

Letter to the Editor

Distinct mutations at the same positions of STAT3 cause either loss or gain of function

To the Editor:

Signal transducer and activator of transcription 3 (STAT3) directly or indirectly regulates the pathways of IL-6, IL-10, IL-11, IL-17, IL-23, G-CSF, M-CSF, and leptin, among others. STAT3 has 24 exons, which are spliced into 3 isoforms, resulting in proteins of 722 or 770 amino acids.¹ Dominant-negative (DN) *de novo* and inherited germline mutations in STAT3 predominate in the DNA-binding domain and Src homology 2 (SH2) domains of STAT3 cause hyper-IgE syndrome (Job's or HIES).^{2,3} Recently, somatic mutations in STAT3 have been found in up to 40% of patients with large granular lymphocytic leukemia,⁴ so far all in the SH2 domain. More recently, germline gain-of-function (GOF) mutations in STAT3 were shown to underlie multiorgan immune disease with immunodeficiency and lymphoproliferation.⁵ STAT3 mutations have also been identified in leukemia,⁶ lymphoproliferative disorders,⁷ aplastic anemia, and autoimmune disorders.^{8,9} Dominant negative STAT3 mutations cause defective IL-6 and IL-10 signaling and diminished DNA binding.² In contrast, GOF mutations are hypermorphic with higher DNA binding, leading to aberrant STAT1 and STAT5 signaling and decreased regulatory T-cell numbers.⁸

Missense mutations and deletions have been reported, affecting at least 89 different positions in STAT3 (see Fig E1 in this article's Online Repository at www.jacionline.org). Most of the reported mutations have been in the DNA-binding domain and the SH2 domains of STAT3, where both hypomorphic and hypermorphic mutations have been reported, with some positions supporting both hypomorphic and hypermorphic functions, depending on the amino acid.

We identified 6 positions in the SH2 domain at which distinct point mutations have been reported to cause either HIES or lymphoproliferation/autoimmunity (Table I). Polyphen-2 (Polymorphism Phenotyping v2) predictions showed that all the substitutions were possibly or probably damaging, except the arginine substitution at position 647, which was predicted to be benign (Table I). We functionally characterized the HIES mutations N647D and K658E and their leukemia and autoimmunity counterparts N647I and K658N. We transfected Cos-7 cells with plasmids encoding wild-type or mutant alleles and stimulated them with Oncostatin M. On stimulating them with Oncostatin M, the levels of phosphorylated STAT3 (Y705; pSTAT3) were higher for GOF N647I and K658N and lower for DN N647D and K658E alleles, compared with the wild-type STAT3 transfected cells (Fig 1, A; for Methods, see this article's Online Repository at www.jacionline.org). Stimulation of transfected Cos-7 cells with various concentrations of IL-6 demonstrated a similarly clear demarcation between hypomorphic and hypermorphic STAT3 alleles (Fig 1, B). Furthermore, transfected Cos-7 cells showed a dose-dependent response in the wild-type and GOF mutants while the response of the DN mutants was profoundly blunted (Fig 1, B). Transfection of

TABLE I. GOF and dominant-negative mutations occurring at the same positions in the STAT3 protein with initial citations

Mutation*	Disease	PolyPhen-2	
		Score	Prediction
S614G ^{E1}	HIES	0.994	Probably damaging
S614R ^{E2}	T-LGL	1.000	
G617E ^{E3}	HIES	1.000	Probably damaging
G617V ^{E1}	HIES	0.998	
G617R ^{E4}	DLBCL	1.000	
G618D ^{E5}	HIES	0.892	Possibly damaging
G618R ^{E2}	NK-LGL	0.983	
N647D ^{E6}	HIES	0.037	Benign
N647I ^{E7}	LGLL	0.395	
Y657C ^{E6}	HIES	1.000	Probably damaging
Y657S ^{E8}	HIES	0.999	
Y657N ^{E9}	HIES	0.999	
Y657ins ^{E10}	γδ T lymphoma		
Y657dup ^{E11}	T-LGL		
Y657_M660dup ^{E12}	IHCA, CHC1021T		
Y657_K658insY ^{E7}	LGLL		
K658M ^{E2}	T-LGL	0.994	Probably damaging
K658N ^{E7}	LGLL/autoimmunity	0.990	
K658Y ^{E12}	IHCA; CHC379T	0.956	Possibly damaging
K658E ^{E13}	HIES	0.926	

The PolyPhen-2 scores and their predictions indicate the effects of single residue changes in the structure and function of STAT3.

DLBCL, Diffuse large B-cell lymphoma; IHCA, inflammatory hepatocellular adenomas; LGLL, large granular lymphocytic leukemia; T-LGL, T-cell large granular lymphocytic leukemia; NK-LGL, natural killer cell large granular lymphocytic leukemia.

*For the references, see this article's Online Repository at www.jacionline.org.

U3A cells was in agreement with the Cos-7 data of the STAT3 alleles (Fig 1, C).

Three-dimensional protein structural analyses showed that the mutated amino acid substitutions radically changed the electrostatic potentials in the mutated positions. Despite the benign prediction by Polyphen-2 analysis, the DN mutation N647D changes the electrostatic potential to be much more negative, which might adversely affect DNA-binding affinity (Fig 1, D). In contrast, the GOF N647I mutation reshapes the binding pocket and removes the localized positive electrostatic potential. The DN missense mutation K658E alters both the local molecular surface shape and the local electrostatic potential from slightly positive to significantly negative, which would probably disrupt binding of STAT3 to DNA. In contrast, GOF K658N does not alter the molecular landscape nearly as much and the electrostatic potential shift is not nearly as strong as the K658E (Fig 1, D).

These structural and functional analyses provide *in vitro* functional confirmation and molecular predictions of different nonsynonymous mutations in STAT3 at exactly the same positions that can be either hypomorphic or hypermorphic. Therefore, GOF and loss-of-function mutations in STAT3 are not so much a function of the region of STAT3 in which they occur as they are a function of their effect on local charge and DNA-binding affinity. Presumably, similar variations occur in regions of STAT3 involved in protein-protein interactions, as well. Rational classification of STAT3 mutations hence requires functional analyses in conjunction with the clinical diagnosis.

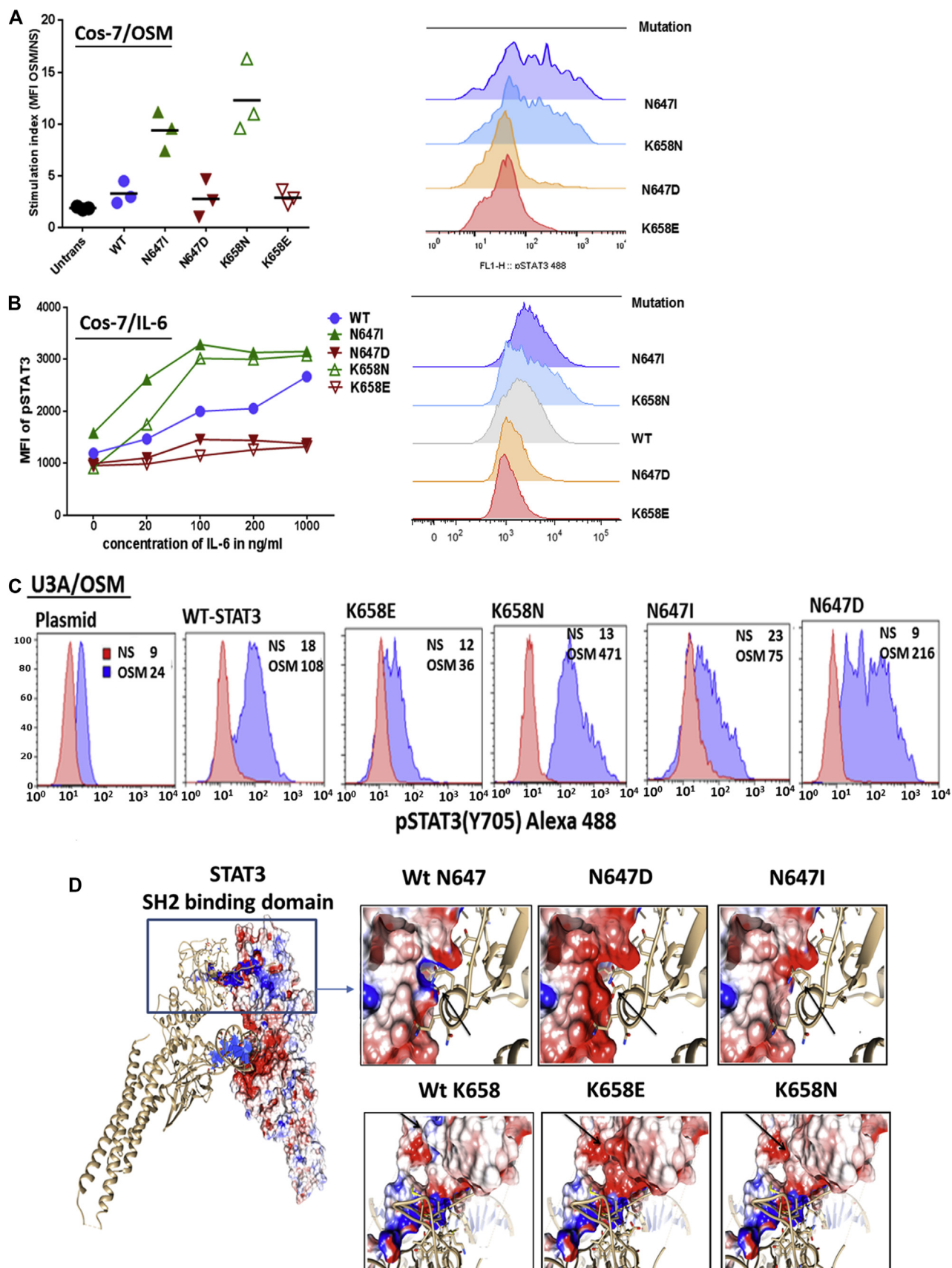


FIG 1. Functional characterization of STAT3 mutations: WT or mutant STAT3 allele transfected Cos-7 (**A** and **B**) or U3A (**C**) cells were assessed for pY(705)STAT3 by flow cytometry after stimulation with Oncostatin M or varying concentrations of IL-6. **D**, The electrostatic potential near the WT or mutated STAT3 residues and their DNA binding. Negative charge is shown in red, positive in blue. MFI, Mean fluorescent intensity; OSM, Oncostatin M; WT, wild-type.

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METHODS

Cell lines and transfections

IL-6R-expressing Cos-7 (Origene Technologies, Rockville, Md) cells and U3A cells (provided by G. Stark, Cleveland Clinic, Ohio) were used. The cells were maintained in complete Dulbecco modified Eagle medium (Life Technologies, Green Island, NY) supplemented with 10% FCS, 2 mM L-glutamine, HEPES, and antibiotics. Transient transfections were carried out using either electroporation (Lonza, Germany) or Lipofectamine 2000 transfection reagent (Life Technologies) following manufacturers' protocol.

Plasmids and constructs

Plasmid-encoding Myc-DDK-tagged wild-type STAT3 transcript variant 1 was purchased from Origene Technologies (RC215836). Point mutations were introduced in the wild-type sequences using point mutagenesis PCR and the resulting PCR products were cloned into the pCM6-XL4 background (BioInnovative, Rockville, Md). Endotoxin-free plasmid preparations were made using Endo Free plasmid kits purchased from Qiagen (Valencia, Calif).

STAT3 phosphorylation assay

Cells transfected with plasmids encoding wild-type or mutant *STAT3* were stimulated with Oncostatin M (50 ng/mL) or IL-6 in increasing concentrations (20, 100, 200, 1000 ng) for 30 minutes at 37°C. Cells were then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pa), permeabilized with 100% methanol, and stained for STAT3 (allophycocyanin [APC]) and phosphoSTAT3 (Alexa Fluor 488, pY705) (BD Biosciences, San Jose, Calif). Fold induction of phosphoSTAT3 (Y705) in OSM- or IL-6-stimulated cells over nonstimulated cells was calculated using the geometric mean of the fluorescent values and the fluorescent values are denoted as mean fluorescent intensity.

All data were acquired on either FACS Calibur or BD LSR Fortessa (BD Biosciences). A minimum of 50,000 counts were acquired within the live cell population gate for each tube. Data were analyzed using FlowJo version 9 (Tree Star Inc, Ashland, Ore).

Protein models

The 3D models of STAT3 were generated using the I-TASSER server. The electrostatic potential was calculated with APBS and displayed using UCSF Chimera.

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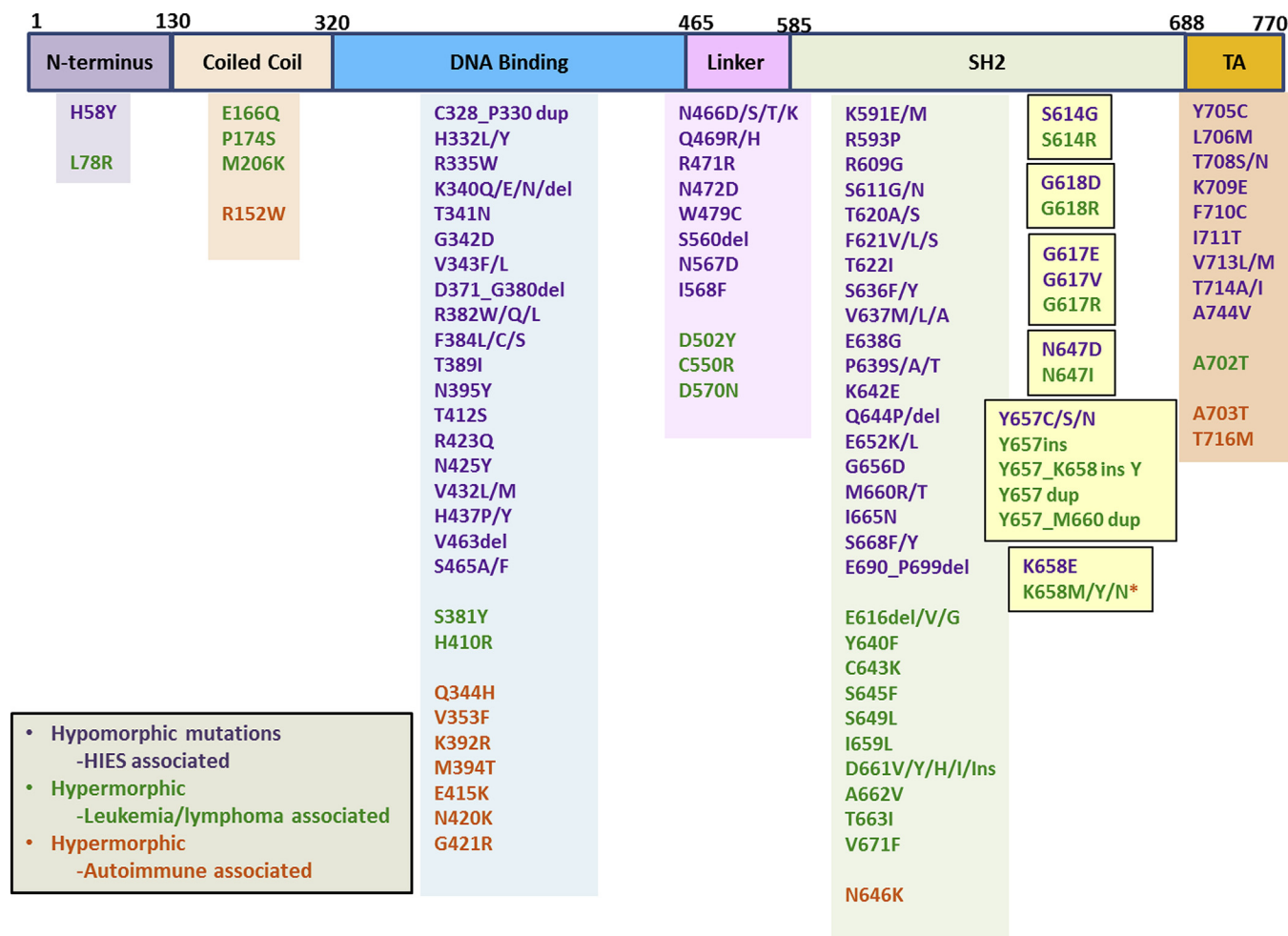


FIG E1. Previously reported *STAT3* mutations by STAT3 domain. Hypomorphic DN mutations found in patients with HIES (Job's) are indicated in *purple*; hypermorphic GOF mutations found in patients with leukemia or lymphoma are indicated in *green*; hypermorphic GOF mutations found in patients with autoimmune disorders are indicated in *orange*.