independent cytokine IFN- α was intact (see **Fig E3, B-K**, in this article's Online Repository at www.jacionline.org).

p.Ala517Pro, p.Asn404Tyr and p.Pro498Leu *IL6ST* variants result in alterations in IL-6 family cytokine signaling.

We have previously reported two HIES causing *IL6ST* variants, p.Asn404Tyr and p.Pro498Leu.^{15, 16} To compare the functional consequences of p.Ala517Pro with the two previously reported HIES-causing variants, we used a GP130-deficient HEK293 cell line.¹⁵ Transfection with a plasmid expressing WT *IL6ST* restored GP130 surface expression as did transfection with plasmids encoding p.Asn404Tyr and p.Pro498Leu mutant forms of *IL6ST*. Notably, the p.Ala517Pro variant was not recognized by GP130 antibody staining (**Fig 3, A**), consistent with absent GP130 surface detection on patient fibroblasts (**Fig 2, A**). This could

suggest a conformational change caused by the p.Ala517Pro variant that precludes epitope binding or differences in protein expression or trafficking to the cell surface. GP130 protein was detectable at equal amounts in cells transfected with WT as well as p.Ala517Pro, p.Asn404Tyr and p.Pro498Leu by immunoblotting using an antibody raised against the C-terminus of GP130 (**Fig 3, B**).

To investigate the expression of GP130 and its variants in different cellular compartments, we next performed live cell confocal imaging using YPet-fused GP130 variants. We used CellMaskTM Deep Red to stain the plasma membrane, co-transfected marker ECFP-KDEL to indicate the endoplasmic reticulum (ER) and mCherry-Rab5a for early endosomes. This analysis suggested that while p.Ala517Pro can be detected at the plasma membrane, a larger fraction is also present in the ER and to some extent in early endosomes suggesting delayed protein maturation and trafficking (**Fig 3, C**). p.Asn404Tyr and p.Pro498Leu variants did not show enhanced ER localization but were increased in early endosomes suggesting that these variants might display enhanced internalization kinetics. We further confirmed all variants are colocalized on the plasma membrane as demonstrated by ImageStream microscopy imaging (see **Fig E4** in this article's Online Repository at www.jacionline.org).

We next investigated the response to GP130-dependent cytokines: IL-6, IL-11, IL-27, OSM, LIF, CT-1, CLC, and CNTF. Transfection with p.Ala517Pro, or p.Asn404Tyr variant showed defective pSTAT3 responses in all GP130-dependent cytokines whereas the effect of the p.Pro498Leu variant was more subtle (**Fig 4, A-H**). Transfection with the three HIES variants collectively show that the p.Pro498Leu variant was the least affected whereas p.Ala517Pro variant was most affected. GP130-independent pSTAT3 response to IFN α was comparable to WT, further indicating that these defects were specific to the IL-6 family (**Fig 3, I**). These

results suggest that all three HIES *IL6ST* variants are expressed on the cell surface but have different pSTAT3 responses to IL-6 and IL-11, compared to LIF and OSM.

p.Ala517Pro and p.Asn404Tyr *IL6ST* variants result in increased conformational flexibility of GP130

We next investigated the molecular mechanism by which *IL6ST* substitutions cause selective cytokine signaling defects. The three variants are located in D4, 5 and 6 of the extracellular portion of GP130 (**Fig 5, A**). The amino acid conservation scores, calculated by the ConSurf server,^{33, 34} for Asn404, Pro498 and Ala517 vary from highly conserved Asn404 and Pro498 to less conserved Ala517 (**Fig 5, B**). No homologues of the pathogenic p.Ala517Pro, p.Asn404Tyr, or p.Pro498Leu variants were detected in any species (**Fig 5, C**).

We hypothesized that changes in GP130 conformation may explain functional defects. Since each of these variants are in close proximity to domain interfaces, with p.Asn404Tyr at the D3-D4 interface; p.Pro498Leu at the D4-D5 interface; and p.Ala517Pro at the D5-D6 interface, the mutations may create steric clashes and/or alterations to local structure that result in disruption of the domain interfaces (**Fig 5, D**). In addition, p.Pro498Leu and p.Ala517Pro could alter the local backbone conformation due to the restricted backbone configuration of proline relative to other amino acids and effects on adjacent residues.³⁵ Indeed, the mCSM server³⁶ suggests that the variants p.Ala517Pro, p.Pro498Leu, and p.Asn404Tyr have $\Delta\Delta G$ of -2.6 kJ/mol, - 4.4 kJ/mol and -2.3 kJ/mol respectively, which suggests that all three variants are destabilizing.

To assess the structural impact of the three recessive mutations on the configuration of GP130, we ran all-atom MD simulations on GP130 models of each variant. The relative

dynamics of the two domains adjacent to each mutation were assessed by aligning each frame of a simulation on one of the domains, then calculating the movement of the second domain with respect to its three principal axes at the start of the simulation (**Fig 6** and see **Fig E5** in this article's Online Repository at www.jacionline.org). In two of the triplicate 500 ns simulations of the p.Ala517Pro variant, which converts a conserved di-proline linker motif³⁷ into a tri-proline sequence, D6 underwent large angular shifts relative to D5, while the angles measured for the WT were relatively stable throughout the simulations (**Fig 6, A and B** and see **Fig E5** and **movie E7** in this article's Online Repository at www.jacionline.org). In one simulation, the altered orientation of D6 in the p.Ala517Pro variant endured over the second half of the trajectory (**Fig 6, B**). Consistent with these observations the overall angular distributions for these simulations were different (**Fig 6, A and B** and see **Fig E5, A and B, movie E7** in this article's Online Repository at www.jacionline.org). The third p.Ala517Pro variant simulation showed angular distributions that were broader than those of the WT, further confirming increased flexibility of D6 relative to D5 caused by the mutation. In contrast, the angular measurements of D4 relative to D5 were comparatively stable throughout the trajectories for both the p.Pro498Leu and WT simulations, resulting in very similar angular distributions, and indicating little alteration of conformational flexibility due to the mutation (**Fig 6, C and D, and Fig E5, A and C, movie E8** in this article's Online Repository at www.jacionline.org). Measurements of the angular displacement of D4 relative to D3 showed that D4 was more dynamic in both the p.Asn404Tyr variant and WT simulations than we observed for the other domain pairings (**Fig 6, E and F, and see movie E9** in this article's Online Repository at www.jacionline.org). Inspection of angular measurements and distributions for the triplicate simulations indicated that the p.Asn404Tyr distributions were more variable than those of the WT (**Fig 6, E and F**), suggesting that the variant undergoes increased conformational flexibility around the D3-D4 interface, accessing

a larger range of inter-domain angles (see **Fig E5, D and E**, in this article's Online Repository at www.jacionline.org).

Pathogenic *IL6ST* variants do not prevent the formation of hexameric IL-6 receptor signaling complexes

The three *IL6ST* variants are not located within the cytokine binding domains of GP130 (D1-D3) but may alter signaling capacity.²⁶ We performed pulldown experiments using Hyper IL-6-Fc, an artificial fusion protein of IL-6 and the soluble ectodomain of IL-6R α ³⁸ fused to an additional human Fc-tag, (see **Fig E6, A**, in this article's Online Repository at www.jacionline.org). While the affinity of the *IL6ST* variants p.Ala517Pro, p.Asn404Tyr and p.Pro498Leu to Hyper IL-6-Fc was slightly reduced as compared to WT, receptor complex formation was present in all *IL6ST* variants tested (see **Fig E6, B**, in this article's Online Repository at www.jacionline.org). These results suggest that hexameric signaling complexes are able to form with each of the variants while downstream signaling is affected.

A structural model for pathogenic *IL6ST* variants

To understand why IL-6 and IL-11 complexes were able to form, yet signaling was perturbed, we constructed models for the hexameric IL-6/IL-6R α /GP130 and IL-11/IL-11R α /GP130 complexes and the trimeric LIF/LIFR/GP130 complex using alignments with existing structural data. Our models of the IL-6/IL-6R α /GP130 or IL-11/IL-11R α /GP130 complexes, suggested that D4-D6 from the two GP130 molecules do not make significant contact with each other in the complex (**Fig 7, A**). The relative conformation of D4-D6 of GP130 is proposed to position the membrane-proximal domains in close proximity to allow signaling,²² and deletion of any of these domains results in an inactive receptor.³⁹ We propose that increased flexibility of the membrane-proximal domains of the two GP130 molecules would

likely alter the relative positions and poses of the membrane-proximal GP130 domains, contributing to loss of signaling by IL-6 and IL-11.

In contrast, the LIF/LIFR/GP130 complex model suggests that the membrane-proximal domain (D8) of LIFR interacts with D5-D6 of GP130. This interaction may be sufficient to stabilize the geometry of GP130 and thus the relative positions of the membrane-proximal domains of GP130 and LIFR (**Fig 7, A**), consistent with the limited effect of the variants on STAT3 activation via LIF. This is in contrast to the Stüve-Wiedemann syndrome causing complete loss-of-function c.1699+4A>G splice variant that deletes exon 13 and prevents stabilization via D6 (**Fig 7, B**).

DISCUSSION

Signaling by cytokines of the IL-6 family including IL-6, IL-11 and OSM has been linked with a number of auto-immune diseases including inflammatory bowel disease,⁴⁰ multiple sclerosis,⁴¹ arthritis,⁴² cytokine release syndromes associated with cancer immune-therapies⁴³ and infections including COVID-19 pneumonia.⁴⁴ Understanding the function of GP130 is therefore of great importance. We describe two novel *IL6ST* compound heterozygous variants in a patient with HIES and compare their impact on signaling with other known *IL6ST* variants. This work highlights the following findings: (i) we expand the spectrum of AR-HIES patients related to *IL6ST*; (ii) we show that there was a selective loss of IL-6 and IL-11 signaling; (iii) using live cell confocal imaging, we show all *IL6ST* AR-HIES variants are expressed at the plasma membrane, as well as the ER and early endosomes; (iv) we demonstrate through MD simulations and structural models, how *IL6ST* missense recessive variants may differentially affect cytokine receptor binding and signaling.

Patient II-1 displayed a typical clinical presentation of AR-HIES with many features in common with the previously reported cases of *IL6ST* or *STAT3* associated HIES^{15, 16} (**Table E4**). All three variants presented with a similar HIES phenotype characterized by high IgE, recurrent bacterial infections and abnormal dentition. All three variants led to a complete loss of IL-6 and IL-11 signaling, a partial to severe loss of IL-27 and OSM, CT-1, CLC and CNTF signaling, and normal to partial loss of LIF signaling. Impaired IL-6 responses likely account for most of the immunological abnormalities. Defective IL-11 signaling is likely to contribute to the skeletal phenotype as patients with *IL11RA* mutations present with craniosynostosis and dental anomalies.^{45, 46} In addition to those previously described features, we noted additional phenotypes. All three patients had some degree of psycho-motor retardation. Mice deficient in the neurotropic members of the GP130 family: CT-1, CLC, and CNTF all display defects in motor neurons.⁴⁷⁻⁴⁹ While we observed a profound defect in CT-1 signaling, CLC and CNTF responses were partially preserved, which might explain why the clinical observed neurological defects were only mild. The lack of pathognomonic features of Stüve-Wiedemann syndrome were consistent with largely preserved LIF activity.⁵⁰

Despite a considerable overlap between AR and AD forms of *IL6ST* in HIES, the reported 12 heterozygous HIES patients with dominant-negative *IL6ST* variants (AD) do not display skeletal features including craniosynostosis.¹⁷ AD-*IL6ST* variants lack recycling motif and all four STAT3 binding sites. Defective internalization causes accumulation of mutant GP130 on the cell surface and GP130-dependent cytokine signaling defects, most profoundly in IL-6 and IL-11. AR-*IL6ST* variants similarly show most profound signaling defects on IL-6 and IL-11, that are most likely to account for the majority of immunological and skeletal defects.

It is of note that there is a quantitative discrepancy of the response between patient primary fibroblasts and p.Ala517Pro transfected GP130-deficient HEK293 cells¹⁶ (**Fig, 2 and 4**). One potential explanation is that the two cell types likely have different receptor expression levels and therefore different stoichiometry compared to GP130. The receptor density at the cell surface likely contributes to the discrepancies in responsiveness.

We observed the most profound signaling defects in the p.Ala517Pro variant followed by the p.Asn404Tyr and p.Pro498Leu variants, which does not correlate with HIES score (37, 40, and 57 respectively); however, the significant destruction of joints in patient II-1 does contribute to the overall disease severity (**Fig 1, D and E**).

MD simulations suggest that p.Ala517Pro and p.Asn404Tyr variants result in increased flexibility in the membrane-proximal domains of GP130. The differential signaling capacity is likely explained by differences in the interaction domains between hexameric and trimeric GP130 complexes. IL-6R α and IL-11R α bind GP130 via the D1-D3 domains, forming a hexameric complex incorporating two GP130 molecules. The HIES variants likely destabilize the GP130 hexameric complex, preventing intracellular signaling. By contrast, binding of GP130 to the LIF-LIFR is mediated via an additional contact region. Interaction between the LIFR and GP130 D5-D6 may be sufficient to compensate and stabilize the geometry of the trimeric receptor complex in the presence of variants p.Ala517Pro, p.Asn404Tyr and p.Pro498Leu. Transmission electron microscopy analysis of a full-length GP130-LIFR complex suggested that complex formation results in engagement of D5 of GP130 with D7 of LIFR and D6 of GP130 and D8 of LIFR.⁵¹ This model is supported by a previously reported *IL6ST* intronic variant (c.1699+4A>G) leading to skipping of exon 13 in D6 that results in complete loss of LIF signaling, further reinforcing the importance of GP130 D6 for LIFR

complex signaling¹⁴ (**Fig 7, B**). As OSMR and IL-27R also have structured membrane-proximal domains, the milder OSM and IL-27 signaling defects may be explained by similar structural effects within the signaling complex. It is of note that OSM can signal through both OSMR and LIFR.⁶ It is likely that the exon skipping *IL6ST* variant (p.Gly484_Pro518delinsArg) causes significant unfolding of the remainder of GP130 D5, removing constraint on the relative geometry of D4 and D6. Thus, if D5 is involved in the GP130-LIFR interface, this component of the signaling complex formation interactions will be lost and the interaction with LIFR may not be able to overcome the very significant structural alterations in GP130 D4-D6.

In summary, we established a genotype-phenotype association of AR-HIES with *IL6ST* variants. We provide a structural model, explaining how increased flexibility in the membrane-proximal region causes IL-6 and IL-11 signaling defects and why contacts between membrane-proximal regions of GP130 and LIFR allow LIF signaling. The structural insight explains why some *IL6ST* variants cause HIES but not Stüve-Wiedemann syndrome.

AUTHOR CONTRIBUTIONS

JAB, MTW, DBZ, and JG performed genetic and clinical evaluations. SM performed bioinformatic analysis and Luminex data analysis. CM performed patient and sample coordination and consent. DB, EAW, YY and CME performed DNA sequencing and analysis. LF and SBM performed RNA-seq and analysis. YH and CZ performed primary fibroblast cell culture. JAB, MTW, PGF and EAA provided scientific oversight. YHC, FK, LG performed functional experiments. RM, MG, TP, VS, CJM and MWP conducted 3D modeling and molecular dynamics analysis. YHC, DS, AL, and HHU wrote the manuscript. All authors

have revised and approved the manuscript. Members of the Undiagnosed Diseases Network are listed in Data S1.

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FIGURE LEGENDS

FIG 1. Clinical and genetic findings of HIES patient with compound heterozygous variants in *IL6ST*. **A**, Family pedigree: *Solid symbol*, patient II-1 with compound heterozygous for variants in *IL6ST*. **B**, Pan-craniosynostosis and midface retrusion. **C**, Left elbow. **D-E**, Left knee showing progressive arthritis at 8 and 9 years. *White arrows* indicate joint destruction. **F**, *Red arrow* indicates compound heterozygous variant of II-1 by Sanger sequencing. **G**, Sashimi plot showing one transcript with exon skipping.

FIG 2. Defective GP130-dependent IL-6, IL-11 and OSM signaling but normal LIF signaling in patient fibroblasts. **A**, GP130 surface expression in fibroblasts from patient II-1 and controls (n=4). **B-G**, pSTAT3 responses in p.Ala517Pro fibroblasts after stimulation with indicated cytokines compared to healthy controls. *Left*, representative histograms. *Middle*, representative titration curves (nonlinear regression) for all cytokines (n=2) except IFN- α . *Black arrows* indicate the cytokine concentration used in representative histograms and quantification. *Right*, Quantification of pSTAT3+ cells after stimulation (n=3-5). **H**, Radar chart summarizing pSTAT3 responses from GP130-dependent cytokine based on *black arrow* from (B-F). **A-G**, Statistical differences were determined by Mann-Whitney *U* test, ** $P \leq 0.01$, and * $P \leq 0.05$.

FIG 3. Localization of the p.Ala517Pro, p. Asn404Tyr and p.Pro498Leu variants within cellular compartments. **A**, GP130 surface expression from GP130-deficient HEK293 cells transfected with empty vector (EV), WT or variants of GP130 (n=4). **B**, Immunoblot analysis

of GP130 in the lysates of GP130-deficient HEK293 cells transfected with WT or *IL6ST* variants. **C**, GP130-deficient HEK293 cells were transfected with YPet-fused *IL6ST* variants. Live cell confocal imaging showing *left*, colocalization of YPet-fused GP130 variants using markers for the ER (ER-ECFP), nucleus (Hoechst), early endosomes (mCherry-Rab5a) and plasma membrane (CellMask); *Right*, quantification.

FIG 4. The p.Ala517Pro variant was associated with profound defect of cytokine specific GP130 function. A-I, pSTAT3 responses in GP130-deficient HEK293 cells transfected with WT or *IL6ST* variants after stimulations with indicated cytokines. *Left*, representative histograms. *Middle*, representative titration curves (nonlinear regression) for all cytokines (n=2) except IFN- α . *Black arrows* indicate the cytokine concentration used in representative histograms and quantification. *Right*, Quantification of pSTAT3+ cells after stimulation (n=3-5). Statistical differences were determined by one-way ANOVA, *** $P \leq 0.001$, ** $P \leq 0.01$, and * $P \leq 0.05$.

FIG 5. Structural mapping of the p.Ala517Pro, p. Asn404Tyr and p.Pro498Leu variants. A, Domain organization of the human *IL6ST* gene highlighting mutation sites. **B-C**, Conservation of GP130: *Color bar* indicates the conservation score, and pie charts represent conservation from the ConSurf Server. *White* indicates the proportion of species with the human WT amino acid at the indicated position; *grey* indicates the proportion of species with substitution to an indicated amino acid; and *black* indicates the proportion of species in which the amino acid site is mutated to any amino acid (*Xaa*). **D**, *Left*, Extracellular domains of GP130 and *red dots* indicate the mutation sites. *Right*, Enlarged domain interfaces for WT and *IL6ST* variants. *Red dotted lines* indicate clashes. *D*, domain.

FIG 6. Molecular dynamics modelling of WT GP130 and the three pathogenic variants.

A, Dynamics of D6 relative to D5 for WT GP130 D4-D6. **B**, Dynamics of D6 relative to D5 for p.Ala517Pro GP130 D4-D6. **C**, Dynamics of D4 relative to D5 for WT GP130 D4-D6. **D**, Dynamics of D4 relative to D5 for p.Pro498Leu GP130 D4-D6. **E**, Dynamics of D4 relative to D3 for WT GP130 D2-D5. **F**, Dynamics of D4 relative to D3 for p.Asn404Tyr GP130 D2-D5. Each panel: *Left*, frames of the structure throughout simulation 1 (overlaid at 100 ns increments), alongside the principal axes of the domain (red, green and cyan) and the central axis of the domain at the start of the simulation (purple); *Middle*, angles measured between the principal axes and the domain axis throughout the trajectory colored according to the color of the principal axes from the *Left*; *Right*, angular distributions over the 500 ns trajectories shown from the *Middle*.

FIG 7. A structural model for pathogenic IL6ST variants. A-B, Model of hexameric IL-6-IL-6R α -GP130, IL-11-IL-11R α -GP130 and trimeric LIF-LIFR-GP130 complex in the context of HIES and Stüve-Wiedemann syndrome. *Red* indicate mutation sites.

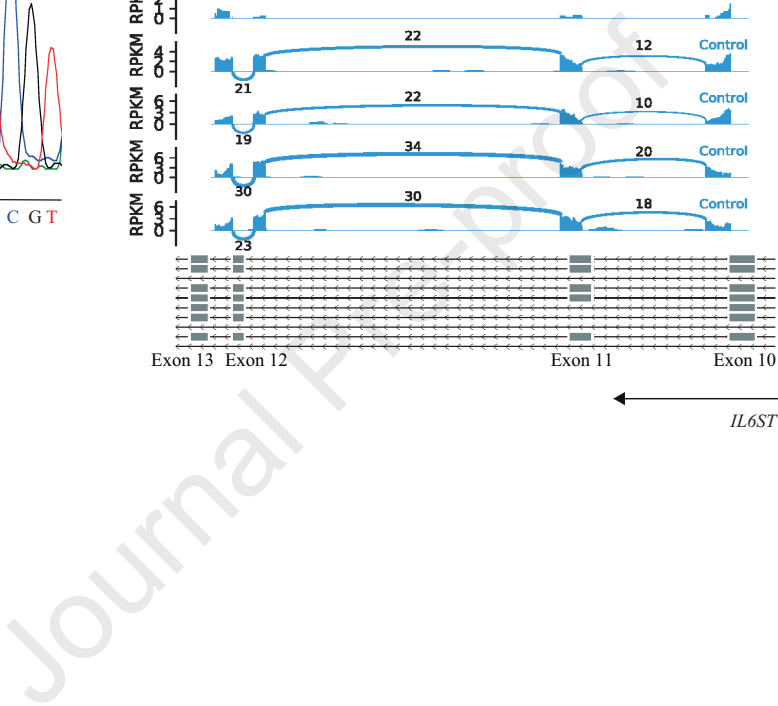
Table 1. Clinical measurements in patient II-1.

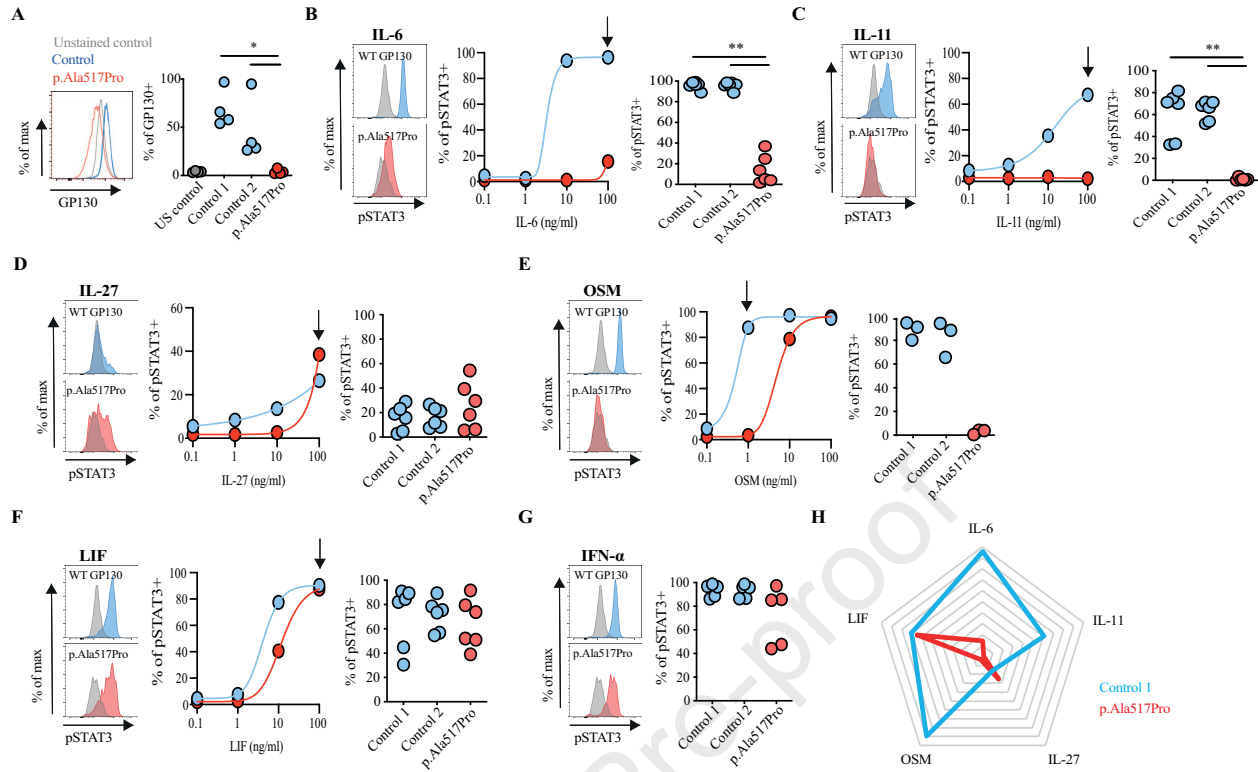
	Patient II-1	Reference
Lymphocyte subset (cells/ μ l) (7 year)		
Lymphocytes	4,429	1,100-5,900
CD3+ T cells	2,967	700-4,200
CD3+CD4+ T cells	1,019	300-2,000
CD3+CD8+ T cells	1,550	300-1,800
CD19+ B cells	531	200-1,600
CD16+CD56+ NK cells	886	90-9,000
IgG (mg/dL) (7 year)	765	608-1229
IgM (mg/dL) (7 year)	32.6	46 - 197
IgA (mg/dL) (7 year)	52	33-200
IgE (IU/ml)	842 (4.2 y)- 1549 (5.5 y)	< 12.1
Strep. Pneumoniae IgG Antibody (23 serotypes, mcg/mL) (8 year)		> 0.3 – 0.50
Serotype 12 (12F)	<0.3	
Serotype 20 (20)	<0.3	
Serotype 54 (15B)	<0.3	
Serotype 70 (33F)	<0.3	
Serotype 17 (17F)	0.3	
Serotype 22 (22F)	0.3	
Serotype 57 (19A)	0.4	
Serotype 2 (2)	0.5	
Serotype 4 (4)	0.5	
Serotype 34 (10A)	0.5	
Serotype 43 (11A)	0.5	
12 Serotypes	> 0.5 - 8.6	
Tetanus Toxoid stimulation index (SI) (8.5 year)	45	> 3
Eosinophils (cells/uL)	320 (8.3 y)- 1430 (8.2 y)	0 - 200
Neutrophil oxidative burst (DHR) (7.5 year)	100%	> 93%
Toll-like receptor function 1-8 (7.5 year)	normal	
T helper IL-17 (7 year)	CD4+ T cells demonstrate normal induction of IL-17 with stimulation (1.2% positive)	
Erythrocyte Sedimentation Rate (mm/h)	2 (8 y) - 75	0 – 15

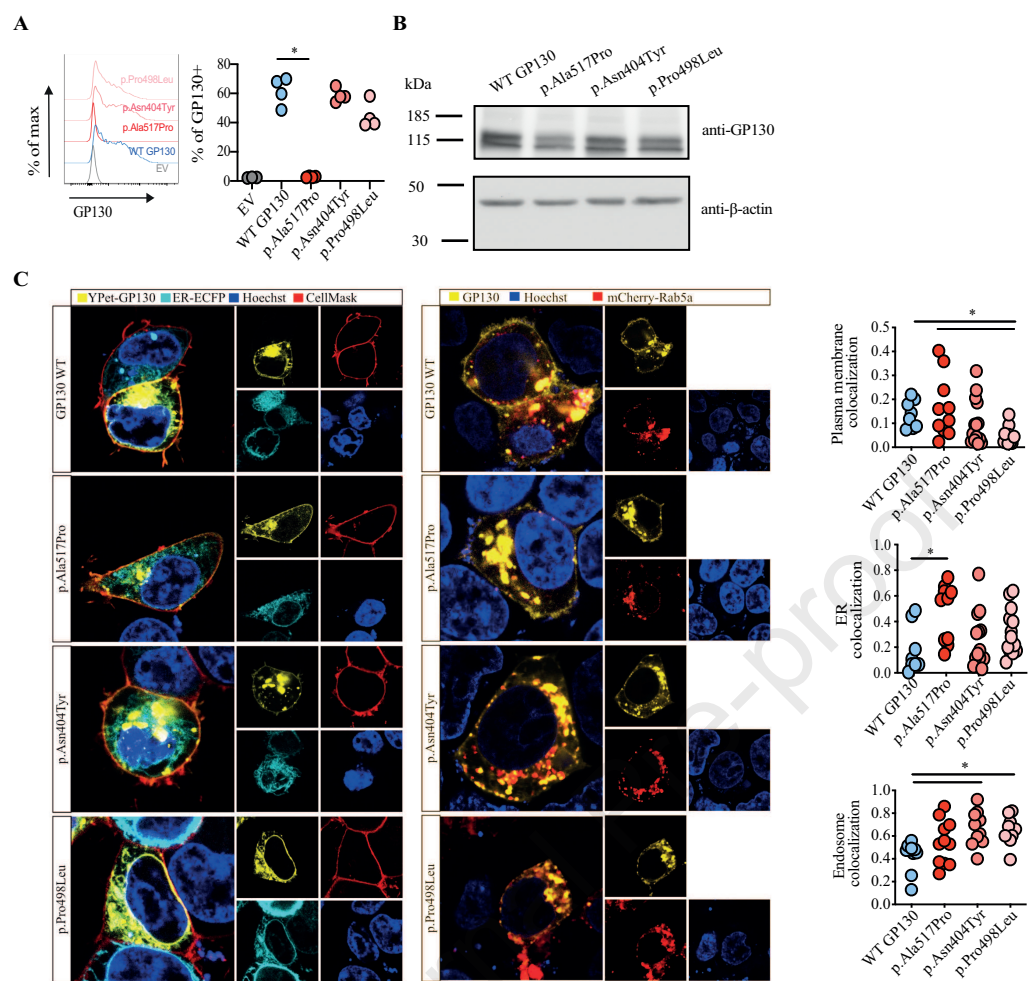
C-reactive protein (mg/dL) and concurrent infection		< 0.9
7 year - Staphylococcus aureus, left knee	16.2	
7.2 year- Staphylococcus aureus, left knee (MRSA+)	3.5	
7.3 year – no infection	0.8	
7.5 year Staphylococcus aureus, left elbow (MRSA+)	1.4	
7.7 year – no infection	< 0.2	
8 year - Streptococcus agalactiae, left knee	1.8	
8.1 year – Streptococcus agalactiae, left knee	1.1	
8.2 year – no infection	< 0.2	
8.3 year – no infection	0.3	
Fibrinogen (mg/dL) (7 year)	478	234-395

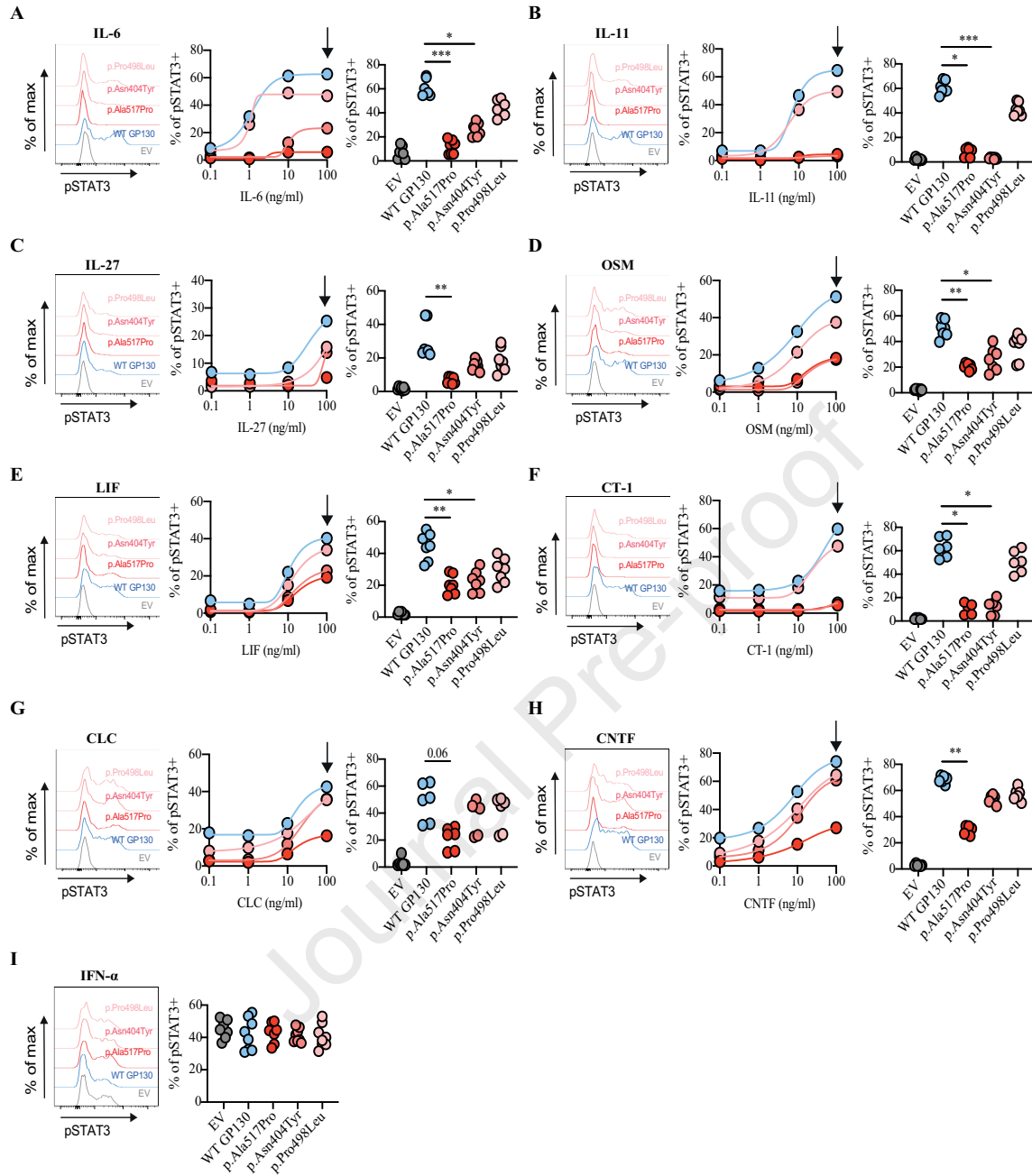
817 Age of patient II-1 when tests were performed. Abnormal results are shown in bold. MRSA, methicillin-
 818 resistant *Staphylococcus aureus*.

819

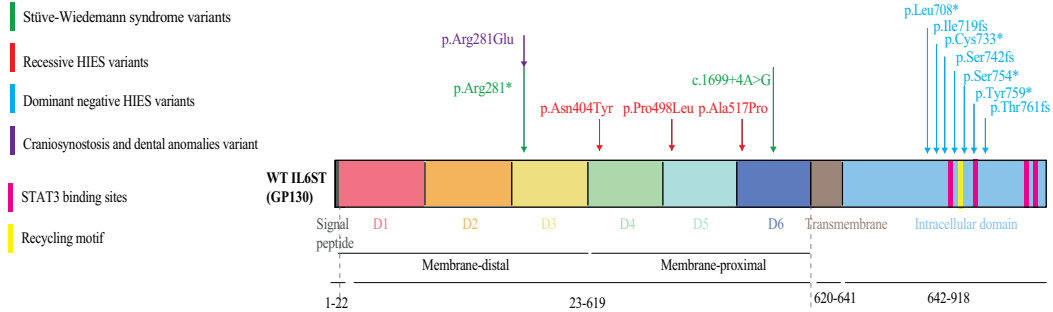






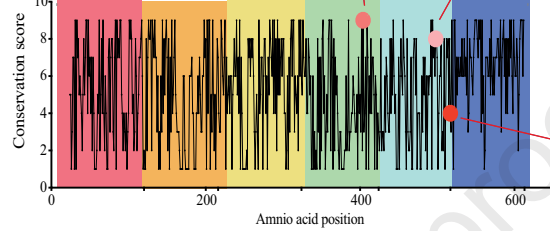
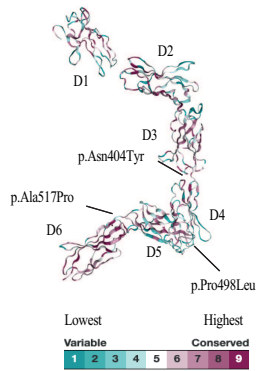


A

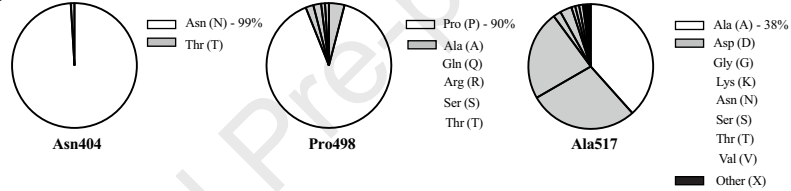


B

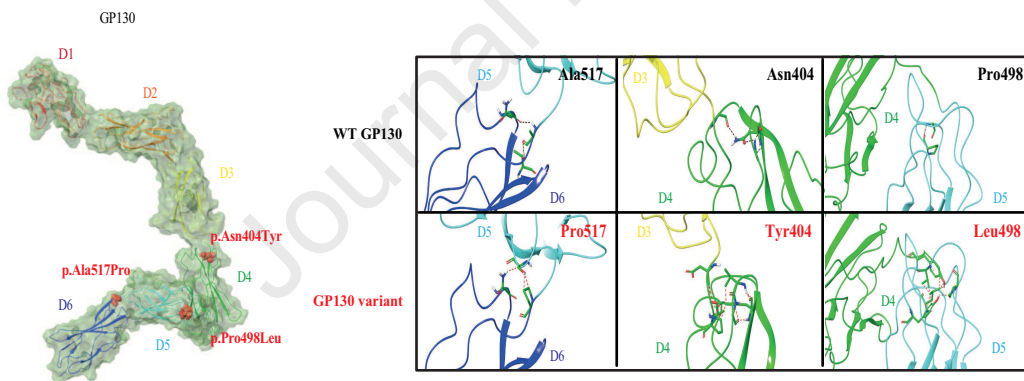
Mapping of the GP130 variants in 3D

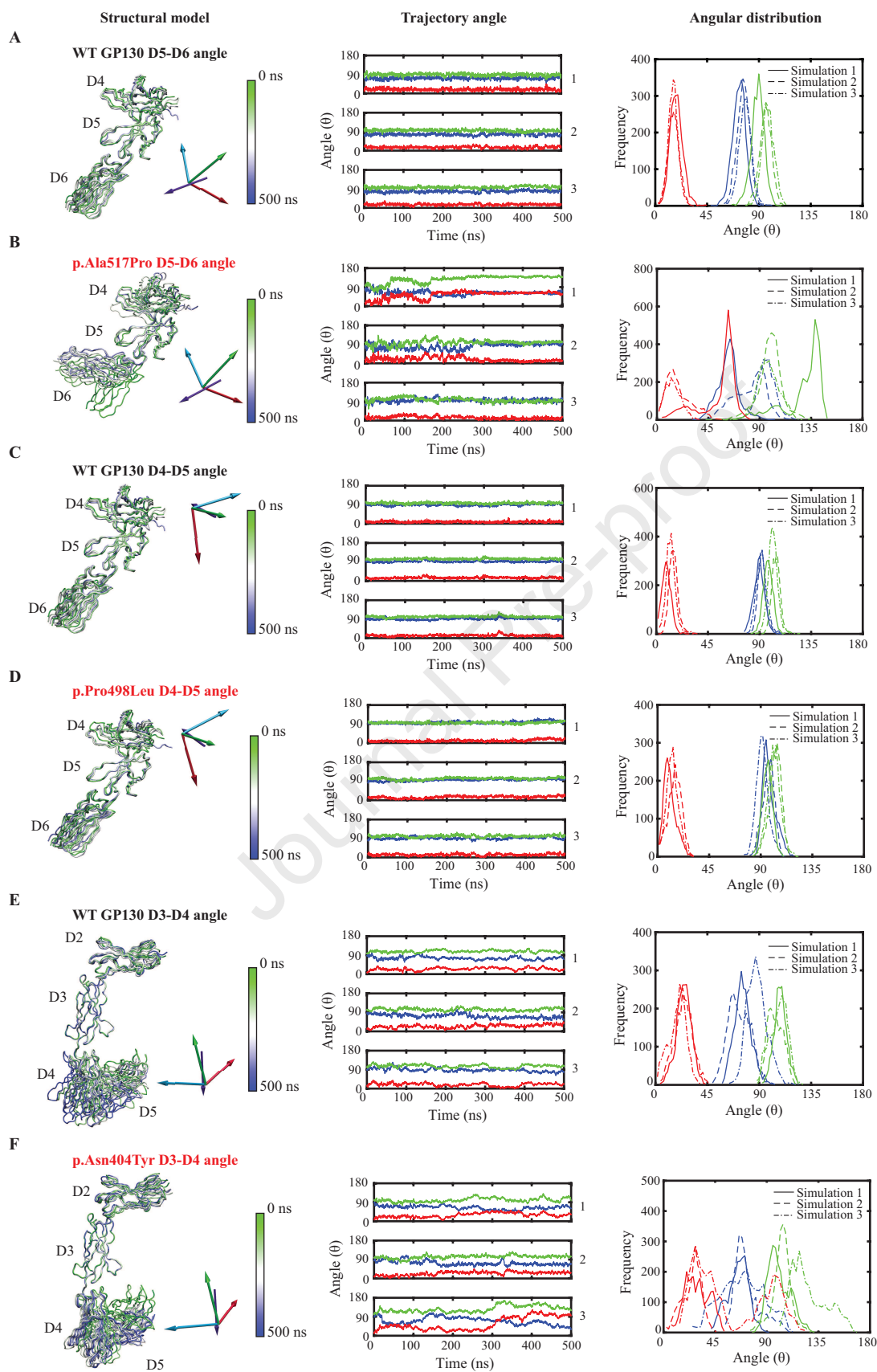


C



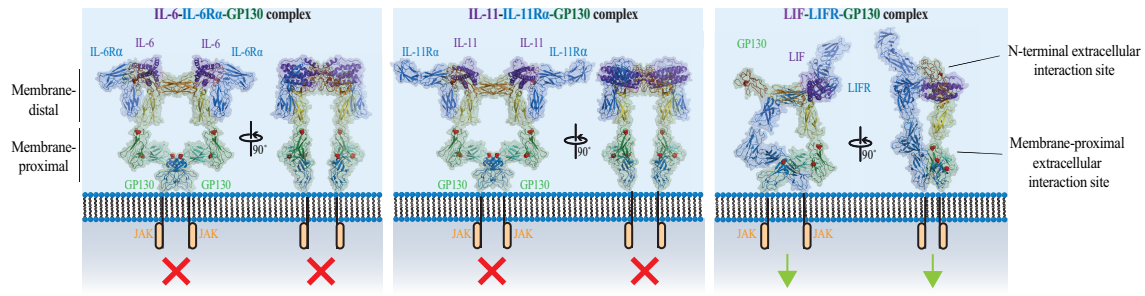
D





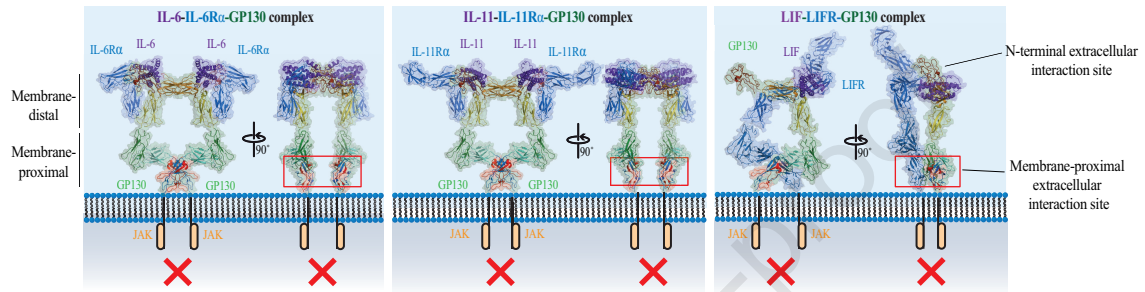
A

HIES variants: p.Asn404Tyr, p.Pro498Leu, p.Ala517Pro •



B

Stüve-Wiedemann syndrome: intronic c.1699+4A>G with exon skipping □



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SUPPLEMENTAL MATERIAL

METHODS

Whole exome and genome sequencing

The genome was covered at an average 40-times. Observed variants of interest were confirmed using an orthogonal sequencing technology (Sanger (dideoxy) sequencing). For Sanger sequencing the following *IL6ST* primers were used:

*IL6ST*_Ex12F: TGTAACGACGGCCAGT CCAGTACACAAAAGACAATGCTT

*IL6ST*_Ex12R: CAGGAAACAGCTATGACC ACATCTTTATTATAAGCGATAAAATG

*IL6ST*_Ex12_SeqbF: TGTAACGACGGCCAGT CCAACTCGCAAAGTAGGACC

*IL6ST*_Ex12_SeqaR: CAGGAAACAGCTATGACC GGTGTCTCCTGAGGCCAATA

The identified variants have been reported to ClinVar: VCV000636252.1 for NM_002184.4 (*IL6ST*): c.1549G>C (p.Ala517Pro); and VCV000636253.1 for NM_002184.4 (*IL6ST*): c.1552+3A>C.

Luminex cytokine measurements

eBioscience/Affymetrix Magnetic bead Kits were used to measure circulating cytokines in patient serum. Beads from human 62-plex kits (Thermo Fisher, Santa Clara) were added to a 96 well plate and washed in a Biotek ELx405 washer. Samples were added and incubated on the plate in duplicate at room temperature for 1 hour followed by overnight incubation at 4°C with shaking at 500-600 rpm. Plates were washed in a Biotek ELx405 washer and stained with biotinylated detection antibody for 75 min at room temperature with shaking. Plate was washed as above and incubated for 30 min at streptavidin-PE followed by wash and reading buffer. Plates were read using a Luminex 200 or a FM3D FlexMap instrument with a lower bound of 50 beads per sample per cytokine. Custom Assay Chex control beads (Radix Biosolutions) were added to all wells.

Immune profiling was performed with 62-plex Luminex assay on serum samples from 20 undiagnosed patients at the Stanford Center for Undiagnosed Diseases. The participants assayed were broadly divided into immune phenotype (n=11) and control (n=9) groups based on their disease presentation. For two patients with immune phenotype samples from 2-3 different time points were collected. Raw MFI values were used for the analysis. Median values of two or three technical replicates were used for further analysis. One control sample failed quality control and was removed. Each patient in our study cohort has a unique rare or undiagnosed disease. We assumed that most individuals have soluble proteins within the physiological range and thus compared each sample against all others in the cohort to scan for outliers.

Evolutionary conservation analysis

The ConSurf Server was used for identification of functional regions and evolutionary conservation of amino acid positions in GP130 (PDB ID:3L5H). For conservation, scores were calculated by the Bayesian method. Model of substitution for proteins was best fit. The average number of replacements between any two sequences in the alignment was performed with an average pairwise distance of 0.857472 (Lower bound: 1.01758e-07; Upper bound: 2.11153; A distance of 0.01 means that on average, the expected replacement for every 100 positions is 1). Multiple Sequence Alignment was performed using MAFFT, homologues being collected from UNIREF90, Homolog search algorithm (HMMER; HMMER E-value: 0.0001, No. of HMMER Iterations: 1, Maximal %ID between Sequences: 95, Minimal %ID for Homologs: 35, 150 sequences that sample the list of homologues to the query).

Molecular dynamics simulations

MD simulations were performed using NAMD 2.1.3b1^{E1} and the CHARMM22 forcefield^{E1},
at 310 K in a water box with periodic boundary conditions. Simulations were analyzed in
VMD 1.9.3.^{E3} For the p.Ala517Pro and p.Pro498Leu mutations, the high-resolution structure
of D4-D6 of GP130 (PDB ID: 3L5I)^{E4} was used as the starting structure. The structure was
solvated (box size $64.7 \times 111.3 \times 63.0$ Å), and ions were added to an approximate final
concentration of 0.15 M NaCl. Simulations were carried out with 10 ps minimization,
followed by 50 ns MD. Mutations were then introduced to this equilibrated model, and a
further simulation was carried out with 10 ps minimization, followed by triplicate 500 ns MD
trajectories. A restraint was applied to restrain protein molecules in the center of the box. For
the p.Asn404Tyr mutation, a model comprising D2-D5 of GP130 was constructed by aligning
D2-D3 of GP130 from PDB ID: 1I1R^{E5} and D4-D5 of PDB ID: 3L5I^{E4} with PDB ID:
3L5H.^{E4} The structure was solvated (box size $69.3 \times 139.9 \times 77.6$ Å), and ions were added to
an approximate final concentration of 0.15 M NaCl. Simulations were carried out with 100 ps
minimization, followed by 50ns MD. Mutations were then introduced to this equilibrated
model, and a further simulation was carried out with 100 ps minimization, followed by
triplicate 500 ns MD trajectories. Triplicate 500ns MD simulations for both models without
mutation were conducted. A restraint was applied to restrain the C α positions of D3 of GP130
at their initial C α position. Interdomain angular distributions were determined using a script
in VMD. Frames in the simulation were aligned on one domain using VMD, to determine
angles between this domain and a second 'mobile' domain. A vector was defined in the
mobile domain which spanned the longest axis in the domain, and the angle between this
vector and the initial principal axes of the domain was measured through the simulation.
Angular distributions were calculated using a script in MATLAB r2019a.

The mCSM server^{E6} was used to calculate the free energy change as a result of the p.Ala517Pro, p.Asn404Tyr and p.Pro498Leu mutations. The structure of GP130 domains D4-D6 with selenomethionine converted to methionines, was used as the input model for the server (PDB ID: 3L5I).^{E4}

3D structural modeling

A model of the hexameric IL-6/IL-6R α /GP130 complex was constructed by alignment of the complete extracellular domains of GP130 (PDB ID: 3L5H)^{E4} with each of the truncated GP130 molecules of the hexamer structure (PDB ID: 1P9M).^{E7} The hexameric IL-11/IL-11R α /GP130 complex was constructed by alignment of IL-11 (PDB ID: 6O4O) and IL11R α (PDB ID: 6O4P)^{E8} with the constructed IL-6 complex. A model of the trimeric LIF signaling complex was constructed by alignment of the complete extracellular domains of GP130 (PDB ID: 3L5H)^{E4} with the truncated GP130 molecule of the LIF/GP130 complex (PDB ID: 1PVH).^{E9} LIFR was placed by overlaying the LIF molecules of the LIF/LIFR complex (PDB ID: 2Q7N)^{E10} and the LIF/GP130 complex. As no structural data are available for D6-D8 of LIFR, these domains were modeled as D4-D6 of GP130 (PDB ID: 3L5H)^{E4} by alignment of D1-D3 of GP130 with D3-D5 of LIFR similar to the model previously described.^{E11}

Computational structural modeling for exon 12 skipping

A structural model of the c.1552+3A>C splice variant was built based on the crystal structure and EM images of the full ectodomain and fibronectin III domains of GP130 (PDB IDs: 3L5H, 3L5I, 3L5J, 3E0G, 1P9M, 1PVH), as described previously.^{E12} The peptide sequence corresponding to exon 12 was deleted.

ImageStream imaging

GP130-deficient HEK293 cells were transfected with YPet-fused *IL6ST* variants. 24 hours after transfection, cells were incubated for 10 min with CellMaskTM Deep Red Plasma membrane stain and Hoechst 33342 solution (ThermoFisher) before ImageStream imaging using an Amnis ImageStream MKII.

GP130 complex isolation and immunoblotting

GP130-deficient HEK293 cells were transiently transfected with plasmids encoding for *IL6ST* WT or variants p.Ala517Pro, p.Asn404Tyr or p.Pro498Leu (pMM using linear polyethyleneimine Max (Polysciences; #24765) as the transfection agent. 24 hour post-transfection cells were serum starved overnight in DMEM without FCS followed by incubation on ice for 30 min and stimulation with recombinant Hyper IL-6-Fc (100 ng/mL). Cells were lysed at 4 °C in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.3% sodium deoxycholate, 1% Triton X-100) supplemented with protease and phosphatase inhibitors. Lysates were cleared by centrifugation at 12,000g for 15 min. Protein A/G beads (Millipore) for immunoprecipitation were washed with PBS and blocked in 1 % BSA in PBS for 30 min prior to usage. Lysates for immunoprecipitates were incubated with blocked Protein A/G beads for 1 hour at 4 °C. Subsequently beads were washed three times in RIPA buffer supplemented with protease and phosphatase inhibitors and beads were incubated for 5 min at 95°C in 2 times reducing Laemmli buffer. All samples were analyzed by 10% bis-tris SDS-PAGE and immunoblotting. The following antibodies were used: anti-GP130 (Santa Cruz Biotechnology; #sc-655), and anti-beta-actin (clone: AC-15). Secondary antibodies were anti-mouse-HRP or anti-rabbit-HRP (Dianova).

Online resources

The following genetic databases were used:

174 CADD (<https://cadd.gs.washington.edu>)
175 ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>)
176 Consurf (<https://consurf.tau.ac.il>)
177 ExPASy Peptide Cutter (https://web.expasy.org/peptide_cutter/)
178 gnomAD (<https://gnomad.broadinstitute.org>)
179 Integrative Genomics Viewer (<http://www.broadinstitute.org/igv>)
180 MaxEntScan (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)
181 MutationTaster (<http://www.mutationtaster.org>)
182 SIFT (<https://sift.bii.a-star.edu.sg>)
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229 Absence of GP130 cytokine receptor signaling causes extended Stuve-Wiedemann syndrome.
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241 **SUPPLEMENTARY FIGURE LEGENDS**

242 **FIG E1. Cytokine measurements in patient II-1. A**, GP130-dependent cytokines. **B**, IL-10,
243 IL-12p40, IL-12p70, IL-17F, IL-22, IL-23, TNF- α and TNF- β . **A-B**, Cytokines were
244 measured from serum samples by Luminex, shown as median fluorescence intensity. *Filled*
245 *red triangle* indicates patient II-1 whereas other symbols represent other samples in the same
246 batch from patients with immune or non-immune phenotypes.

247

248 **FIG E2. Exome reanalysis filtering scheme and confirmation of compound**
249 **heterozygosity in patient II-1. A**, Overview of exome sequencing reanalysis. Confidence
250 filter: kept variants with call quality at least 20.0 in cases or at least 20.0 in controls AND
251 outside top 5.0% most exonically variable 100 base windows in healthy public genomes
252 (1000 genomes) AND outside top 1.0% most exonically variable genes in healthy public
253 genomes (1000 genomes). Allele frequency filter: excluded variants observed with an allele
254 frequency greater than or equal to 0.1% of the genomes in the 1000 genomes project or
255 greater than or equal to 0.1% of the NHLBI ESP exomes (All) or greater than or equal to
256 0.1% of the ExAC Frequency or greater than or equal to 0.1% of the gnomAD Frequency.
257 Predicted deleterious filter: kept variants (up to 20 bases into intron) that are experimentally
258 observed to be associated with a phenotype: pathogenic, possibly pathogenic or disease-
259 associated according to ClinVar or established gain-of-function in the literature or gene
260 fusions or inferred activating mutations by Ingenuity or predicted gain-of-function by BSIFT
261 OR Frameshift, in-frame indel, or stop codon change. Autosomal dominant refers to
262 heterozygous in case but absent from control samples whereas autosomal recessive indicates
263 compound heterozygous or homozygous in case but not in control samples. **B**, Integrative

genomics viewer shows compound heterozygous variants within the *IL6ST* locus for patient II-1 but not the mother.

FIG E3. Exon 12 skipping causes complete loss of GP130 function. **A**, *In silico* model of the hexameric structure of the extracellular region of GP130 in complex with IL-6 and IL6R α . Comparison of the D5/D6 interface between WT and exon 12 skipping c.1552+3A>C variant. *Red ribbon* highlights a significantly reduced portion of D5 at the interface and connection to D6, resulting in a weaker interdomain interface. D, domain. **B**, GP130 surface expression from GP130-deficient HEK293 cells transfected with empty vector (EV), WT or *IL6ST* variants (n=3). **C-K**, pSTAT3 responses in GP130-deficient HEK293 cells transfected with WT or *IL6ST* variants after stimulations with indicated cytokines. *Left*, representative histograms. *Middle*, representative titration curves (nonlinear regression) for all cytokines (n=1) except IFN- α . *Black arrows* indicate the cytokine concentration used in representative histograms and quantification. *Right*, Quantification of pSTAT3+ cells after stimulation (n=3). Statistical differences were determined by one-way ANOVA, ** $P \leq 0.01$, and * $P \leq 0.05$.

FIG E4. WT, p.Ala517Pro, p.Asn404Tyr, and p.Pro498Leu variants are expressed on the plasma membrane. GP130-deficient HEK293 cells were transfected with YPet-fused *IL6ST* variants. Microscopy images by ImageStream show colocalization of YPet-expressing GP130 using a marker for plasma membrane (CellMask).

FIG E5. Molecular dynamics simulations of WT GP130 and the three pathogenic variants show marked changes in interdomain conformational flexibility for the p.Ala517Pro and p.Asn404Tyr variants. **A**, Overlay of simulation frames at 100 ns

intervals for the three 500 ns WT GP130 D4-D6 simulations aligned on D5. **B**, Overlay of simulation frames at 100 ns intervals for the three 500 ns p.Ala517Pro GP130 D4-D6 simulations aligned on D5. **C**, Overlay of simulation frames at 100 ns intervals for the three 500 ns p.Pro498Leu GP130 D4-D6 simulations aligned on D5. **D**, Overlay of simulation frames, at 100 ns intervals for the three 500 ns WT GP130 D2-D5 simulations aligned on D3. **E**, Overlay of simulation frames at 100 ns intervals, for the three 500 ns p.Asn404Tyr GP130 D2-D5 simulations aligned on D3. Frames are colored per simulation.

FIG E6. Impaired GP130-Hyper IL-6 interaction in p.Ala517Pro, p. Asn404Tyr and p.Pro498Leu variants. **A**, Schematic workflow for pulldown. **B-C**, Pulldown analysis of GP130-deficient HEK293 cells transfected with WT, or p.Ala517Pro, p.Asn404Tyr or p.Pro498Leu variants. *Left*, representative immunoblots for cells incubated with or without Hyper IL6-Fc. *Right*, quantification of three independent experiments.

SUPPLEMENTAL MOVIE LEGENDS:

MOVIE E7: Animation of 500 ns MD simulation of WT GP130 and the GP130 p.Ala517Pro (A517P) variant.

MOVIE E8: Animation of 500 ns MD simulation of WT GP130 and the GP130 p.Pro498Leu (P498L) variant.

MOVIE E9: Animation of 500 ns MD simulation of WT GP130 and the GP130 p.Asn404Tyr (N404Y) variant.

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351 Martin, Martin G. Martin, Julian A. Martínez-Agosto, Shruti Marwaha, Jacob McCauley,
352 Allyn McConkie-Rosell, Colleen E. McCormack, Alexa T. McCray, Elisabeth McGee,
353 Heather Mefford, J. Lawrence Merritt, Matthew Might, Ghayda Mirzaa, Eva Morava, Paolo
354 M. Moretti, Marie Morimoto, John J. Mulvihill, David R. Murdock, Mariko Nakano-Okuno,
355 Avi Nath, Stan F. Nelson, John H. Newman, Sarah K. Nicholas, Deborah Nickerson, Shirley
356 Nieves-Rodriguez, Donna Novacic, Devin Oglesbee, James P. Orengo, Laura Pace, Stephen
357 Pak, J. Carl Pallais, Christina GS. Palmer, Jeanette C. Papp, Neil H. Parker, John A. Phillips
358 III, Jennifer E. Posey, Lorraine Potocki, Barbara N. Pusey, Aaron Quinlan, Wendy Raskind,
359 Archana N. Raja, Deepak A. Rao, Genecee Renteria, Chloe M. Reuter, Lynette Rives, Amy
360 K. Robertson, Lance H. Rodan, Jill A. Rosenfeld, Natalie Rosenwasser, Maura Ruzhnikov,
361 Ralph Sacco, Jacinda B. Sampson, Susan L. Samson, Mario Saporta, C. Ron Scott, Judy
362 Schaechter, Timothy Schedl, Kelly Schoch, Daryl A. Scott, Prashant Sharma, Vandana
363 Shashi, Jimann Shin, Rebecca Signer, Catherine H. Sillari, Edwin K. Silverman, Janet S.

Sinsheimer, Kathy Sisco, Edward C. Smith, Kevin S. Smith, Emily Solem, Lilianna Solnica-
Krezel, Rebecca C. Spillmann, Joan M. Stoler, Nicholas Stong, Jennifer A. Sullivan,
Kathleen Sullivan, Angela Sun, Shirley Sutton, David A. Sweetser, Virginia Sybert, Holly K.
Tabor, Cecelia P. Tamburro, Queenie K.-G. Tan, Mustafa Tekin, Fred Telischi, Willa
Thorson, Cynthia J. Tifft, Camilo Toro, Alyssa A. Tran, Brianna M. Tucker, Tiina K. Urv,
Adeline Vanderver, Matt Velinder, Dave Viskochil, Tiphannie P. Vogel, Colleen E. Wahl,
Stephanie Wallace, Nicole M. Walley, Chris A. Walsh, Melissa Walker, Jennifer Wambach,
Jijun Wan, Lee-kai Wang, Michael F. Wangler, Patricia A. Ward, Daniel Wegner, Mark
Wener, Tara Wenger, Katherine Wesseling Perry, Monte Westerfield, Matthew T. Wheeler,
Jordan Whitlock, Lynne A. Wolfe, Jeremy D. Woods, Shinya Yamamoto, John Yang,
Guoyun Yu, Diane B. Zastrow, Chunli Zhao, Stephan Zuchner.

Table E1. Rare sequence variants identified in exome variant reanalysis and prioritized for manual review. Prior *STAT3* sequencing was negative. Singleton WES identified a variant of unknown clinical significance in *FGFR2* (c.749-4C>A) which was thought to be non-diagnostic by the clinical team. Reanalysis of WES data prioritized 32 variants for additional review. Of these, 13 variants were discarded upon manual review due to: allele frequency greater than 0.1% (7 variants); location in repeat region and likely sequencing artifact or normal variation (4 variants); or heterozygous variant in a gene associated with autosomal recessive disease and did not match disease phenotype (2 variants).

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Gene	RefSeq	Variant	Zygosity
420			
<i>TNFRSF1B</i>	NM_001066.2	c.1075G>A (p.G359R)	Heterozygous
421			
<i>SYNC</i>	NM_030786.2	c.1427C>G (p.T476R)	Heterozygous
422			
<i>FCGR2B</i>	NM_004001.4	c.856-1G>A	Heterozygous
423			
<i>CCR2</i>	NM_001123041.2	c.817G>T (p.G273C)	Heterozygous
424			
<i>IL6ST</i>	NM_002184.3	c.1549G>C (p.A517P)	Heterozygous
425			
<i>ADAM19</i>	NM_033274.4	c.2570C>T (p.P857L)	Heterozygous
426			
<i>REV3L</i>	NM_002912.4	c.5519A>G (p.Y1840C)	Heterozygous
427			
<i>MDM2</i>	NM_002392.5	c.1235G>T (p.S412I)	Heterozygous
428			
<i>MYCBP2</i>	NM_015057.4	c.5795-3_5795-2insT	Heterozygous
429			
<i>ADAM10</i>	NM_001110.3	c.1150G>A (p.E384K)	Heterozygous
430			
<i>ABCC1</i>	NM_004996.3	c.3901C>T (p.R1301C)	Heterozygous
431			
<i>TNFRSF13B</i>	NM_012452.2	c.854C>T (p.P285L)	Heterozygous
432			
<i>CASC3</i>	NM_007359.4	c.1013G>A (p.R338Q)	Heterozygous
433			
<i>GRAMD1A</i>	NM_020895.4	c.78_83dupGCCCCC (p.P27_P28dup)	Heterozygous
434			
<i>EDN3</i>	NM_207034.2	c.677C>T (p.S226F)	Heterozygous
435			
<i>FIP1L1</i>	NM_001134937.1	c.1441_1442delAG (p.R487fs*3)	Heterozygous
436			
<i>CAPN3</i>	NM_000070.2	c.2235C>T (p.Y745Y)	Heterozygous
437			
<i>ASPN</i>	NM_017680.4	c.150_152dupTGA (p.D50dup)	Heterozygous
438			
<i>ASPN</i>	NM_017680.4	c.150_152delTGA p.D50del	Heterozygous

Table E2. Summary of *IL6ST* variant curation

Position (hg19)	5:55248081	5:55248075	445
HGVS DNA Reference NM_002184.4	c.1549G>C	c.1552+3A>C	446
HGVS Protein Reference	p.Ala517Pro	p.(Gly484_Pro518delinsArg)	447
Inheritance	Unknown (in <i>trans</i>)	Maternal	
In Silico Prediction			448
SIFT	Damaging		
MutationTaster	Disease Causing		449
CADD	25.1	21.8	
GERP RS	5.0599	4.7699	450
MaxEntScan		Broken WT Donor Site	
(% variation)	0.273	-71.75 (5' Motif)	451
TraP Score		0.959	
gnomAD v.2			
Overall Allele Count	2/280,866	0 (>20X coverage)	452

Table E3. RNAseq expression of cytokine signaling-associated gene transcripts from blood of patient II-1.

Expression outliers are bolded (z-score ≥ 2)

Gene symbol	Expression z-score
<i>EBI3</i>	-2.5019411
<i>TYK2</i>	-1.1576718
<i>STAT1</i>	-1.0407459
<i>CLCF1</i>	-0.9745476
<i>IL11RA</i>	-0.6549557
<i>IL6R</i>	-0.4648575
<i>TNF</i>	-0.4584135
<i>IL27</i>	-0.4368653
<i>PTPN11</i>	-0.2083372
<i>IL6ST</i>	-0.187
<i>CRLF1</i>	-0.1814083
<i>OSM</i>	-0.1652972

<i>STAT3</i>	-0.1155172 457
<i>IL27RA</i>	0.71507001
<i>IL10</i>	1.15036224 458
<i>IL23A</i>	1.32700983
<i>CBL</i>	1.38219264 459
<i>SOCS3</i>	1.47664179
<i>JAK2</i>	1.5693058 460
<i>JAK1</i>	1.8060475
<i>IL17D</i>	2.79295591 461

Table E4. Clinical features of HIES patients with *IL6ST* variants.

	p.Ala517Pro (II-1)	p.Asn404Tyr	p.Pro498Leu
Ancestry	Native American, Italian, Scottish	South Asian	Turkish
Consanguinity	None	Yes	Yes
<i>IL6ST</i> variants	c.1549G>C; p.Ala517Pro c.1552+3A>C (compound heterozygous)	p.Asn404Tyr (homozygous)	p.Pro498Leu (homozygous)
Gender	Male	Female	Male
Age	10	7	12
Craniosynostosis	+	+	+
Congenital contractures	Fingers, elbows	Fingers, elbow	Fingers
Scoliosis	-	+	+
Short stature	+	+	+
Pulmonary infections	Osteomyelitis, pneumonia, otitis media, septic arthritis, empyema	Bronchiectasi s, keratitis, pneumonia, periorbital cellulitis	Keratitis, pneumonia, empyema, pneumatocel e, otitis media
Candidiasis	Infantile non-	-	-

	invasive candida infection			470
Neurological	Mild motor delays	Mild delays	motor	Mild motor delays 471
Immune	High IgE (1523 IU/mL), eosinophilia (1.4K/ μ L), food allergies	High IgE (>5000 IU/mL), intermittent eosinophilia		High IgE (>2000 IU/mL), eosinophilia, food allergies 472
Skin abscesses or eczema	-	Eczema		Eczema 473
Retained primary teeth	+	+		+
Others	Midface retrusion, high arched palate, telecanthus, long palpebral fissures, prognathism, pneumothorax, fractures with minor trauma, chronic destructive arthritis	Pneumothorax, superior sagittal sinus thrombosis, L-MCA venous infarct, R-hemiplegia, congenital hip dislocation, fungal lesions		Pneumothorax, hip dislocation and ankle and knee edema, destructive arthropathy 474
NIH HIES score	37	40		57 481

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