

PROTEIN STABILIZATION IMPROVES STAT3 FUNCTION IN AUTOSOMAL DOMINANT HYPER-IgE SYNDROME

Short Title: Stabilizing STAT3 in AD-HIES

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Key Points:

- Most AD-HIES mutations destabilize STAT3 protein, contributing to impaired STAT3 function.
- STAT3 function is improved in immune cells from patients and a mouse model of AD-HIES by small molecule protein stabilizers.

ABSTRACT

AD-HIES is caused by dominant negative mutations in *STAT3*; however, the molecular basis for mutant *STAT3* allele dysfunction is unclear and treatment remains supportive. We hypothesized that AD-HIES mutations decrease *STAT3* protein stability and that mutant *STAT3* activity can be improved by agents that increase chaperone protein activity. We used computer modeling to characterize the effect of *STAT3* mutations on protein stability. We measured *STAT3* protein half-life ($t_{1/2}$) and determined levels of *STAT3* phosphorylated on tyrosine (Y) 705 (pY-*STAT3*) and mRNA levels of *STAT3* gene targets in EBV-transformed B (EBV) cells, human peripheral blood mononuclear cells (PBMC), and mouse splenocytes incubated without or with chaperone protein modulators—HSF1A, a small-molecule TRiC modulator, or geranylgeranylacetone (GGA), a drug that upregulates heat shock protein (HSP) 70 and HSP90. Computer modeling predicted that 81% of AD-HIES mutations are destabilizing. *STAT3* protein $t_{1/2}$ in EBV cells from AD-HIES patients with destabilizing *STAT3* mutations was markedly reduced. Treatment of EBV cells containing destabilizing *STAT3* mutations with either HSF1A or GGA normalized *STAT3* half-life, increased pY-*STAT3* levels, and increased mRNA levels of *STAT3* target genes up to 79% of control. In addition, treatment of human PBMC or mouse splenocytes containing destabilizing *STAT3* mutations with either HSF1A or GGA increased levels of cytokine-activated pY-*STAT3* within human CD4⁺ and CD8⁺ T cells and numbers of IL-17-producing CD4⁺ mouse splenocytes, respectively. Thus, most AD-HIES *STAT3* mutations are destabilizing; agents that modulate chaperone protein function improve *STAT3* stability and activity in T cells and may provide a specific treatment.

INTRODUCTION

Autosomal dominant hyper-IgE syndrome (AD-HIES), or Job's Syndrome, is a life-shortening primary immunodeficiency and multi-system disorder characterized by developmental abnormalities, poor wound healing, and recurrent infections¹⁻⁷. There is no specific therapeutic intervention for AD-HIES patients^{8,9}. While a haploidentical donor hematopoietic stem cell transplant (HSCT) recently performed in an adolescence with AD-HIES restored immune function¹⁰, an earlier attempt at bone marrow transplant was reported not to be successful⁸ and syngeneic HSCT in a mouse model of AD-HIES only partially restored resistance to bacterial infection¹¹.

AD-HIES is caused by dominant negative mutations in signal transducer and activator of transcription 3 (STAT3), a transcription factor that plays a central role in the signal transduction pathway of multiple cytokines, growth factors, and other peptide hormones^{2-4,6,7,12}. In resting cells, STAT3 is located predominantly within the cytoplasm dimerized N-terminal-to-N-terminal (head-to-head)¹³. In peptide hormone-stimulated cells, STAT3 is recruited to phosphotyrosyl (pY) peptide motifs within activated receptor complexes through its Src homology 2 (SH2) domain, becomes phosphorylated at Y705, then dimerizes C-terminal-to-C-terminal (tail-to-tail). Tail-to-tail STAT3 homodimers accumulate within the nucleus, where they bind DNA and activate the promoters of genes involved in tissue injury repair, inflammation, and immunity¹⁴. *STAT3* mutations associated with AD-HIES are usually single amino acid missense mutations or single in-frame deletions that occur at 46 residues within the protein, predominantly within the DNA binding domain (DBD) or the SH2 domain of STAT3^{2-4,6,7}. The dominant-negative effect of these mutations results from dilution of functional WT:WT tail-to-tail dimers by a 3-fold excess of dysfunctional dimers—mutant (M):mutant (M) and M:WT. A key issue remaining in

understanding the molecular pathogenesis of AD-HIES is the specific molecular mechanism for dysfunction for each of the mutant *STAT3* alleles and whether these mechanisms overlap and can be targeted for therapeutic benefit.

Our lab and others have shown that *STAT3* requires chaperones to achieve its native conformation and function within the cell^{12,15,16}. Specifically, *STAT3* requires interaction with the eukaryotic protein folding machine or chaperonin, tailless-complex polypeptide-1 (TCP-1) ring complex (TRiC), for its biogenesis and folding¹⁶. Multiple additional chaperones play a role in optimizing *STAT3* protein function, especially heat shock proteins (HSP) 90 and HSP70, which raises the possibility that some mutated *STAT3* proteins may be less stable and exceed chaperone capacity, resulting in misfolded *STAT3* protein. Our results strongly support the hypotheses that AD-HIES *STAT3* mutations reduce *STAT3* function by decreasing *STAT3* stability within the cell and that *STAT3* function can be improved in cells from AD-HIES patients and a mouse model of AD-HIES by upregulating chaperone protein activity.

METHODS:

Stability modeling. To predict the impact of AD-HIES mutations on *STAT3* stability, 5 protein stability predictors [PoPMuSiC 2.1¹⁷, I-Mutant 2.0¹⁸, MU Pro¹⁹, SDM²⁰, and DFIRE²¹] were used to analyze the 77 *STAT3* mutations identified in AD-HIES patients that result in single amino acid residue substitutions or deletions. In addition, the functional importance of each mutated *STAT3* residue was estimated using real-valued evolutionary trace methodology²². See Supplemental Data for additional details.

Plasmids. Site-directed mutagenesis was performed for AD-HIES mutations R382W, V463del, V637M, and Y657S using a kit (QuikChange II; Agilent Technologies) and the pSG5

vector containing the human Flag-tagged STAT3 cDNA (kind gift of Shuo Dong PhD, Baylor College of Medicine). Primers were designed using QuikChange Primer Design Program (www.genomics.agilent.com/primerDesignProgram.jsp). The sequence of each STAT3 mutant-containing plasmid was confirmed by sequencing.

In vitro transcription and translation. *In vitro* transcription and translation reactions (50 μ L) were carried out using a rabbit reticulocyte lysate (RRL) system, as described²³. Briefly, RRL (TNTT7 Quick Coupled Transcription/Translation System; Promega) reactions included [³⁵S]-methionine and 1 μ g of pSG5 vector containing the insert of interest and were incubated for 30 min at 30 °C, then terminated by the addition of 2mM puromycin, 5 mM EDTA, and 1 mM azide.

TRiC immunoprecipitation. TRiC immunoprecipitation was performed, as described¹⁶. See Supplemental Data for more details.

EBV-transformed B (EBV) cells and PBMC. Venous blood samples were obtained from AD-HIES and non-AD-HIES patients at Texas Children's Hospital (TCH) in Houston, TX, and at the National Institutes of Health (NIH) using IRB approved protocols. PBMC were isolated from anticoagulated blood by Ficoll-Hypaque sedimentation and a portion infected with EBV to generate EBV cells, as described²⁴. See Supplemental Data for more details.

Cycloheximide chase assays. Cycloheximide was purchased from Sigma-Aldrich and dissolved in cell media. EBV cells were treated with CHX (100 μ M) to stop new protein synthesis, as previously described²⁵. Total STAT3 protein levels were measured over time using a Luminex bead-based assay and normalized to GAPDH, which has a half-life of > 72 hours, as previously described²⁶.

Luminex assays. Luminex assays for pY-STAT3, total (t) STAT3, GAPDH, activated

caspase 3, HSP70, and HSP90 was performed, as described²⁷. See Supplemental Data for more details.

Quantitative RT-PCR. Quantitative RT-PCR was performed, as described²⁷. See Supplemental Data for more details.

Cytokines, HSF1A, and GGA. Recombinant human IL-6 and IL-21 were obtained from R&D Systems. The chemical structure of HSF1A was obtained²⁸ and custom synthesized by ChemBridge Corporation. HSF1A was dissolved using DMSO to a concentration of 25 mg/ml. GGA was purchased from Sigma-Aldrich and suspended in DMSO at 10 mg/ml. See Supplemental Data for more details.

Phosphoflow. PBMCs from healthy donors and AD HIES patients were isolated by Ficoll-Paque density gradient (GE Healthcare), cryopreserved in fetal bovine serum (GE Biosciences) containing 10% dimethyl sulfoxide (Fisher Scientific) and stored at -140°C until further use. After thawing, PBMCs were resuspended in RPMI 1640 supplemented with 10% FBS, glutamine, penicillin and streptomycin (GIBCO) and incubated for 48 hours in the presence of GGA, HSF1A or vehicle (DMSO) at a final concentration of 3 µM added daily to the cultures. After 2 days, PBMCs were washed, resuspended, rested for 2 hours in serum-free RPMI 1640 supplemented with GGA, HSF1A or vehicle, then stained and analyzed as described in Supplemental Data.

Th17 cell polarization and IL-17 intracellular staining. Total CD4⁺ T cells were enriched from splenocytes isolated from mut-Stat3¹¹ and cage-control, wild type (WT) mice (6-10 weeks old) using a MojosortTM mouse CD4⁺ T cell isolation kit (BioLegend). CD4⁺ cells were incubated under Th17 cell polarizing conditions²⁹, washed, stimulated for 5 hours with the cell activation cocktail (BioLegend), permeabilized, and stained with anti-IL-17A APC (clone

eBio17B7, eBioscience). Data was collected with BD LSR II and analyzed with Flowjo software (Tree Star).

Statistical analysis. STATA 11.2 was used for statistical analysis. See Supplemental Data for more details.

RESULTS

Computer modeling predicts that most *STAT3* AD-HIES mutations are destabilizing and separates them into three categories--functional, structural, and structural-functional.

We identified 87 unique *STAT3* mutations reported in the literature^{2-4,6,7,30-38}. Seventy-four mutations are single amino acid missense mutations (involving 46 residues), 3 mutations are in-frame deletions, and 10 mutations contain larger deletions or duplications. We analyzed the 77 single amino acid substitutions and in-frame deletions using 5 computer-based programs to predict their impact on protein stability (**Table S1**). Each individual program predicted that most mutations are destabilizing; a composite analysis of all 5 programs predicted that 62 (81%) mutations were destabilizing (**Table S1**).

We next used Evolutionary Trace (ET) methodology to predict the functional importance of the *STAT3* residues mutated in AD-HIES³⁹. Based on its location within the *STAT3* crystal structure⁴⁰ and other considerations, each *STAT3* mutation has a high ET ranking, indicating a strong effect on *STAT3* function (**Table S2, Fig. S1**). We completed a second ET analysis for *STAT3* mutations located within the SH2 domain, which allowed us to differentiate the predicted effect of a mutation on SH2 domain stability from its aggregate effect on *STAT* protein function (**Table S2**). Combining the ET ranking, solvent accessibility, and distance from the DNA binding site or SH2 dimerization interface, we determined that 20 mutations were predicted to

impair DNA binding, 25 mutations were predicted to impair dimerization, and 20 mutations were predicted to impair the folding of STAT3 (**Table S2**).

Combining this analysis with the stability predictions, we categorized each of the 77 mutations into one of three groups: functional, structural, or structural-functional (**Table S3**). Functional (F) mutations affect a domain-specific function of STAT3, such as DNA binding or tail-to-tail dimerization, without affecting STAT3 stability. This category includes 15 mutations, such as the most common AD-HIES mutation, R382W, which is thought to directly impair DNA binding⁴⁰. Structural (S) mutations destabilize and impair the folding of STAT3, thereby indirectly affecting STAT3 functions such as DNA binding, phosphotyrosylation, or dimerization. This category includes 20 mutations, including the second most common AD-HIES mutation, V463del, located in the DBD, as well as several mutations in the SH2 domain “hot spot” at residue Y657, i.e. Y657S, Y657C, and Y657N. Structural-functional (S-F) mutations are predicted to be both destabilizing and to directly impair a domain-specific STAT3 function. This category includes 42 mutations, such as V637M, the most common mutation in the SH2 domain and the third most common AD-HIES mutation, overall (**Fig. 1, Table S3, Fig. S2**).

STAT3 protein half-life and activity within AD-HIES patient EBV cells supports the computer-based categorization and stability predictions of STAT3 mutations.

To test our computer-based categorization and stability predictions, we determined STAT3 protein half-life, a surrogate marker for stability, using cycloheximide (CHX) chase assays in EBV cells from 8 AD-HIES patients and 2 controls (**Fig. 2; Table 1**). Total STAT3 protein levels were measured over time after addition of CHX, which inhibited new protein synthesis. The half-life of STAT3 protein within EBV cells containing STAT3 S or S-F mutations

was reduced by 79% compared to control cells ($p < 0.05$; **Fig. 2B** and **C**; **Table 1**). In contrast, the half-life of STAT3 protein in EBV cells containing the *STAT3* F mutation, R382W, was similar to control cells (**Fig. 2A**; **Table 1**). These results strongly support our computer-based categorization of AD-HIES *STAT3* mutations and their predicted effect on STAT3 protein stability.

Similar to results reported previously⁴¹, cytokine-induction of suppressor of cytokine signaling (SOCS) 3, a gene transcriptionally activated by STAT3, was reduced by 75% compared to controls in EBV cells in both the DBD mutation sub-group and the SH2 domain mutation sub-group (**Fig. S3A**), which could be attributed, in the case of the SH2 domain subgroup, to reduced levels of cytokine-induced pY-STAT3 (**Figs. S3C** and **S3D**) but not to reduced levels of total STAT3 (**Fig. S3B**). Re-examination of the SOCS3 induction data after subgrouping AD-HIES EBV cells into F, S, and S-F categories revealed that EBV cells in each subgroup demonstrated reduced SOCS3 induction, as expected (**Fig. S3E**). Of note, however, EBV cells in the STAT S-F mutation subgroup not only had reduced pY-STAT3 levels to explain reduced SOCS3 induction (**Figs. S3G** and **S3H**), but their reduced pY-STAT3 levels could be explained by reduced levels of total STAT3 protein (**Fig. S3F**). Thus, our categorization system revealed a new mechanism for reduced STAT3 function in AD-HIES patient cells, i.e. reduced levels of total STAT3 protein.

STAT3 S mutant proteins have decreased interaction with TRiC and decreased biogenesis *in vitro*; STAT3 biogenesis and function can be improved by modulating TRiC levels or activity.

We recently demonstrated that interaction with TRiC is required for optimal STAT3 biogenesis and function¹⁶. To determine if impaired interaction with TRiC contributes to

decreased stability and function of some mutant STAT3 proteins, ³⁵S-labeled mutant and WT STAT3 proteins were expressed in rabbit reticulocyte lysates (RRL) and subjected to TRiC immunoprecipitation. STAT3 S mutant proteins, V463del and Y657S, demonstrated markedly reduced TRiC binding compared with STAT3 WT protein ($p < 0.001$; **Fig. 3A**). In contrast, STAT3 F mutation R382W protein and S-F mutation V637M protein each had equivalent TRiC binding compared to WT protein. In addition to reduced binding to TRiC, the level of STAT3 S mutation Y657S protein produced in RRL was reduced to 47% of WT protein. Of note, addition of exogenous purified bovine TRiC (120 nM) normalized levels of STAT3 S mutation Y657S protein, indicating that the impaired biogenesis of STAT3 S mutant protein by RRL could be overcome by increasing the level and/or activity of TRiC (**Fig. 3B**).

HSF1A is a small molecule reported to ameliorate protein misfolding in a cell line model of polyQ-mediated neurodegeneration by binding to and modulating TRiC function²⁸. To determine if HSF1A could increase STAT3 levels and/or function, EBV cells from control and AD-HIES patients were incubated with HSF1A (3 μ M) for 48 hours (conditions demonstrated to be optimal for maximizing cytokine-induced pY-STAT3 levels in EBV cells with STAT3 S mutation Y657S; **Fig. S4**). Cytokine-induced SOCS3 mRNA levels in EBV cells containing S mutation V463del, T622I, or Y657S increased from 5.4-23% of WT to 38-62% of WT ($p < 0.01$, **Fig. 4A**). Cytokine-induced SOCS3 mRNA levels also were increased by HSF1A in EBV cells containing S-F mutation R423Q, S611N, V637M, or N647D EBV cells from 20-34% of WT to 45-94% of WT ($p < 0.01$; **Fig. 4A**). Cytokine-induced SOCS3 mRNA levels also were increased by HSF1A in EBV cells containing F mutation R382W, but the magnitude of improvement was less than seen in S and S-F mutations, as expected ($p < 0.01$; **Fig. 4A**). To further confirm these results, we measured mRNA levels of additional STAT3 target genes, Bcl-6 and PRDM1 (**Fig.**

S5). HSF1A treatment of EBV cells containing S mutation Y657S or V463del increased cytokine-induced mRNA levels of Bcl-6 and PRDM by 4 to 11-fold. HSF1A treatment in EBV cells containing S-F mutation V637M also increased mRNA levels of Bcl-6 and PRDM by 2.3 to 3.3-fold.

All cell lines demonstrated increased total STAT3 levels after treatment with HSF1A (**Fig. 4B**) and all cell lines but two (S-F mutation V637M and F mutation R382W) demonstrated increased levels of cytokine-stimulated pY-STAT3 (**Fig. 4C,D**) indicating that HSF1A improves STAT3 activity, at least in part, by increasing total STAT3 levels in the cell. We next repeated the STAT3 protein half-life experiments with Y657S EBV cells after 48 hours of treatment with 3 μ M HSF1A. Remarkably, HSF1A normalized the half-life of STAT3 in EBV cells containing S mutation Y657S (**Fig. 4E-F**).

Geranylgeranylacetone (GGA) improved STAT3 function in AD-HIES patient EBV cells.

In addition to TRiC, STAT3 requires interaction with HSP70 and HSP90 for optimal function in the cell^{12,15}. GGA is an antiulcer drug that induces expression of HSP70 and HSP90. When given to rats during intracerebral hemorrhage, GGA increased pY-STAT3 levels without affecting total STAT3, leading to decreased cell apoptosis and improved functional recovery⁴². EBV cells from control and AD-HIES patients were incubated with GGA (3 μ M) for 48 hours (conditions demonstrated to be optimal for maximizing cytokine-induced pY-STAT3 levels in EBV cells with S mutation Y657S; **Fig. S6**). Cytokine-induced SOCS3 mRNA levels in EBV cells with S mutation V463del, T622I or Y657S were increased from 5.4-23% of WT to 29-104% of WT with GGA treatment ($p < 0.01$). Cytokine-induced SOCS3 mRNA levels in EBV cells with S-F mutations R423Q, S611N, V637M, or N647D also were increased from 20-34% of WT to 34-71% of WT after GGA treatment ($p < 0.01$; **Fig. 5A**). In addition, cytokine-induced

SOCS mRNA levels in EBV cells containing F mutation R382W increased from 2.4% to 20% of WT with GGA treatment ($p < 0.01$; **Fig. 5A**). GGA treatment also increased levels of cytokine-induced mRNA levels of Bcl-6 and PRDM1 by 3 to 12 fold in EBV cells with S mutation Y657S or V463del and by 1.8 to 3 fold in EBV cells containing the S-F mutation V637M (**Fig. S7**).

Unlike HSF1A, treatment with GGA increased total STAT3 levels only in EBV cells containing *STAT3* WT or *STAT3* S-F mutation S611N (**Fig. 5B**). In contrast, treatment with GGA increased cytokine-stimulated pY-STAT3 in all cell lines tested except one containing S-F mutation V637M (**Fig. 5C,D**). We next performed STAT3 protein half-life experiments with Y657S EBV cells after 48 hours of GGA treatment. Like HSF1A, GGA normalized STAT3 protein half-life in EBV cells containing S mutation Y657S (**Fig. 5E,F**).

As expected, GGA treatment increased levels of HSP70 protein by 1.9 fold and levels of HSP90 protein by 1.3 fold in EBV cells (**Fig. 5G**; $p < 0.05$ for both), suggesting that increases in both HSPs contributed to increased STAT3 protein half-life and function. To further support the conclusion that the effect of GGA was mediated directly by its effects on HSP levels, especially levels of HSP70, we expressed GFP-HSP70 in EBV cells from a normal control (WT), or EBV cells containing *STAT3* mutations V463del or Y657S, and measured levels of IL-21-induced SOCS3 mRNA (**Fig. S9**). Ectopic expression of GFP-HSP70 resulted in increased IL-21-induced expression of SOCS3, especially in V463del EBV cells, supporting the conclusion that GGA increased STAT3 function by increasing HSP70 activity.

HSF1A and GGA increased levels of cytokine-induced pY-STAT3 in CD4⁺ and CD8⁺ T cells.

To determine if either HSF1A or GGA could improve STAT3 activity in primary immune cells from AD-HIES patients, PBMCs were isolated from a patient with V463del,

the second most common STAT3 mutation overall and the most common *STAT3* S mutation, and a healthy control. PBMCs were incubated in HSF1A or GGA in triplicate aliquots and examined by multiparametric flow cytometry for level of pY-STAT3 following stimulation with IL-21. Preincubation with HSF1A (**Fig. 6A**) or GGA (**Fig. 7A**) increased the percentage of IL-21 stimulated pY-STAT3-positive CD4⁺ T cells by 31% and 29% ($p=0.0013$ and 0.0052 , respectively) and their MFI by 9% and 7% ($p=0.0028$ and 0.02 , respectively). Pretreatment with HSF1A or GGA also increased the percentage of pY-STAT3-positive CD8⁺ T cells by 60% and 32% ($p=0.0003$ and 0.011 , respectively) and their MFI by 24% and 14 % ($p=0.0004$ and 0.043 , respectively). In contrast, preincubation of healthy control PBMCs with HSF1A did not change the percentage of pY-STAT3-positive cells or their MFI in either CD4⁺ or CD8⁺ T lymphocytes upon stimulation with IL-21, while preincubation with GGA, actually decreased pY-STAT3 frequencies and MFI in both CD4⁺ and CD8⁺ T cells ($p<0.03$ for all). This effect of GGA to reduce pY-STAT3 frequencies and MFI was observed in about 50% of PBMC from healthy controls and indicates that the ability of GGA to increase the frequency of pY-STAT3-positive cells and the levels of pY-STAT3 per cell following IL-21 stimulation is specific to patients cells containing a mutant STAT3 allele.

To determine the robustness of these observations, PBMC from 4 different AD-HIES patients with S mutations (3 patients with V463del and 1 patient with F621V) and 7 healthy controls were incubated in medium containing HSF1A (3 μ M), GGA (3 μ M), or vehicle for 48 hours and stimulated with IL-21 for 30 min. Each patient was tested in at least 4 independent experiments. Pretreatment with HSF1A (**Fig. 6B**) or GGA (**Fig. 7B**) increased the percentage of IL-21-stimulated pY-STAT3 positive CD4⁺ T cells by 28% and 35% ($p\leq 0.01$ for both) and their MFI by 8% and 9% ($p=0.0069$ and 0.0055 , respectively). HSF1A and GGA also increased the

percentage of pY-STAT3-positive CD8⁺ T cells by 39% and 45% (p=0.01 for both) and their MFI by 13% for both (p=0.0069 and 0.01, respectively). In contrast, preincubation of healthy control PBMC with either PM had no effect indicating, as above, that the IL-21-stimulated increase in frequency of pY-STAT3-positive cells and the levels of pY-STAT3 per cell is specific to patient cells containing a mutant STAT3 allele.

HSF1A and GGA increase numbers of IL-17-producing CD4⁺ cell in cultures of mut-*Stat3* mouse splenocytes.

To determine whether GGA or HSF1A treatment can improve Th17 numbers, we incubated splenic CD4⁺ T cells isolated from the mut-*Stat3* or cage-control, wild type (WT) mice under conditions that support Th17 polarization in the absence or presence of GGA or HSF1A (**Fig. 8**). All cells within the mut-*Stat3* mouse contain 2 copies of the V463del *STAT3* transgene, in addition to 2 wild-type *Stat3* alleles; the mut-*Stat3* mouse phenocopies AD-HIES patients, including their reduced ability to generate Th17 cells¹¹. As expected, the percentage of IL-17 producing CD4⁺ T cells in cell cultures from mut-*Stat3* (3.12±2.10) were reduced compared to those from WT mice (6.34±3.37). Co-incubation of CD4⁺ cells from mut-*Stat3* mouse spleens with GGA or HSF1A increased IL-17 producing cells by 49% (p=0.028) and 59% (p=0.029), respectively. In contrast, co-incubation of CD4⁺ cells from WT mouse spleens with GGA or HSF1A increased IL-17 producing cells by only 21% for GGA (p=0.048) and not significantly for HSF1A (4%; p=0.428), respectively.

DISCUSSION

Using computer modeling, we assigned each *STAT3* mutation reported in AD-HIES patients that results in a single amino acid substitution or deletion (77 of 87 known mutations) to one of three categories—functional (F), structural (S), or structural-functional (S-F). Mutations falling into the S and S-F categories were predicted to be destabilizing. Measurement of *STAT3* protein half-life within EBV cells from AD-HIES patients confirmed the computer-based stability predictions. *STAT3* S mutant proteins also demonstrated decreased interaction with TRiC, the major eukaryotic chaperonin, and were synthesized at a reduced rate in RRL, which was normalized by addition of exogenous purified TRiC. Importantly, treatment of EBV cells containing *STAT3* S or S-F mutations with HSF1A, a TRiC-activity modulator, or GGA, an inducer of HSP70 and HSP90, substantially increased cytokine-stimulated *STAT3* transcriptional activity in AD-HIES EBV cells. Of note, HSF1A and GGA also improved the ability of *STAT3* within AD-HIES patient CD4⁺ and CD8⁺ T cells to undergo cytokine-stimulated activation and the increased the number of IL-17-producing CD4⁺ T cells within cultures of mut-*Stat3* splenocytes. These findings support the use of chaperone protein modulators as a novel treatment approach for AD-HIES patients, particularly those with *STAT3* S and S-F mutations.

The dominant-negative effect of *STAT3* mutations likely results from dilution of fully functional WT:WT tail-to-tail dimers by a 3-fold excess of dysfunctional dimers—mutant (M):mutant (M) and M:WT. In cells containing a DBD mutation, the dysfunctional M:M and M:W dimers are tail-to-tail dimers unable to bind DNA with sufficient affinity to achieve gene activation⁴. In cells containing an SH2 domain mutation, dysfunctional M:M and M:W dimers are locked in the head-to-head conformation since they are unable to form stable tail-to-tail dimers⁴³. Alternatively, the dominant negative effect of AD-HIES *STAT3* mutations may be due,

in part, to misfolding, aggregation, mistrafficking, or increased degradation of both the mutant and WT STAT3 protein. Retinitis pigmentosa and neurodegenerative diseases are examples of such diseases caused by autosomal dominant mutations⁴⁴.

Our understanding of the molecular basis for dysfunction of each of the 87 AD-HIES *STAT3* mutant alleles is evolving. *STAT3* mutant dysfunction was initially linked to dysfunction of the domain containing the mutation, e.g. *STAT3* protein within EBV cells from AD-HIES patients with DBD mutations demonstrated impaired DNA binding but normal cytokine-induced *STAT3* tyrosine phosphorylation⁴; *STAT3* protein within EBV cells from AD-HIES patients with SH2 mutations demonstrated reduced cytokine-induced pY-*STAT3* levels and/or dimerization but normal DNA binding⁶. However, subsequent reports using EBV cells with DBD mutations demonstrated variable levels of cytokine-induced pY-*STAT3* ranging from equivalent to significantly less than WT EBV cells⁴⁵; an explanation for this additional abnormality was not provided. Our results indicate that *STAT3* dysfunction within EBV cells containing DBD mutations extends beyond impaired *STAT3* DNA binding to include reduced cytokine-induced pY-*STAT3* levels, as well as reduced total *STAT3* levels, both of which can be attributed to our finding that most DBD *STAT3* mutations are destabilizing. Likewise, we showed that EBV cells containing S mutations, Y657S and T622I, within the SH2 domain have both impaired cytokine-induced tyrosine phosphorylation, as well as decreased levels of total *STAT3* compared with WT, which has not been reported previously. Thus, our findings indicate that the molecular basis for defective *STAT3* function in AD-HIES not only involves dysfunction of the domain in which the mutated residue is found, but also may be due to the effect that the mutation has on overall *STAT3* protein stability.

In this study, we examined the effects of two modulators of chaperone proteins, HSF1A and GGA, on STAT3 protein activity in EBV cells and PBMC from AD-HIES patients. HSF1A increased cytokine-induced expression of STAT3 target genes in 7 of 8 patients' EBV cells by up to 79% of WT. In addition, HSF1A increased the percentages of cytokine-stimulated pY-STAT3 levels and the MFI per cell in CD4⁺ and CD8⁺ T cells within PBMC of AD-HIES patients. HSF1A is a benzyl pyrazole derivative that binds to TRiC and was shown to alleviate aggregation and cytotoxicity of polyQ proteins expressed in neuronal precursor cells, as well as in a fruit fly model of polyQ-dependent toxicity²⁸. Our results showed that HSF1A increased STAT3 transcriptional activity in AD-HIES patients' EBV cells by increasing levels of pY-STAT3 in 6 of 8 patients' EBV cells. The increase in pY-STAT3 levels was mediated largely through an increase in the level of total STAT3 protein, which is consistent with the possibility that HSF1A improved the ability of TRiC to fold nascent mutant STAT3 protein.

GGA has been shown to bind to the substrate-binding domain of HSP70, which downmodulates its activity and increases proteostasis stress. Proteostasis stress has been shown to promote heat shock factor (HSF) 1 activation resulting in induction of HSP expression, primarily HSP70, and to a lesser degree HSP90²⁸. Similar to HSF1A, GGA increased the STAT3 transcriptional activity in AD-HIES patients' EBV cells by increasing levels of cytokine-stimulated pY-STAT3 in 8 of 8 patients' EBV cells. However, unlike HSF1A, GGA increased total STAT3 levels in only 1 of 8 patients' EBV cells suggesting its effects are not mediated through improved synthesis and folding of nascent STAT3 protein, rather through improved function of native STAT3. Importantly, GGA also increased levels of cytokine-stimulated pY-STAT3 in CD4⁺ and CD8⁺ cells within PBMC of AD-HIES patients and increased IL-17A-producing CD4⁺ cell in cultured splenocytes from *mut-Stat3* mice. GGA has been used safely as

an anti-ulcer drug for over 30 years in Japan; adverse reactions include headache, nausea, anorexia, diarrhea, constipation, skin rash, itching, and elevated liver enzymes, which are treated symptomatically and are self-limited. Thus, GGA merits further development into a potential treatment for AD-HIES either alone or in combination with HSCT. HSCT alone may resolve the immune cell deficiencies in AD-HIES¹⁰, but residual STAT3 dysfunction, most likely in non-immune cells, appears to cause persistent problems^{8,11,46}. Alternative approaches to increasing STAT3 levels and/or function in AD-HIES, such as gene therapy, pose the risk of increased rates of malignancy⁴⁷⁻⁴⁹.

In addition to suggesting novel approaches to AD-HIES, we believe that the findings reported here will have broader implications as modulation of protein stability may be beneficial in other primary immunodeficiencies that result from mutations in transcription factors, such as STAT1^{50,51} and STAT5b⁵², that are dominant-negative, because they function as homodimers, and may be destabilizing.

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AUTHORSHIP CONTRIBUTIONS: Claire E. Bocchini: Experiment design and performance, data interpretation, and manuscript writing; Karen Nahmod: Experiment design and performance, data interpretation and manuscript writing; Sang Kim: Experiment design and performance, data interpretation, and manuscript writing; Panagiotis Katsonis: Evolutionary trace analysis and manuscript writing; Moses M. Kasembeli: Experiment performance; Alexandra Freeman: Experiment performance; Olivier Lichtarge: Evolutionary trace analysis and manuscript writing; George Makedonas: Experiment design and manuscript writing; David J. Tweardy: Experiment design, data interpretation, and manuscript writing.

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TABLES

Table 1. Computer modeling accurately predicts STAT3 protein half-life in EBV cells generated from 8 of 8 AD-HIES patients.

Mutation Category	Genotype (N) ¹	Stability prediction (stable=0; unstable=2)	Measured $t_{1/2} \pm SD$ (hr)
None	wt/wt (N=8)	Stable (0)	25 ± 2
Functional	R382W/wt (N=4)	Stable (0.4)	25.5 ± 3.3
Structural	V463del/wt (N=6)	Destabilizing (1)	6.8 ± 4.8^3
	Y657S/wt (N=6)	Significantly destabilizing (1.8)	5.5 ± 4.1^3
	T622I/wt (N=4)	Destabilizing (0.6)	18 ± 5.8^2
Structural-functional	R423Q/wt (N=4)	Destabilizing (0.6)	20 ± 2.3^2
	S611N/wt (N=4)	Destabilizing (1.4)	7 ± 3.6^3
	N647D/wt (N=4)	Destabilizing (0.8)	18.5 ± 3^2
	V637M/wt (N=4)	Destabilizing (1)	5.3 ± 4.5^3

¹ N = number of experiments performed with each EBV cell.

² $p < 0.05$ compared with WT

³ $p < 0.0001$ compared with WT

FIGURE LEGENDS

Figure 1. Schematic model of STAT3 delineating the domain location and category assignment for each AD-HIES single amino acid missense mutation or in-frame deletion.

Functional (F) mutations are shown in blue; structural (S) mutations are shown in red; structural-functional (S-F) mutations are shown in purple.

Figure 2. STAT3 protein half-life measurements in EBV cells from AD-HIES patients.

Single representative experiments are shown for EBV cells with WT *STAT3* and *STAT3* F mutation R382W (A), EBV cells with *STAT3* S mutations V463del or Y657S (B), and EBV cells with *STAT3* S-F mutations R423Q or V637M (C). *STAT3* protein half-life was determined as time after CHX addition until *STAT3* levels decreased to 50% of starting level, as indicated by vertical lines.

Figure 3. STAT3 proteins containing S mutations have decreased interaction with TRiC. *In*

vitro transcription/translation reactions were performed in RLL containing ³⁵S-methionine and equivalent amounts of cDNA constructs encoding WT or mutant *STAT3* without or with exogenous bovine TRiC (120nM; TRiC) as indicated. In the top half of panels A and B, an equivalent fraction of each reaction was separated by SDS-PAGE, in the case of panel A, either before (Input) or after immunoprecipitation with anti-TRiC (TRiC-IP; column E) or control antibody (column C). Gels were dried and autoradiographed. In the bottom half of panels A and B, bands from multiple experiments (N as indicated or ≥3 where noted) were quantitated by densitometry and the mean ± SD plotted for each *STAT3* cDNA construct. Differences in means of mutant constructs from WT are indicated by asterisks (*, ** p<0.001, Student's t-test).

Figure 4. HSF1A increased STAT3 function in AD-HIES patient EBV cells. EBV cells were treated with HSF1A (3 μ M) for 48 hr then stimulated with IL-21 (50 ng/ml). SOCS3 expression was measured by Q-RT-PCR 120 minutes after stimulation (A). Total and phosphotyrosylated STAT3 and GAPDH were measured by Luminex 30 minutes after stimulation and results shown (B-D). STAT3 protein half-life determined (E-F; $n \geq 4$ for each experiment). Comparisons of means marked by symbols *, **, #, ##, ^, ^^, x, xx, ∞∞, v, vv, +, &, &&, §, §§ indicate $p < 0.01$; symbol ∞ indicates $p < 0.05$ (ANOVA A-D; Student's t-test for F).

Figure 5. GGA increased STAT3 function in AD-HIES patient EBV cells. EBV cells were treated with GGA (3 μ M) for 48 hr then stimulated with IL-21 (50 ng/ml). SOCS3 expression was measured by Q-RT-PCR 120 minutes after stimulation (A). Total and phosphotyrosylated STAT3 and GAPDH were measured by Luminex 30 minutes after stimulation (B-D). STAT3 protein half-life in a single representative experiment (E) and in multiple experiments (F; $N=3$). Comparisons of means marked by symbols *, **, #, ^, x, v, +, &, §, indicate $p < 0.01$; symbol ∞ indicates $p < 0.05$. Levels of HSP70 and HSP90 were determined without and with GGA in two experiments (G); symbols *, # indicate $p < 0.05$.

Figure 6. HSF1A increased STAT3 function in AD-HIES patient PBMC. PBMC from a patient with *STAT3* S mutation V463del or a healthy control were each preincubated in triplicate aliquots with HSF1A and stimulated with IL-21, as above (G). PBMC from three patients with *STAT3* S mutation V463del, one patient with *STAT3* S mutation F621V, and 7 healthy controls were incubated with HSF1A and stimulated with IL-21, as above, each on 4 or more occasions

(H). Results shown are percentage of pY-STAT3-positive cells and MFI in CD4⁺ and CD8⁺ T cells determined by multiparametric flow cytometry [ANOVA (G), paired T-test (H)].

Figure 7. GGA increased STAT3 function in AD-HIES patient PBMC. PBMC from a patient with *STAT3* S mutation V463del or a healthy control were each preincubated in triplicate aliquots with GGA and stimulated with IL-21, as above (H). PBMC from three patients with *STAT3* S mutation V463del, one patient with *STAT3* S mutation F621V, and 7 healthy controls were incubated with GGA and stimulated with IL-21, as above, each on 4 or more occasions (I). Results shown are percentage of pY-STAT3-positive cells and MFI in CD4⁺ and CD8⁺ T cells determined by multiparametric flow cytometry [ANOVA (H), paired T-test (I)].

Figure 8. GGA and HSF1A increased IL-17A producing cells within CD4⁺ splenocytes from mut-Stat3 mice. Representative flow cytometry analysis (panel A) of TCR⁺ CD4⁺ T cells in splenocytes from mut-Stat3 or wild type (WT) mice; IL-17A intracellular staining was performed upon isolation without PMA/Ionomycin stimulation (Unstimulated) or after incubation in Th17 polarizing conditions either without proteostasis modulators (no PMs) or with GGA (3 μM) or HSF1A (3 μM) and stimulation with PMA/Ionomycin. In panel B, the percentage of IL-17A producing TCR⁺CD4⁺ cells measured in the presence of GGA or HSF1A was normalized to the percentage in the absence of proteostasis modulators and the mean and SD (vertical bars) plotted for each mouse in both the mut-Stat3 and WT groups (n=4; p values determined using paired t-test are shown).

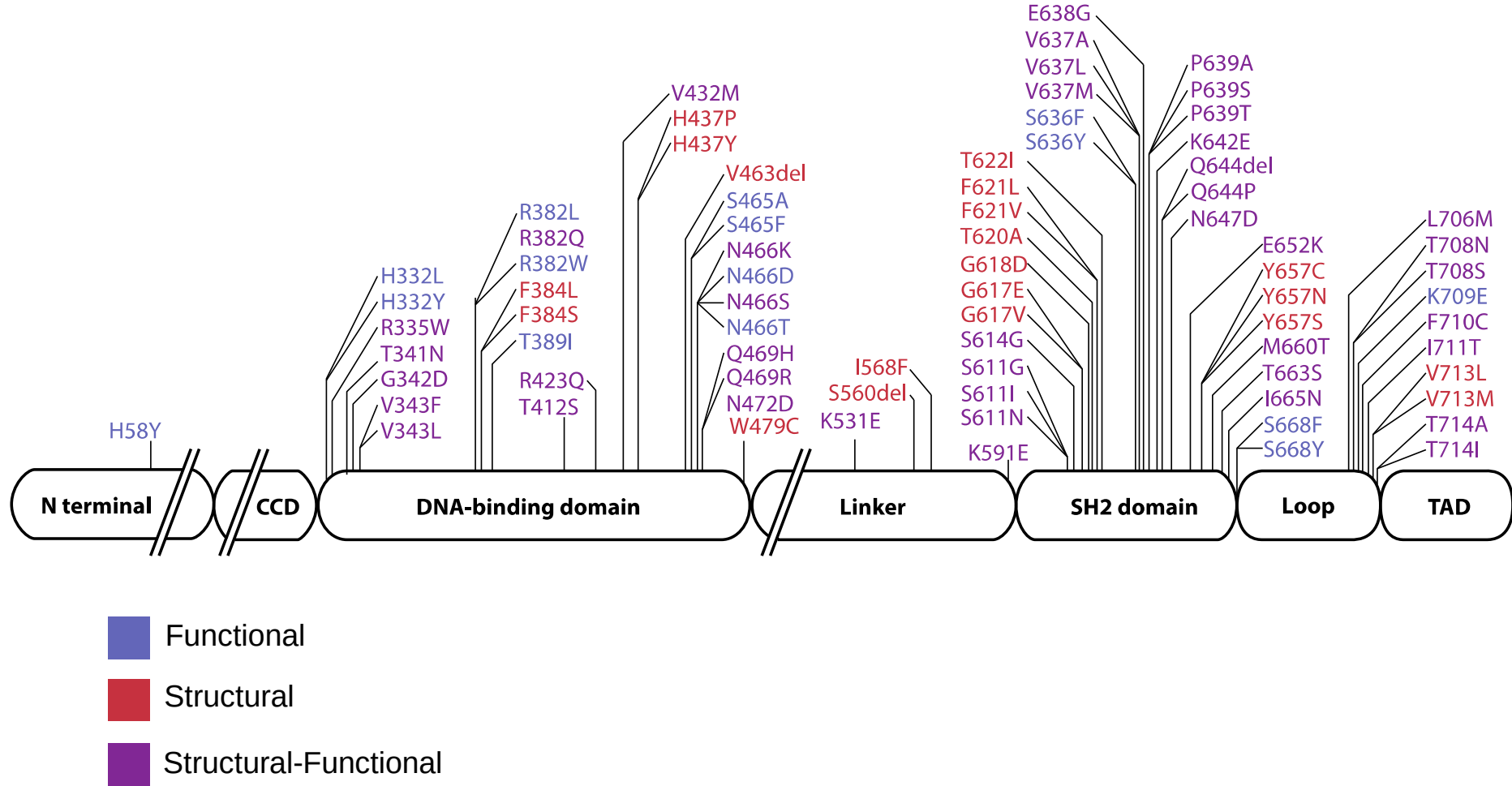


FIGURE 1

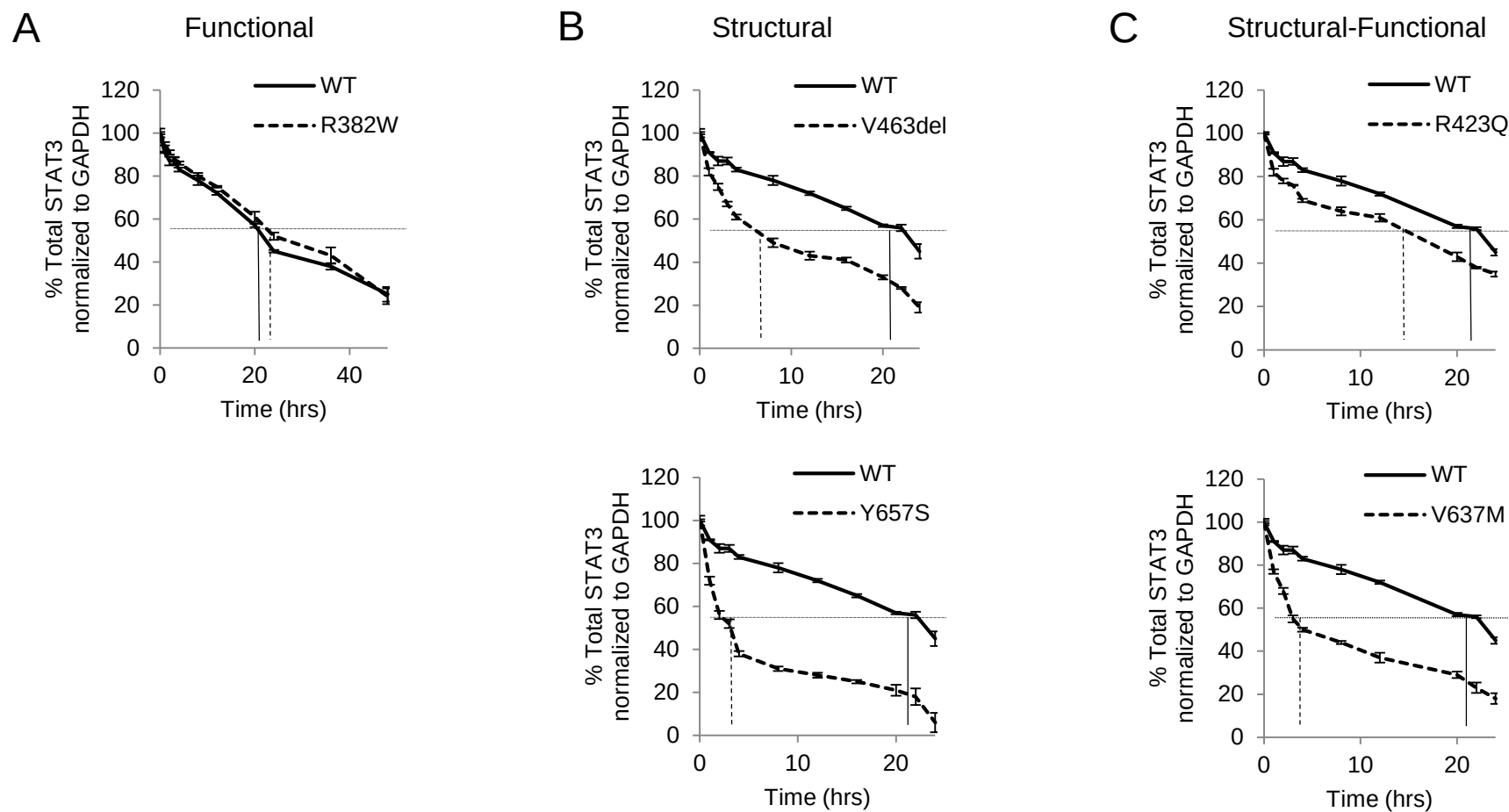
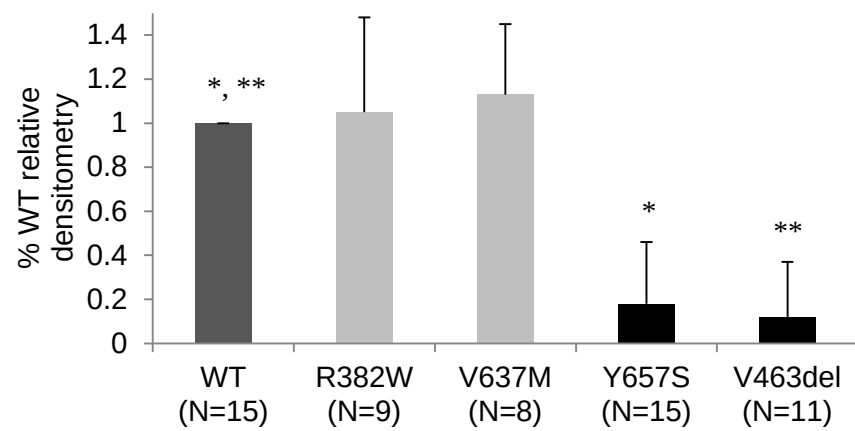
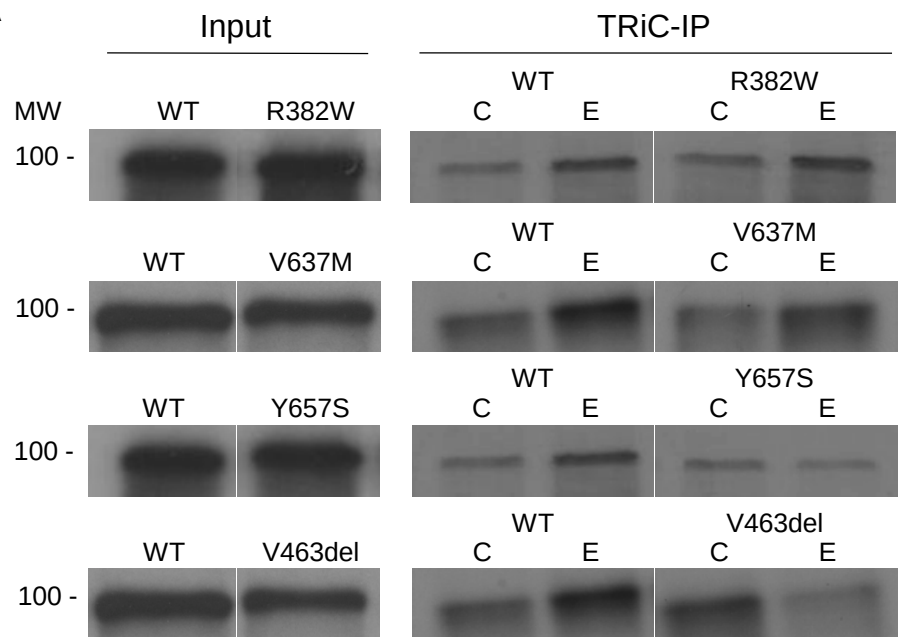


FIGURE 2

A



B

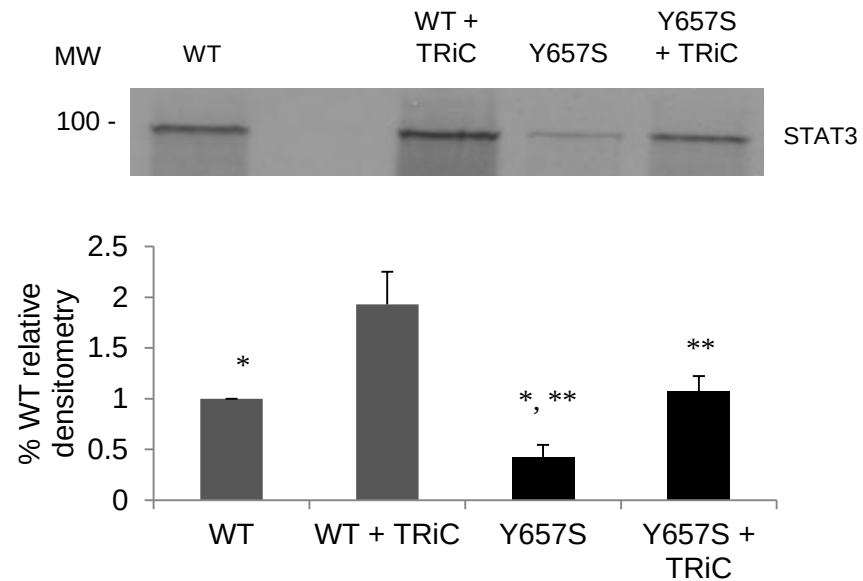


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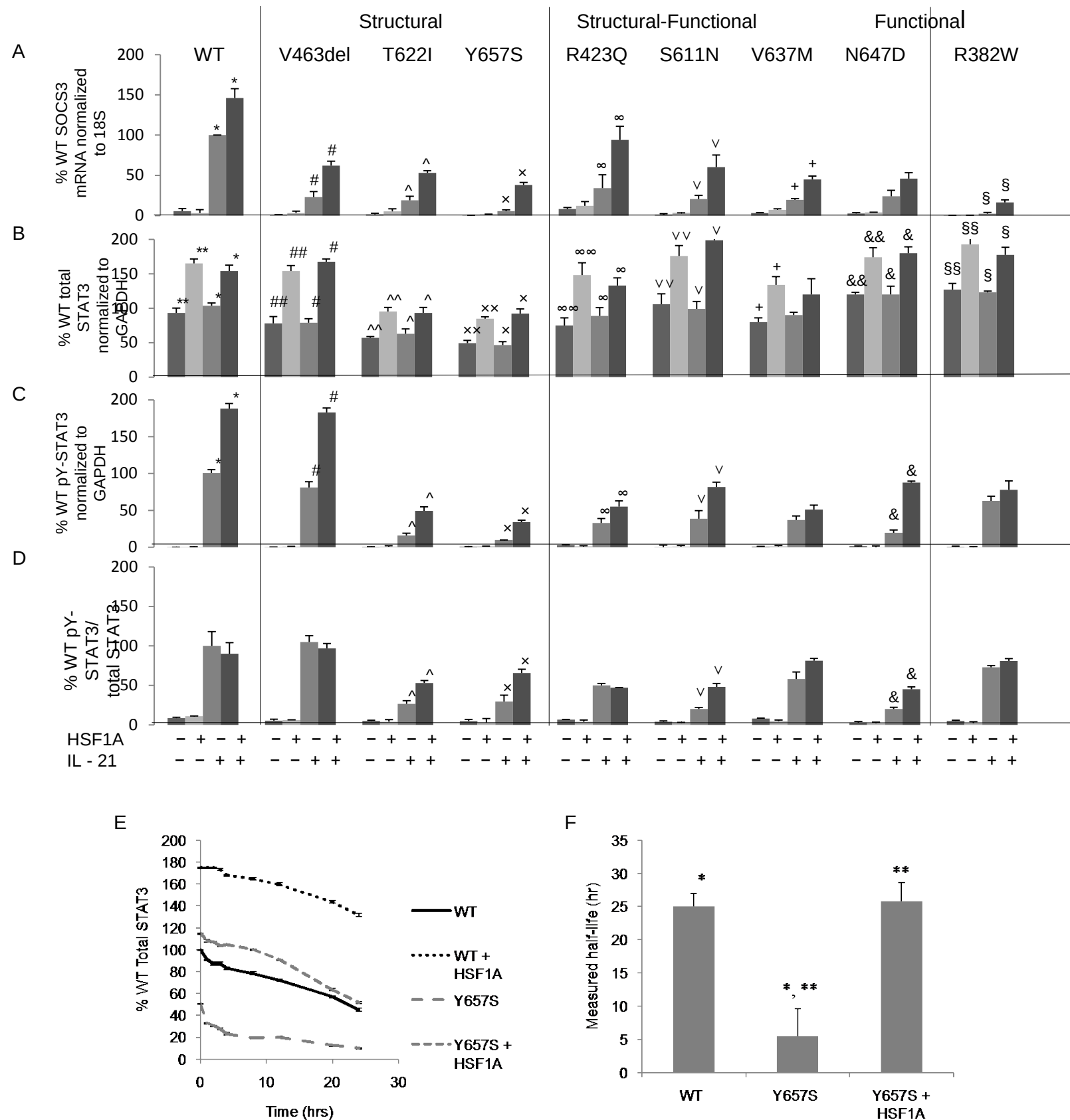


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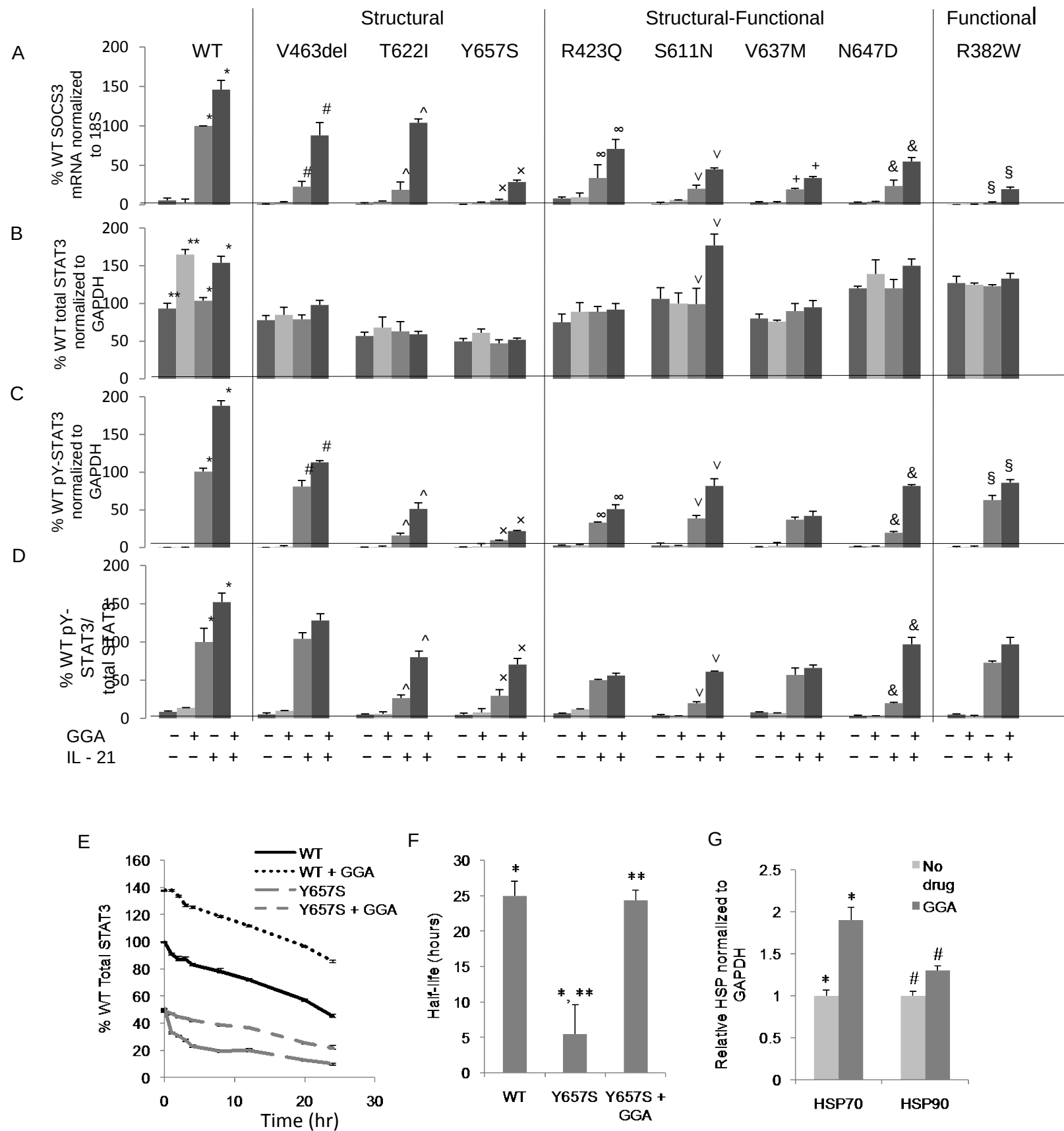


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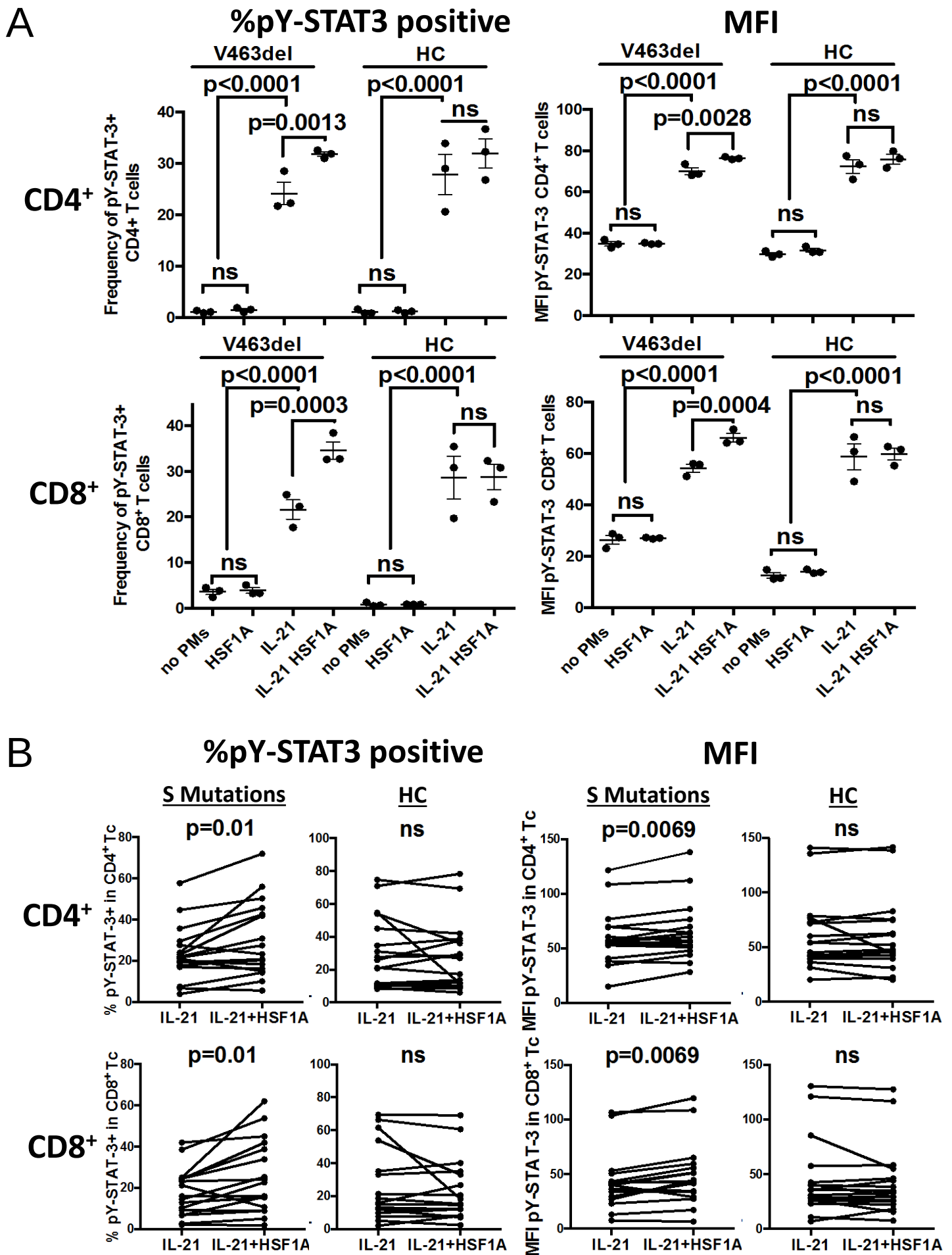


FIGURE 6

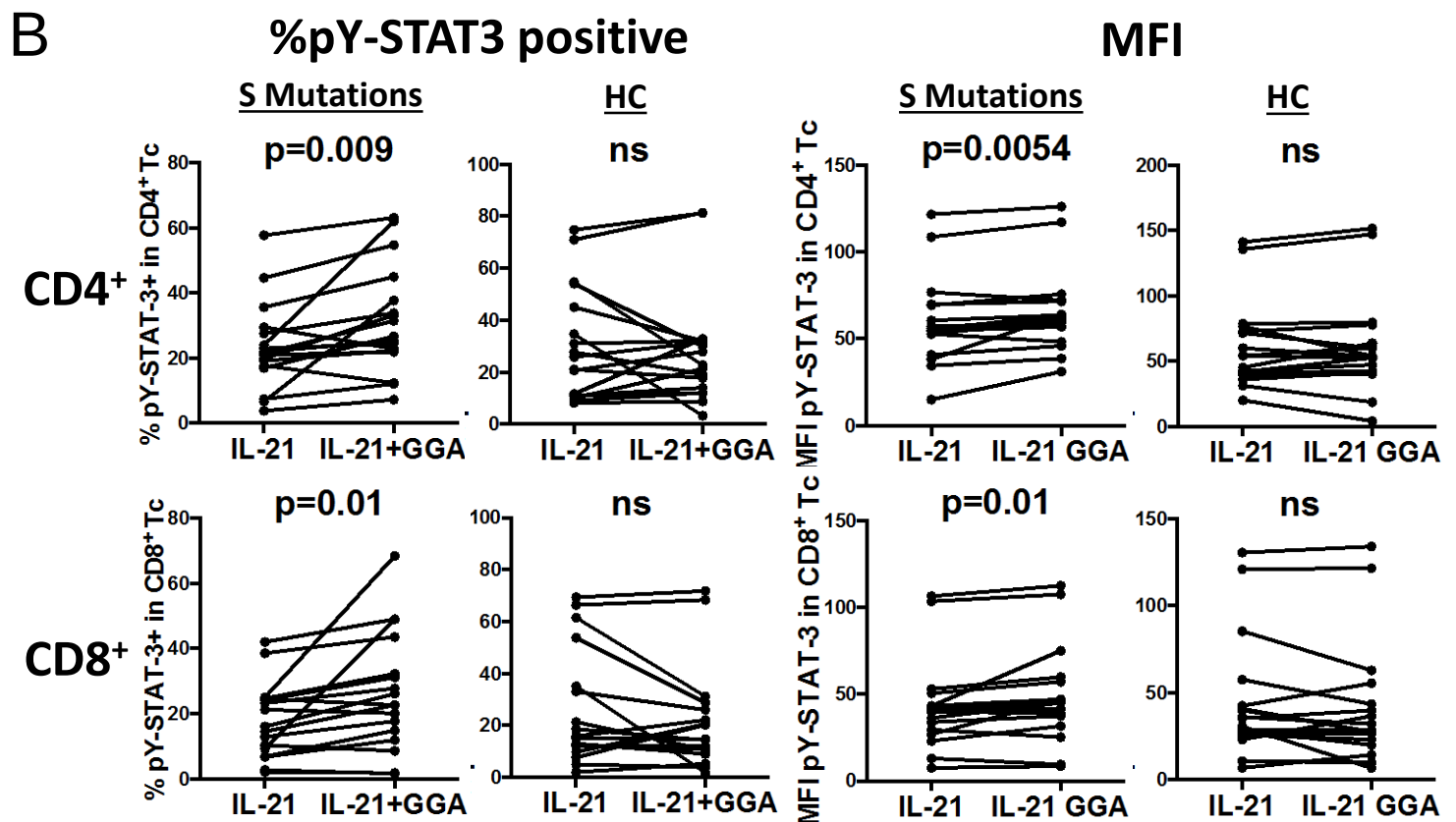
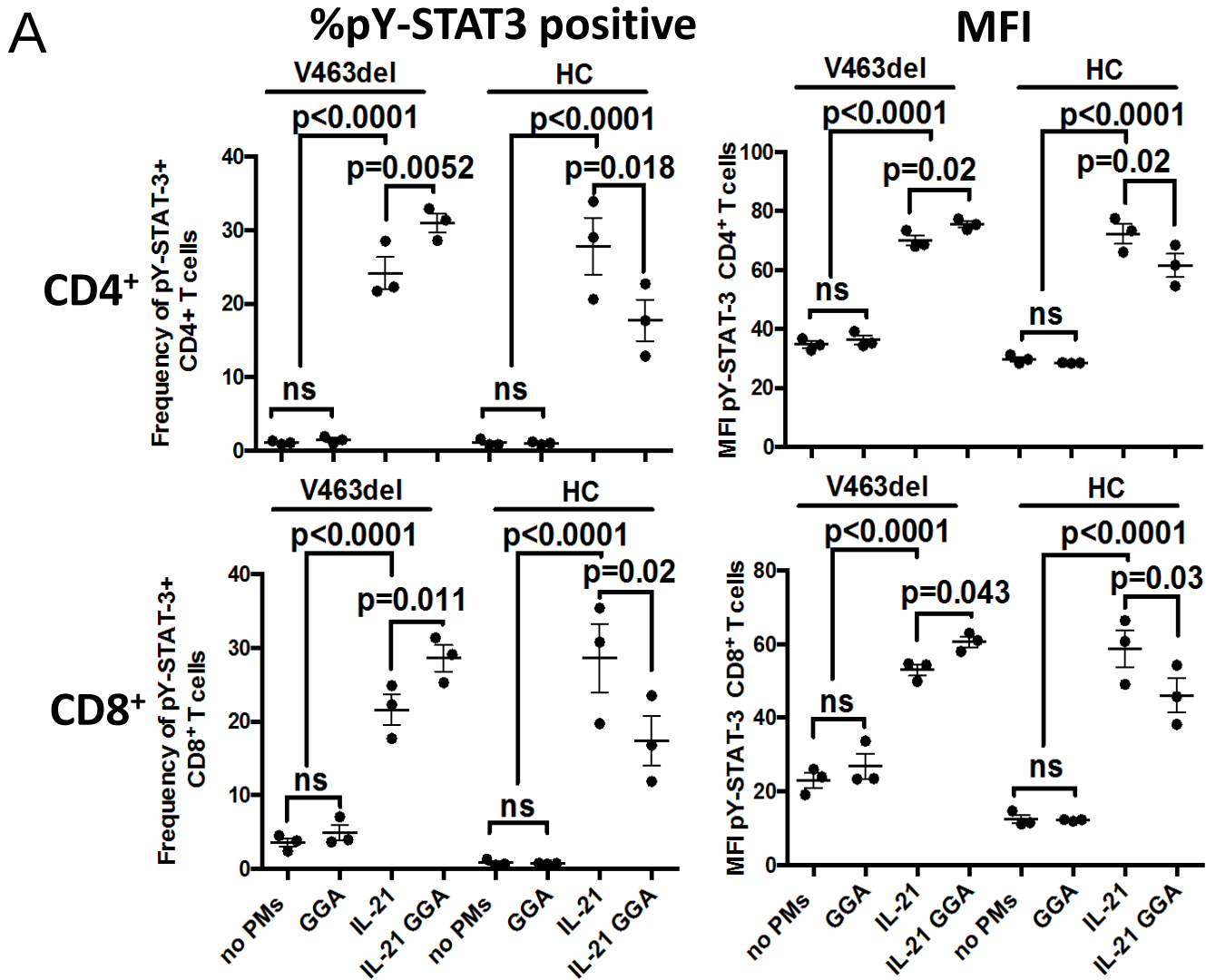


FIGURE 7

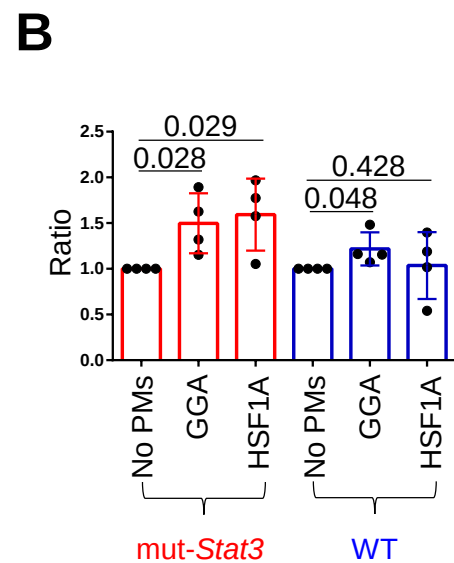
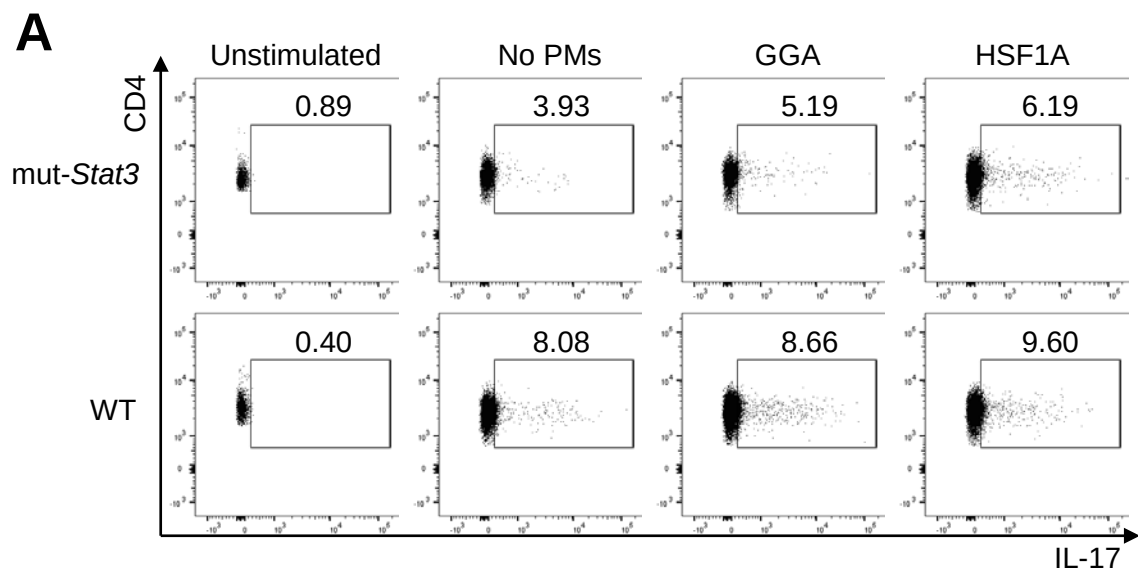


FIGURE 8



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Protein stabilization improves STAT3 function in autosomal dominant hyper-IgE syndrome

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