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Mutations underlying autosomal dominant hyper-IgE syndrome impair distinct stages of STAT3 signaling

Running title: Structure/function analysis of STAT3 mutations

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Key Points

1. Distinct loss-of-function mutations in *STAT3* causing autosomal-dominant hyper-IgE syndrome impair STAT3 signaling at different stages.
2. Defects impacting distinct stages of STAT3 signaling all result in impaired DNA binding and target gene transcription.

Abstract

Autosomal dominant hyper IgE syndrome (AD-HIES) is a primary immunodeficiency caused by heterozygous dominant negative mutations in the gene encoding Signal transducer and activator of transcription 3 (*STAT3*). These mutations cause a multisystemic disorder characterized immunologically by susceptibility to infection with pathogens such as *C. albicans* and *S. aureus* due to defective Th17 responses, and a failure to generate effective humoral immune responses. Interestingly, disease-causing mutations have been identified throughout all functional domains of STAT3 including the DNA binding, SH2 and transactivation domains. However, no phenotype-genotype correlations have been observed. To better understand how different mutations all result in the same clinical and functional outcome we sought to characterize which stage(s) of STAT3 signaling was impaired by each of these mutations and if this was consistent for mutations in different domains. We used B-cell lines from AD-HIES patients or cell lines transfected with mutant *STAT3* to determine the abilities of mutant STAT3 proteins to undergo phosphorylation, cytokine receptor docking, dimerization, nuclear translocation, bind DNA and induce gene transcription. Our analysis revealed that while all mutations resulted in a distal impairment in STAT3 binding to DNA, the block occurred at different proximal stages in signaling: transactivation domain mutant had reduced phosphorylation, SH2 domain mutants either were not phosphorylated or had reduced dimerization, and DNA binding domain mutants had defective nuclear translocation or DNA binding. Thus, this study reveals the biochemical mechanisms underlying dysfunctional STAT3 signaling in AD-HIES and also identifies residues and regions of domains that are critical for normal STAT3 function.

Introduction

Heterozygous dominant negative loss-of-function (LOF) mutations in *STAT3* cause autosomal dominant hyper IgE syndrome (AD-HIES), a disease characterized by increased susceptibility to infection at mucosal surfaces with *Staphylococcus aureus*, *Streptococcus pneumonia* and *Candida albicans*, eczema, extremely elevated levels of serum IgE, poor humoral immune responses, and non-immunological features including defects in musculoskeletal, dental, vascular, and connective tissue systems.[1-3] *STAT3* encodes a latent cytosolic transcription factor, Signal transducer and activator of transcription 3 (STAT3), that has a key role in signaling downstream of multiple cytokine receptors, including those for IL-6, IL-10, IL-21 and IL-23.[4]

In canonical STAT3 signaling, binding of a cytokine to its receptor induces conformational changes to the cytoplasmic region of the receptor, resulting in activation and auto-phosphorylation of associated Janus activating kinases (JAKs).[5,6] STAT3 can then dock with the receptor and be phosphorylated at tyrosine 705 (Y705) by the associated JAK. After phosphorylation STAT3 forms multimers and translocates to the nucleus, where it binds DNA and induces gene transcription.[7,8]

STAT3 is comprised of several key functional domains each with a specific role in intracellular signaling. The N-terminal domain is required for the formation of unphosphorylated dimers and nuclear localization.[9] The DNA binding domain (DNA BD) is where the interaction/interface between STAT3 and target genes takes place.[10] The Src Homology 2 (SH2) domain is important in multimerization of different STAT3 molecules[10] and interaction with cytokine receptors.[10,11] Finally, the transactivation (TA) domain contains two residues – tyrosine 705 (Y705), serine 727 (S727) – that are phosphorylated and regulate signaling.[6,8]

STAT3 mutations detected in AD-HIES cause multiple cellular defects that underlie disease pathogenesis in affected individuals. For example, these patients lack Th17 cells and have reductions in MAIT cells; furthermore production of IL-17A and IL-17F by the residual MAIT cells in these patients is impaired[12-15]. These defects most likely explain susceptibility to fungal infections and the development of chronic mucocutaneous candidiasis[12-15]. They also have defects in antibody production and secondary antibody responses, presumably resulting from the combined effects of defects in the ability of B cells to respond to the STAT3 dependent cytokines IL-10 and IL-21[16,17] and reduced numbers of T follicular helper cells.[18]

While our knowledge of the cellular defects that cause AD-HIES has increased dramatically in recent years our understanding of the molecular impact of the various *STAT3* mutations remains limited. More than 60 different *STAT3* mutations have been reported in AD-HIES.[2,3,19] While they most commonly occur in the DNA BD and SH2 domain, mutations have been detected in all functional domains of STAT3. Interestingly there is no relationship between genotype and phenotype, indicating a similar clinical presentation and outcome irrespective of the nature of the disease-causing mutations.[20,21] This raises the question of which stage do these diverse mutations impair STAT3 signaling to manifest the same clinical and cellular phenotype.

To explore this, we have examined the consequences of distinct disease-causing mutations in the DNA BD, SH2 and TA domains on each stage of STAT3 signaling, specifically docking with cytokine receptors, tyrosine phosphorylation, dimer formation, nuclear translocation, DNA binding and gene transcription. We found that while the various mutations affect STAT3 signaling at discrete stages, they all yield same end result, that being impaired binding to DNA, and subsequent gene transcription.

Methods

EBV-transformed lymphoblastoid cell lines (EBV-LCL)

EBV-LCLs were established from PBMCs isolated from normal healthy donors or patients with confirmed mutations in *STAT3.*[17]

Assessing STAT3 Phosphorylation

HEK-293T cells (2×10^6 cells/well) were seeded in 6 well plates (Corning) in DMEM + 5% FCS, incubated overnight at 37°C in 5% CO_2 and then transfected using Lipofectamine 3000 (Life technologies) with 3.5 µg of a pCDNA3.1 plasmid containing either wild-type (WT) or mutant *STAT3* engineered to include an N-terminal FLAG tag. Transfected HEK-293T cells were cultured for 48 hours, harvested by incubating with 1 mM EDTA at 37°C, washed in PBS, resuspended and rested for 2 hours in DMEM without FCS, and then stimulated in the absence or presence of 10 ng/ml IL-6 for 30 minutes. Cells were fixed with 2% paraformaldehyde, permeabilised with ice-cold 90% methanol and stained with phycoerythrin-conjugated anti-phosphorylated STAT3 (pSTAT3) (pY705, BD, 612569) and FITC-conjugated anti-FLAG mAbs (Sigma-Aldrich, F4049). Samples were acquired on a FACSCanto (BD). Phosphorylation of WT and mutant STAT3 was determined by gating on FLAG positive cells.

Lysate preparation and Western blotting

Whole-cell lysates of either EBV-LCLs from healthy donors or AD-HIES patients, or of HEK-293T cells transfected with FLAG tagged WT or mutant *STAT3* constructs were prepared using 1% NP-40 in 10 mM Tris-HCl, 150 mM NaCl, 0.1% NaN_3, pH 7.8, supplemented with phosphatase/protease inhibitors (Aprotinin, Na_3VO_4, NaF, PMSF and EDTA). Protein concentrations were determined by BCA Assay (ThermoFisher). Samples were electrophoresed through NuPAGE 4-12% Tris-Bis gels (Life Technologies) and then transferred to Immobilon-FL membranes (Millipore). Membranes were blocked using Odyssey blocking buffer (LiCor Biosciences) and probed with mAbs against FLAG (Sigma-Aldrich, F3165), STAT3 (Santa Cruz Biotechnology, C-20), pSTAT3 (Y705) (Cell Signaling, 3E2) and GAPDH (Santa Cruz Biotechnology, 6C5). IRDye® 680LW or IRDye® 800CW Donkey anti-Mouse/Rabbit secondary antibodies (LiCor Biosciences) were used for detection. Membranes were scanned with the Odyssey Clx (LiCor Biosciences) and analysed using ImageStudio.

Cytokine receptor peptide immunoprecipitation of STAT3

Whole-cell lysates of EBV-LCLs or transfected HEK-293T were generated as above. Lysates were pre-cleared by incubation with M-280 Dynabeads (Life Technologies). Phosphorylated

and unphosphorylated peptides from IL-21R containing the well-characterised STAT3 binding motif YXXQ (ie aa 515-526 PPRS**Y**LR**Q**WVVI), or a control IL-21R sequence (395-406 EDDG**Y**PALDLDA, Mimotopes, Australia), were conjugated to Dynabeads and then incubated with whole cell lysate for 2 hours. Bound proteins were eluted from the Dynabeads and subjected to electrophoresis and Western blotting using mAbs against STAT3 or FLAG.

STAT3 dimerization assay

HEK-293T cells were co-transfected with 3.5 µg of pCDNA3.1 plasmids containing WT STAT3 with an N-terminal MYC tag and either WT or mutant variants of STAT3 with an N-terminal FLAG tag. Cells were stimulated with 10 ng/ml IL-6 for 30 minutes and whole-cell lysates then prepared as above. Immunoprecipitations were performed using anti-FLAG® M2 magnetic beads (Sigma-Aldrich, M8823). Washes were performed in TBS and captured proteins were eluted in 0.1 M Glycine HCl, pH 3.0. Western blots were performed on the input and immunoprecipitated output with antibodies against MYC (Santa Cruz Biotechnology, 9E10), FLAG and GAPDH.

Nuclear Localisation and DNA binding assays

EBV-LCLs from healthy donors or AD-HIES patients with *STAT3* mutations were rested in RPMI1640 for 2 hours. Cells were then either unstimulated or stimulated with IL-21 (10 ng/ml) for 15, 30 or 60 minutes. Nuclear and cytoplasmic fractions were then prepared (NE-PER™ kit, Thermo Scientific) and subjected to SDS-PAGE and Western blotting using antibodies against STAT3, pSTAT3 (Y705), GAPDH and Histone H3 (Cell Signaling, D1H2). DNA binding was assessed using a TransAM STAT3 transcription factor binding kit (Active Motif) on nuclear extracts.

Real-Time PCR

EBV-LCLs from healthy donors or AD-HIES were unstimulated or stimulated with IL-21 for 3 hours. RNA was isolated and reverse transcribed with oligo-dT. Expression of the STAT3 target gene (*SOCS3* 5': AGACTTCGATTCGGGACCA, 3': AACTTGCTGTGGGTGACCA) and two housekeeping genes (*18S* 5': GCAATTATTCCCCATGAACG, 3': GGGACTTAATCAACGCAAGC; *RPL13A* 5': CAAGCGGATGAACACCAAC, 3': TGTGGGGCAGCATACCTC) were assessed by real-time PCR (LightCycler 480 Probe Master Mix and System, Roche).

Results

Differential effects of *STAT3* mutations on cytokine-induced phosphorylation of STAT3

To begin to dissect how mutations in *STAT3* compromise its function we selected 6 mutations previously identified in our cohort of AD-HIES patients that localize to the DNA BD (R382W, H437P, S465F), SH2 domain (Q644P, Y657N) or TA domain (L706M) (Figure 1).[17] One of the initial steps in STAT3 activation following cytokine binding to its receptor is phosphorylation at Y705 by active JAK proteins. To test how each *STAT3* mutation affected tyrosine phosphorylation, HEK-293T cells were transfected with plasmids containing cDNA encoding either WT STAT3 or one of the above-described mutant STAT3 proteins (Figure 1). Stimulation with IL-6 resulted in strong phosphorylation of WT STAT3 as well as of STAT3 harbouring mutations in the DNA BD and the STAT3 SH2 domain Q644P mutant (Figure 2A, B). In contrast, the STAT3 SH2 domain Y657N mutant and the TA domain L706M mutant both failed to undergo significant phosphorylation in response to IL-6 (Figure 2A, B). Thus, only mutations localized in the proximity of the Y705 residue (Figure 1) affected cytokine-induced STAT3 phosphorylation.

Mutations in the SH2 domain of STAT3 affect binding to cytokine receptors

The defect in inducing phosphorylation at Y705 in STAT3 by the Y657N and L706M mutations may be caused by an inability of these mutants to associate with the cytoplasmic domain of STAT3-activating cytokine receptors. The interaction between STAT family members and the cytoplasmic domain of cytokine receptors is a necessary step for phosphorylation induced by receptor-bound JAKs. STAT3 interacts with the YXXQ motif present in the cytoplasmic domain of cytokine receptors including gp130, IL-10R and IL-21R.[22-25] Once bound to this motif STAT3 can be phosphorylated by the JAK associated with the receptor. To assess if the reduction in phosphorylation of STAT3 Y657N and L706M resulted from an inability to interact with cytokine receptors we performed peptide pull down experiments. Peptide sequences containing the YXXQ motifs in human IL-21R, together with a control sequence from IL-21R containing a tyrosine but lacking the STAT3 binding motif were tested against lysates from both WT and mutant LCLs. WT STAT3 and STAT3 with mutations in the DNA binding domain and the TA domain exhibited comparable docking to the IL-21R peptide containing the YXXQ motif (Figure 3A). However, for the STAT3 SH2 domain Y657N mutant there was a trend of reduction in binding. As 50% of the STAT3 in LCLs from the patients will be WT, and these may minimize the defect in receptor binding, we performed similar experiments by over-expressing mutant STAT3 plasmids in HEK-293T cells. This analysis confirmed significantly reduced binding of STAT3 mutant Y657N to IL-21R (Figure 3B). We also tested another STAT3 SH2 domain mutation Q644P in HEK-293T cells, as we were unable to maintain a

viable EBV-LCL line from a patient with this mutation, as observed previously.[26] While this mutation tended to reduce binding of STAT3 to IL-21R, this was not significant. Thus, the Y657N SH2 domain mutation was the only lesion that significantly impaired STAT3 binding to cytokine receptors.

Mutations in the SH2 domain, but not DNA BD or TA domains, impair STAT3 dimerisation

Following receptor recruitment and phosphorylation, STAT3 can form parallel dimers which are dependent on interactions between SH2 domains of the two different STAT3 molecules.[10] Formation of STAT3 dimers can also occur independently of phosphorylation through a mechanism dependent on the N terminal domain.[9] However phosphorylation of STAT3 is necessary for the formation of active parallel dimers[27] and is needed for responses to cytokines.[28] To test the ability of mutant STAT3 proteins to form dimers with WT STAT3, HEK-293T cells were co-transfected with two plasmids - one containing Myc-tagged WT STAT3 and the other Flag-tagged WT STAT3 or STAT3 harbouring one of the mutations described above. This was performed to recapitulate the setting of heterozygous *STAT3* mutations in cells from AD-HIES patients. The cells were then stimulated with IL-6 and STAT3 multimers captured by co-immunoprecipitation using anti-Flag mAb. The relative levels of Flag and Myc tagged STAT3 were measured by Western blot as an indication of dimerization. The levels of dimer formation between WT STAT3 and those with mutations in the DNA BD or TA domain were comparable to those observed for WT/WT STAT3 proteins. However both SH2 domain mutants significantly impaired formation of STAT3 dimers, with the Y657N mutation having the greatest effect (Figure 4B, C).

STAT3 mutations impair nuclear translocation of phosphorylated STAT3

Translocation of STAT3 to the nucleus is crucial for its role as a transcription factor. STAT3 can translocate to the nucleus in both phosphorylated and unphosphorylated forms[29] however the phosphorylated form is required for canonical STAT3 activity in cytokine signaling.[28] To measure translocation of phosphorylated STAT3 from the cytoplasm to the nucleus we prepared nuclear and cytoplasmic extracts from IL-21-stimulated EBV-LCLs derived from healthy controls and AD-HIES patients and compared the levels of phosphorylated STAT3 in each fraction.

The levels of cytoplasmic pSTAT3 in EBV-LCLs from healthy controls were greatest after 15 minutes of stimulation with IL-21 and then declined at later times. The kinetics of phosphorylation of cytoplasmic STAT3, as well as the magnitude of this response, in EBV-

LCLs with DNA BD mutations were comparable to those from healthy controls (Figure 5A). However the Y657N SH2 and the L706M TA domain mutations significantly reduced the level of detectable pSTAT3 in the cytoplasm at the peak of the response to IL-21 (Figure 5A). This is consistent with our data that assessed total STAT3 phosphorylation in transfected HEK-293T cells, which found reductions for these SH2 and TA domain mutations but not those affecting the DNA BD (Figure 2). When we examined nuclear fractions, the appearance of pSTAT3 in normal EBV-LCLs followed similar kinetics as that for cytoplasmic pSTAT3 (Figure 5A, B). However, in contrast to the findings for cytoplasmic pSTAT3, two of the three DNA BD mutations (R382W, H437P) significantly retarded translocation of pSTAT3 from the cytoplasm to the nucleus (Figure 5B). Consistent with reductions in cytoplasmic pSTAT3 in EBV-LCLs with SH2 (Y657N) and TA (L706M) domain mutations there were also significant reductions in the levels of pSTAT3 in the nucleus of these cells (Figure 5B). Interestingly, the S465F DNA BD mutation had no effect on nuclear pSTAT3 translocation.

Mutations in the DNA BD, SH2 or TA domains all compromise binding of STAT3 to target sites in DNA and impair target gene induction

Once in the nucleus STAT3 binds specific target sites in DNA and regulates gene transcription.[6] The effect of different mutations in *STAT3* on its ability to bind DNA was tested using nuclear extracts from normal or STAT3 mutant EBV-LCLs that had been stimulated with IL-21 for different periods of time. WT STAT3 exhibited strong binding to a canonical target sequence that was maximal after 15-30 minutes of IL-21 stimulation (Figure 6A), consistent with the kinetics of STAT3 phosphorylation (Figure 5). Conversely all *STAT3* mutant proteins showed a clear and significant decrease in their ability to bind DNA (Figure 6A). The induction of the STAT3 target gene *SOCS3* in response to IL-21 was then assessed by qPCR. While IL-21 strongly induced *SOCS3* in normal EBV-LCLs, the levels of expression of *SOCS3* in all *STAT3* mutant EBV-LCLs were significantly reduced (Figure 6B). Thus, impaired DNA binding of mutant STAT3 proteins manifests as reduced gene transcription.

Discussion

Signaling through STAT3 is an important process required for the function of a wide range of biological systems. This is demonstrated by the embryonic lethality of germline *Stat3* gene-targeted mice[30] and the multi-systemic consequences, including immunodeficiency, of loss-of function mutations in *STAT3* that cause AD-HIES in humans.[1] However, little is known about how disease-causing mutations in *STAT3* specifically impair intracellular signaling downstream of STAT3-activating cytokines. While several studies have examined the effects of mutations in separate domains of STAT3 on signaling pathways[3,31,32], few studies have systematically compared the impact of a range of mutations that are located in different functional domains of STAT3. Furthermore, there are caveats to some of the studies that addressed this question. For instance, although He et al[32] examined *STAT3* with mutations in the DNA BD or SH2 domain, they transfected cell lines with a single WT or mutant *STAT3* rather than co-transfecting a WT and mutant cDNA.[32] Since AD-HIES is an autosomal dominant disease, to understand how *STAT3* mutations cause disease it is necessary to examine STAT3 function in model systems that reproduce the heterozygous nature of the mutation. By adopting such an approach, we have now been able to shed new light on the biochemical mechanisms underlying cellular dysfunction in AD-HIES due to *STAT3* mutations.

Previous studies showed that mutations in the SH2 domain, but not the DNA BD, of *STAT3* affect phosphorylation.[2,3,20,31] We found that SH2 domain mutations do not always perturb STAT3 phosphorylation, as revealed by intact phosphorylation of the Q644P mutant (Figure 2). This indicates that the diverse range of mutations that cause AD-HIES do not all impair STAT3 function at the same stage of signaling, even if the mutations are in the same domain (Figure 7). This also highlights that single domains have multiple roles in the biological function of STAT3 signaling. The defect in phosphorylation due to the SH2 domain mutant (Y657N) is most likely due to its inability to interact with the cytoplasmic region of receptors for STAT3-activating cytokines. In the absence of STAT3 docking to the cytokine receptor, the associated JAK's would not be able to interact with STAT3, therefore impairing phosphorylation. The mutation in the TA domain (L706M) also abolished STAT3 phosphorylation, despite intact recruitment to STAT3-activating cytokine receptors. This indicates that its lack of phosphorylation was due to a different mechanism. Since this mutation is in close proximity to the site of phosphorylation at Y705 it is possible that steric interference prevents it interacting with active JAKs, thereby impairing phosphoryation.

The ability of STAT3 with mutations in the DNA BD to form dimers with WT STAT3 was previously demonstrated by Minegishi et al.[3] Our findings that mutations in the DNA BD additional to those studied previously[3] do not impair dimer formation are consistent with these observations. In contrast to DNA BD domain mutations, mutations in the SH2 domain reduced dimer formation by ~40-50%, thus identifying these residues as critical for interactions between phosphorylated STAT3 dimers. Interestingly, a recent study suggested SH2 domain mutations (S611N, F621V) abolished STAT3 dimerization.[32] However, this study tested dimerization between mutant proteins, rather than mutant and WT STAT3, a situation that does not replicate the physiological setting of an autosomal dominant mutation.[32] Given our results, it is likely that the reductions in dimerization we observed may be due to the structural location of the mutations; when STAT3 forms dimers the two SH2 domain mutations we investigated would be located at the interface between the two STAT3 molecules (Figure 1).[10] Amino acid substitution in the SH2 mutants may result in changes in charge, which could affect the assembly or stability of STAT3 dimers.

Translocation of STAT3 to the nucleus is required for it to function as a transcription factor. Four of the five *STAT3* mutations that we studied resulted in a reduction in nuclear pSTAT3. For the SH2 and the TA domain mutations this reduction is most likely due to the upstream impairment in phosphorylation and dimer formation (Figures 2, 4). For the other two mutants this suggests an imbalance between nuclear import and export. Several nuclear export sequences have been identified in the DNA binding domain of STAT3.[33] It is possible that the reduction in nuclear pSTAT3 due to the R382 and H437 mutations results from increased nuclear export. This may be due to conformational changes providing greater access to the export sequences. Furthermore, other residues in the DNA BD (R414, R417) are required for nuclear localization with mutations at these sites resulting in impaired nuclear import after stimulation[34] and in greater nuclear retention of unphosphorylated STAT3.[35]

After gaining entry into the nucleus, binding to DNA is a crucial step in STAT3 signaling. We showed a reduction in DNA binding activity by all STAT3 mutants present in nuclear extracts from EBV-LCLs. As some of the *STAT3* mutations affected nuclear translocation, the observed reduction in DNA binding reflects not only an impaired ability of mutant STAT3 to bind its target sequences but also decreases in the amount of pSTAT3 present in the nucleus. Molecular modeling of the structural location of various *STAT3* mutations revealed that the DNA BD mutations are clustered at the interface between STAT3 and DNA (Figure 1). Furthermore, the R382 residue has been shown to be structurally important for the stability of the loops in the DNA BD.[10] Alongside the reduction in DNA binding, all of the mutations

resulted in a ~50% reduction in the induction of the *STAT3* target gene *SOCS3*. This indicates that the defect in DNA binding results in a functional impairment in the ability to induce gene transcription.

In conclusion our study gives insight into dysfunctional STAT3-dependent signaling in AD-HIES, and allowed for the dissection and identification of specific stages of STAT3 signaling affected by these disease-causing mutations. This not only highlights which aspects of STAT3 signaling are impacted by these mutations but also identifies residues and regions of domains in STAT3 that are critical for normal function. This gives insight not only for loss-of function of STAT3 in AD-HIES but could indicate critical residues or regions of STAT3 could be targeted in cases where STAT3 is overactive, such as in germline or somatic gain-of function mutations in *STAT3* resulting in multisystemic autoimmunity[36,37] or hematological malignancies, respectively.[38,39]

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Author Contributions

S.J.P. designed the research, performed experiments, analyzed and interpreted results, and wrote the manuscript; H.L. performed experiments, analyzed and interpreted results; E.K.D and S.G.T. designed the research, analyzed and interpreted results, and wrote the manuscript.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

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Figure Legends

Figure 1. Autosomal dominant hyper IgE syndrome-causing *STAT3* mutations. (A) Schematic representation of STAT3 protein, with the mutations studied here indicated by black lines. (B) Crystallographic structure of a STAT3 dimer bound to DNA (Protein Data Bank, 1BG1[10]). Sites of the mutations are represented by red spheres, phosphorylated tyrosine 705 (Y705) is indicated by black spheres.

Figure 2. Phosphorylation of WT and mutant STAT3 at tyrosine 705. HEK-293T cells were transfected with Flag-tagged WT *STAT3* or *STAT3* harbouring the following mutations: R382W, H437P, S465F, Q644P, Y657N or L706M. Cells were left untreated or stimulated with IL-6 for 30 mins. Phosphorylation of STAT3 was determined by fixing and permeabilising the cells and labeling with Abs specific for Flag and pSTAT3 (Y705). (A) Representative histograms of phosphorylated STAT3 induction after IL-6 stimulation. (B) Quantification of phosphorylated STAT3 induction, geometric MFI. (Two way ANOVA with Dunnett post-test, comparison between wild type IL-6 stimulated and the various mutants. ***p< 0.001. mean ± SEM, n=6)

Figure 3. Mutation at Y657N disrupts docking of STAT3 to the IL-21 receptor. Lysates from normal donors or AD-HIES patients EBV-LCLs (A), or HEK-293T transfected with Flag-tagged WT or mutant STAT3 (B) were generated. A peptide pull down was performed using magnetic beads coupled to STAT3 binding motifs (IL-21R 515-526) or a control sequence (IL-21R 395-406). Western blotting was performed against STAT3 or Flag. (One way ANOVA with Dunnett post-test. *p < 0.05. mean ± SEM, n=3-5)

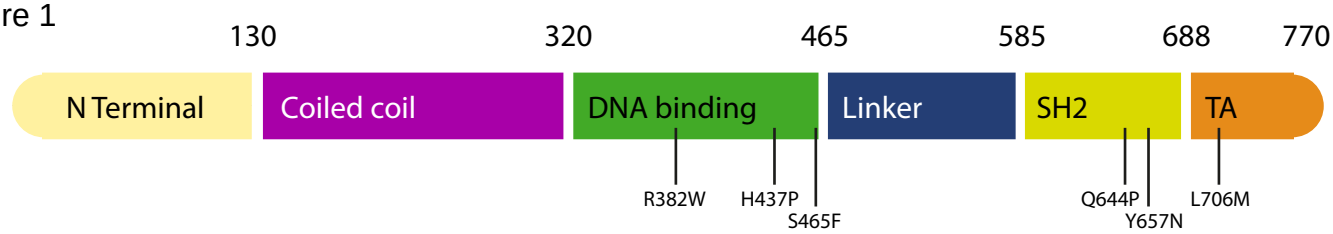
Figure 4. Mutations in the SH2 domain compromise STAT3 dimerisation. HEK-293T cells were co-transfected with plasmids containing Myc-tagged WT STAT3 and either Flag-tagged WT or mutant STAT3. After 48 hours, cells were harvested, rested for 2 hours and then stimulated with 10 ng/ml IL-6 for 30 minutes. Cell lysates were prepared and subjected to immunoprecipitation with anti-Flag Ab, and analysis by Western blotting for the presence of Myc-tagged WT or mutant STAT3. (A) Diagram of the gel lanes. (B) Input whole cell lysates immunoblotted for Myc, Flag or GAPDH (left) and immunopreciptiation of lysates with anti-Flag beads, immunoblotted for Myc and Flag (right). (C) Quantification of the ratio of WT to mutant STAT3, normalized to the WT:mutant ratio of the input. (One way ANOVA with Dunnett post-test, comparison between wild type and the various mutants. **p < 0.01, *p < 0.05. mean ± SEM, n=5)

Figure 5. Impaired nuclear translocation of mutant STAT3 proteins. EBV-LCLs from normal donors or AD-HIES patients were stimulated with IL-21 for 0, 15, 30 or 60 minutes. Cytoplasmic (A) and nuclear (B) fractions were isolated and the abundance of phosphorylated STAT3 determined by immunoblotting for pY705 STAT3, Histone H3 and GAPDH. (Two way ANOVA with Dunnett post-test, comparison between 15 minute time points, ***p<0.001, **p< 0.01, *p< 0.05. mean ± SEM, n=4). Quantification was assessed by determining the ratio of intensity of mutant pSTAT3 against the healthy control stimulated with IL-21 for 15 minutes.

Figure 6. Mutations in all functional domains of STAT3 impair binding to DNA and target gene induction. (A) EBV-LCLs from normal donors or AD-HIES patients were stimulated with IL-21 for 0, 15, 30 or 60 minutes. Nuclear extracts were prepared and binding of WT and mutant STAT3 to consensus DNA binding sites determined using the TransAM kit (ActiveMotif). Values were normalized to normal controls at the 15 minute time point. (Two way ANOVA with Dunnett post-test, comparison between 15 minute time points, ***p< 0.001, **p< 0.01, *p< 0.05. mean ± SEM , n=3-4). (B) EBV-LCLs from normal donors or AD-HIES patients were stimulated with IL-21 or media only for 180 minutes. *SOCS3* transcripts were determined by qPCR. (Two way ANOVA with Dunnett post-test, comparison between IL-21 stimulated, **P < 0.01, *P < 0.05. mean ± SEM , n=4)

Figure 7. Summary of the stage of signaling disrupted by various AD-HIES *STAT3* mutations. A diagram showing the stages of signaling affected by each *STAT3* mutation. IL-21 bound to IL-21R (Protein Data Bank, 3TXG[40]) used as an example of a STAT3 signaling cytokine receptor. Phosphorylation of tyrosine 705 (pY705) is represented in the STAT3 structure with black spheres (Protein Data Bank, 1BG1[10]). Orange box surrounding the mutation indicates TA domain, yellow box indicates SH2 and green box indicates DNA binding domain.

Figure 1


130
320
465
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770
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DNA binding
Linker
SH2
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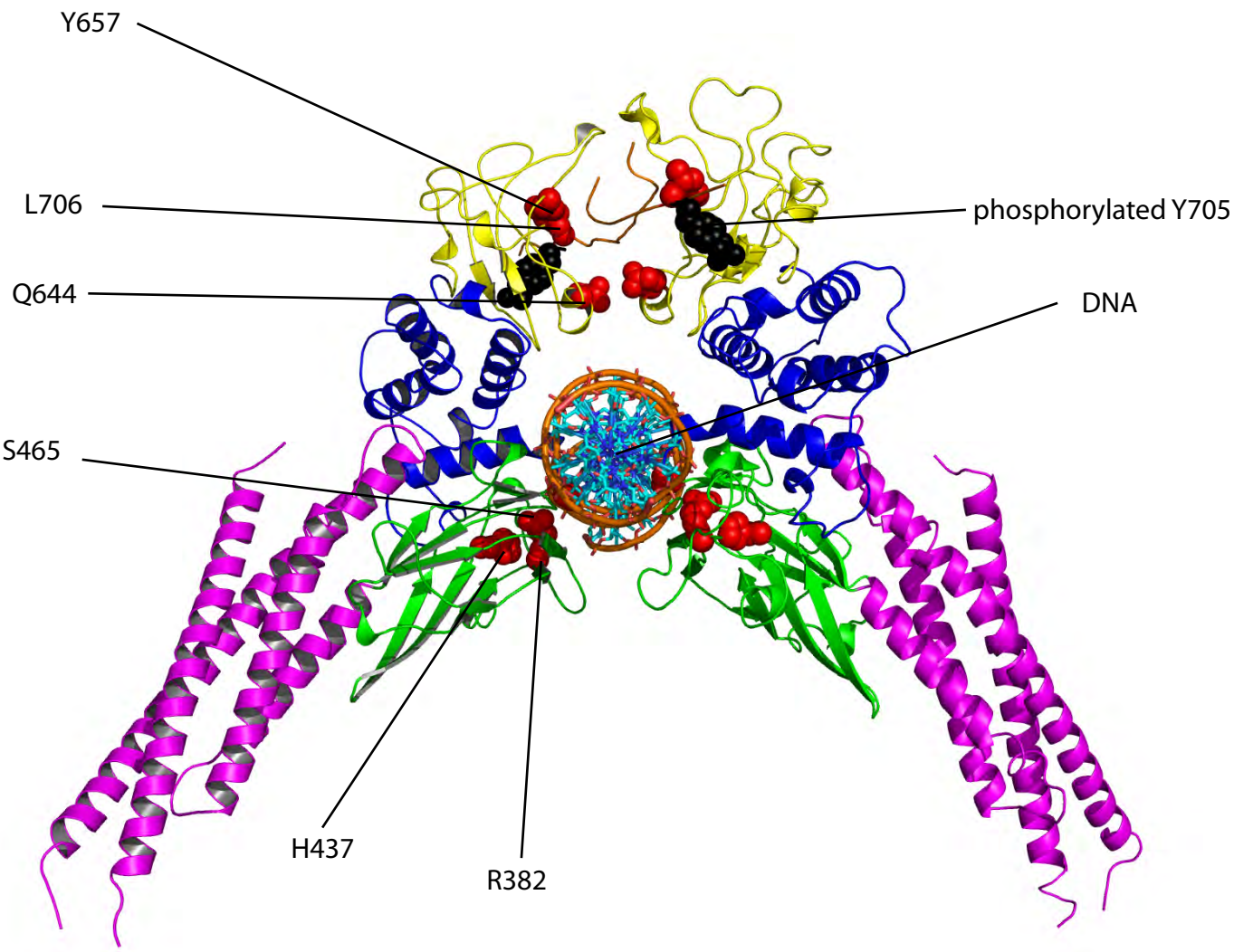
L706M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S465
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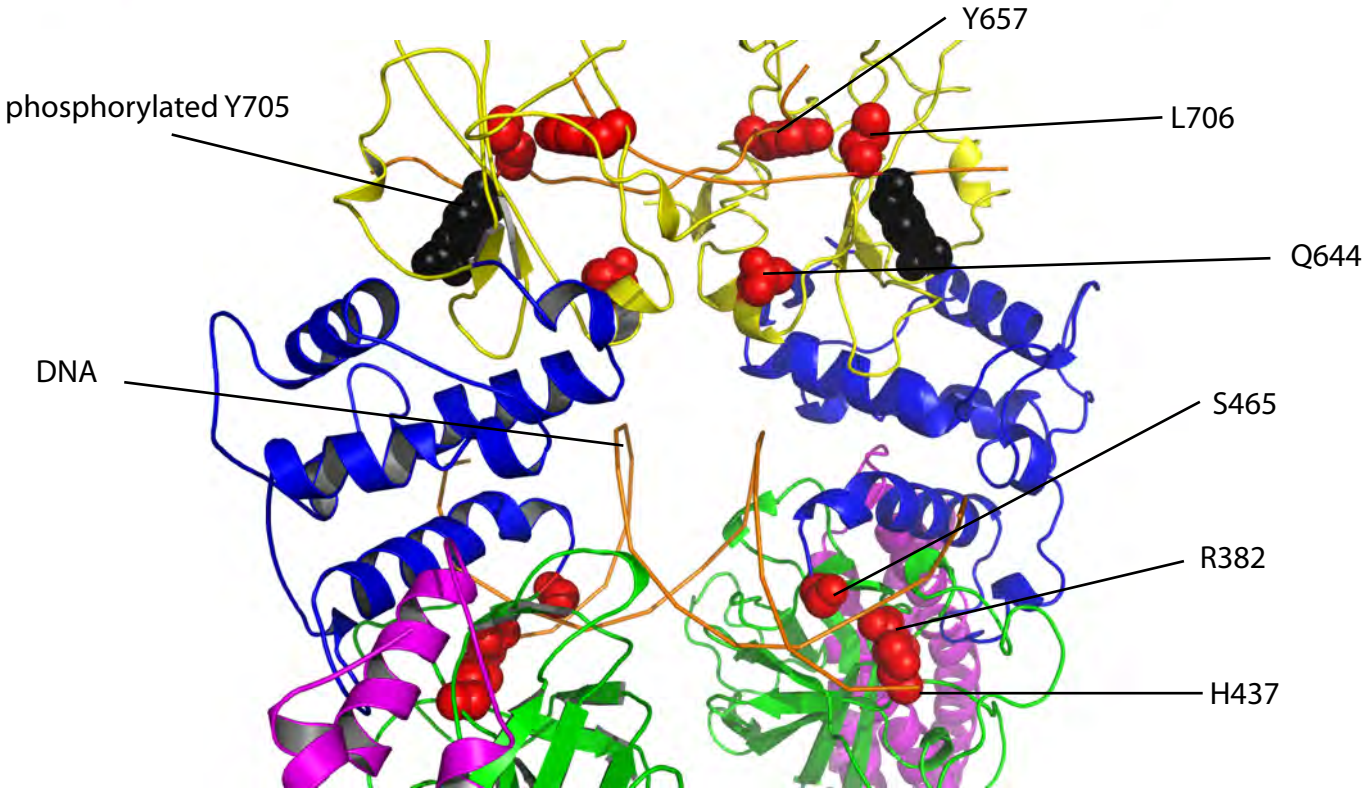
R382
phosphorylated Y705
DNA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H437

Figure 2

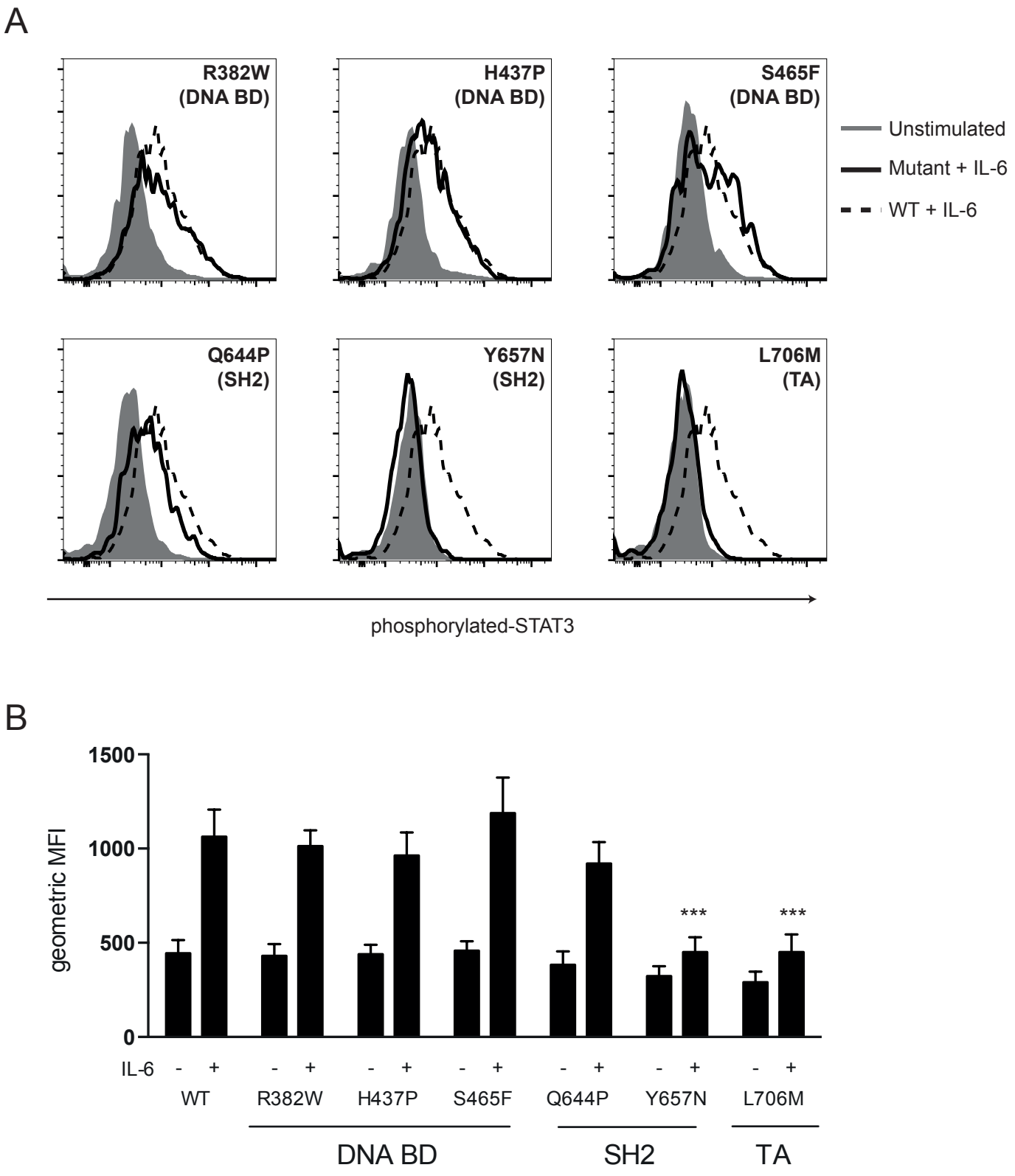
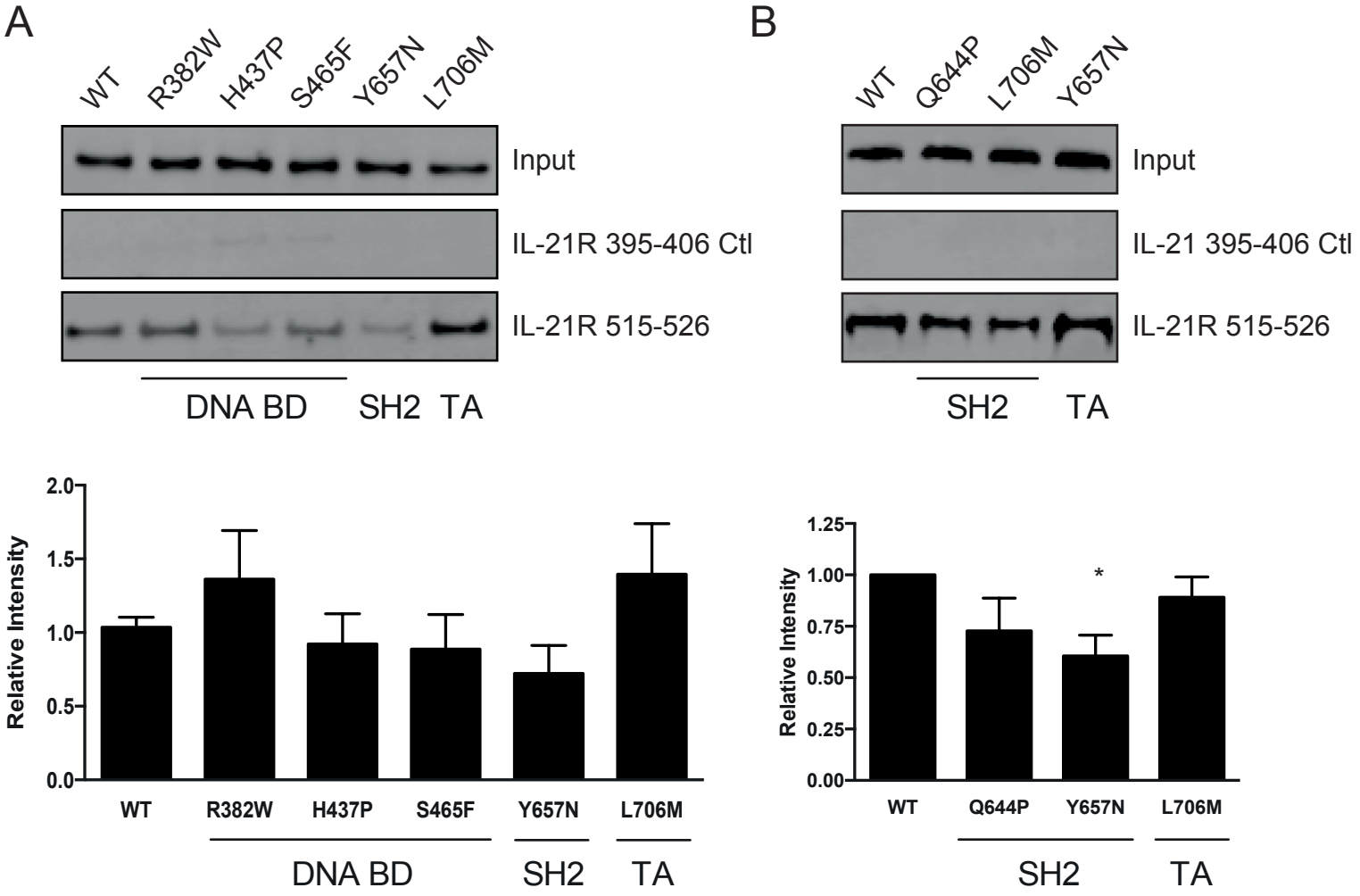

A
R382W
(DNA BD)
H437P
(DNA BD)
S465F
(DNA BD)
Q644P
(SH2)
Y657N
(SH2)
L706M
(TA)
Unstimulated
Mutant + IL-6
WT + IL-6
phosphorylated-STAT3
B
geometric MFI
1500
1000
500
0
***
***
IL-6 - + - + - + - + - + - + - +
WT
R382W
H437P
S465F
Q644P
Y657N
L706M
DNA BD
SH2
TA

Figure 3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WT
R382W
H437P
S465F
Y657N
L706M
Input
IL-21R 395-406 Ctl
IL-21R 515-526
DNA BD
SH2
TA
B
WT
Q644P
L706M
Y657N
Input
IL-21 395-406 Ctl
IL-21R 515-526
SH2
TA
Relative Intensity
2.0
1.5
1.0
0.5
0.0
WT
R382W
H437P
S465F
Y657N
L706M
DNA BD
SH2
TA
Relative Intensity
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1.00
0.75
0.50
0.25
0.00
*
WT
Q644P
Y657N
L706M
SH2
TA

Figure 4

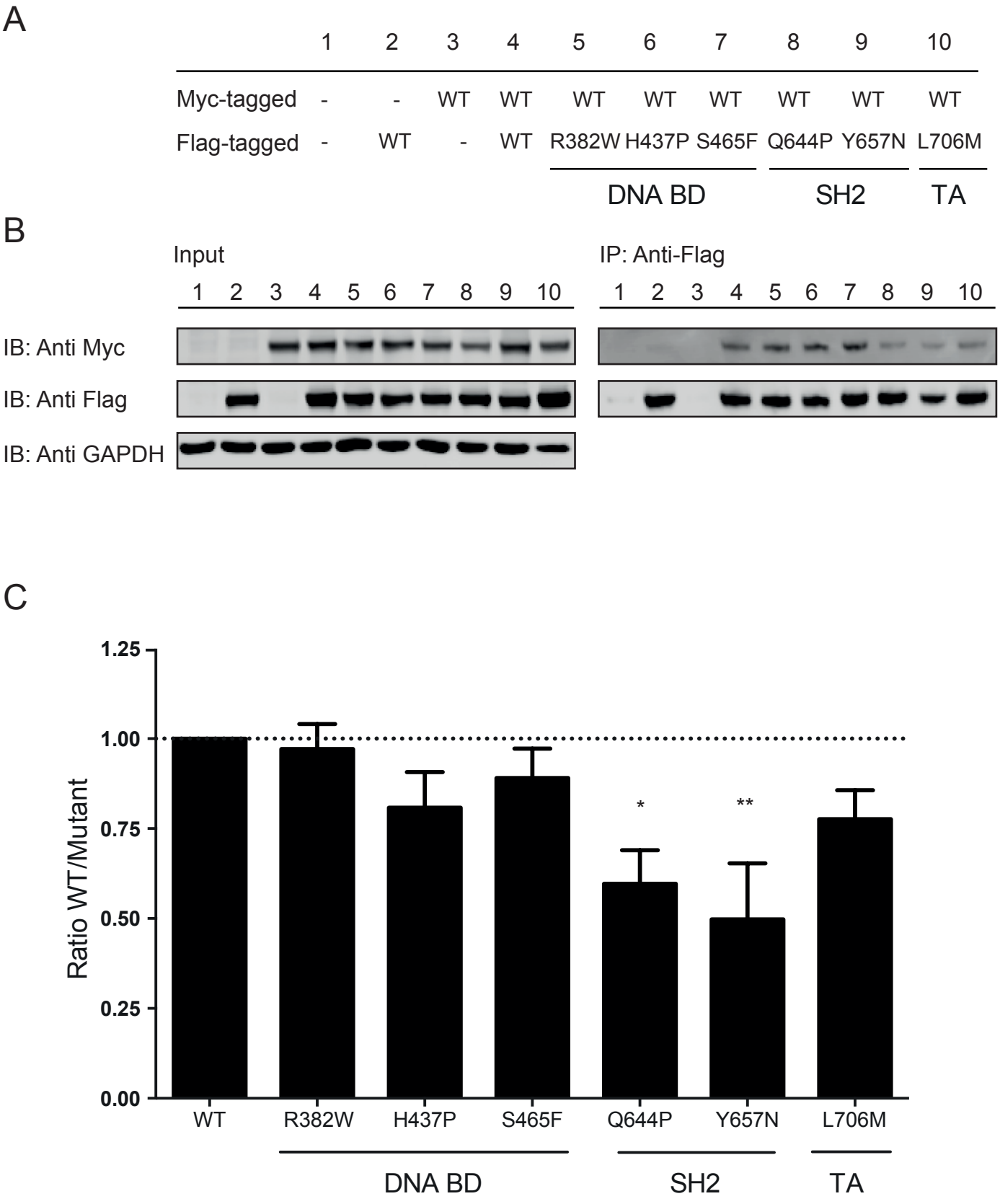
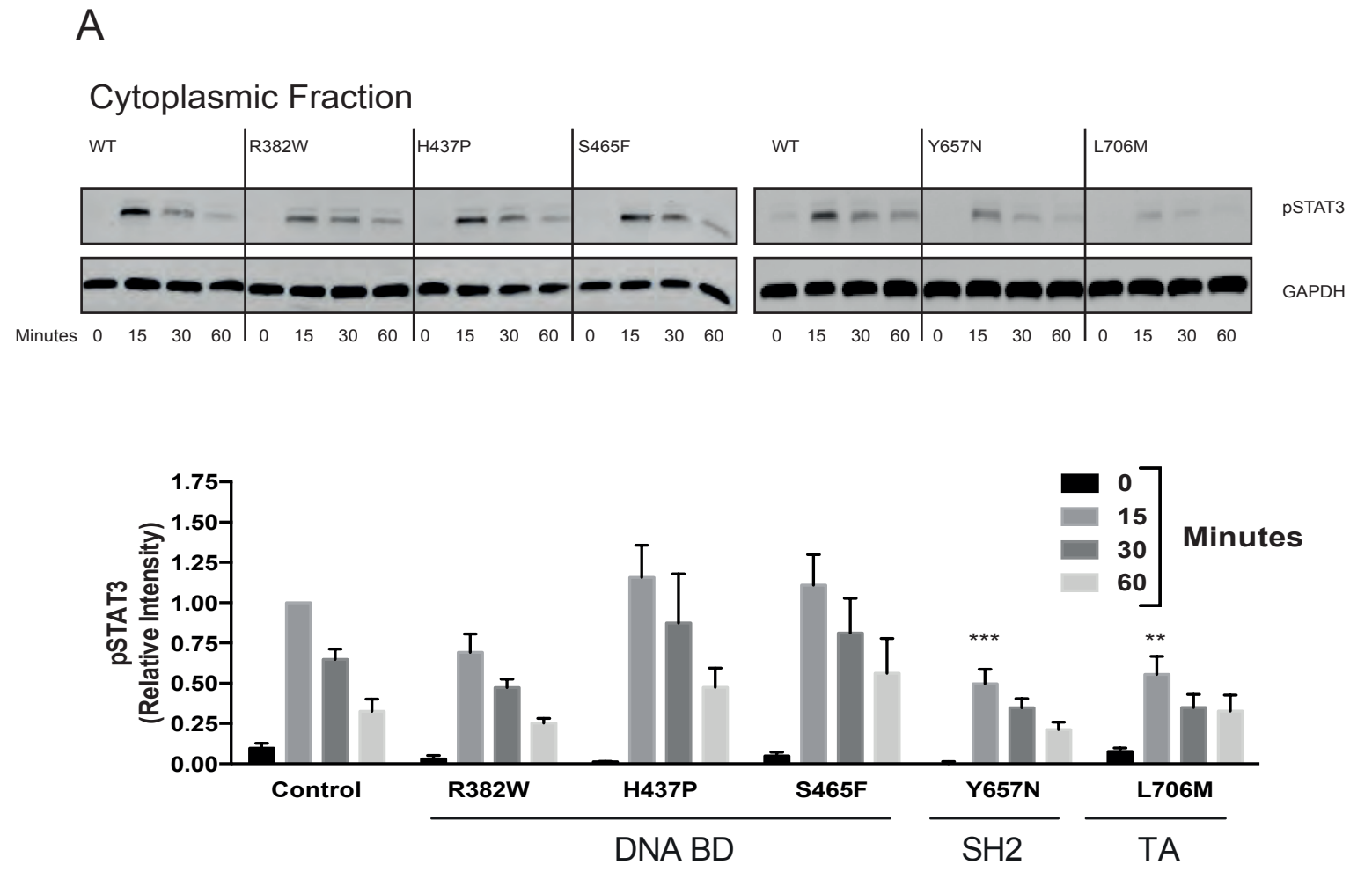

A
1 2 3 4 5 6 7 8 9 10
Myc-tagged - - WT WT WT WT WT WT WT WT
Flag-tagged - WT - WT R382W H437P S465F Q644P Y657N L706M
DNA BD
SH2
TA
B
Input
IP: Anti-Flag
1 2 3 4 5 6 7 8 9 10
IB: Anti Myc
IB: Anti Flag
IB: Anti GAPDH
C
Ratio WT/Mutant
1.25
1.00
0.75
0.50
0.25
0.00
*
**
WT
R382W
H437P
S465F
Q644P
Y657N
L706M
DNA BD
SH2
TA

Figure 5


A
Cytoplasmic Fraction
WT
R382W
H437P
S465F
WT
Y657N
L706M
pSTAT3
GAPDH
Minutes 0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60
pSTAT3 (Relative Intensity)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
15
30
60
Minutes
***
**
Control
R382W
H437P
S465F
Y657N
L706M
DNA BD
SH2
TA

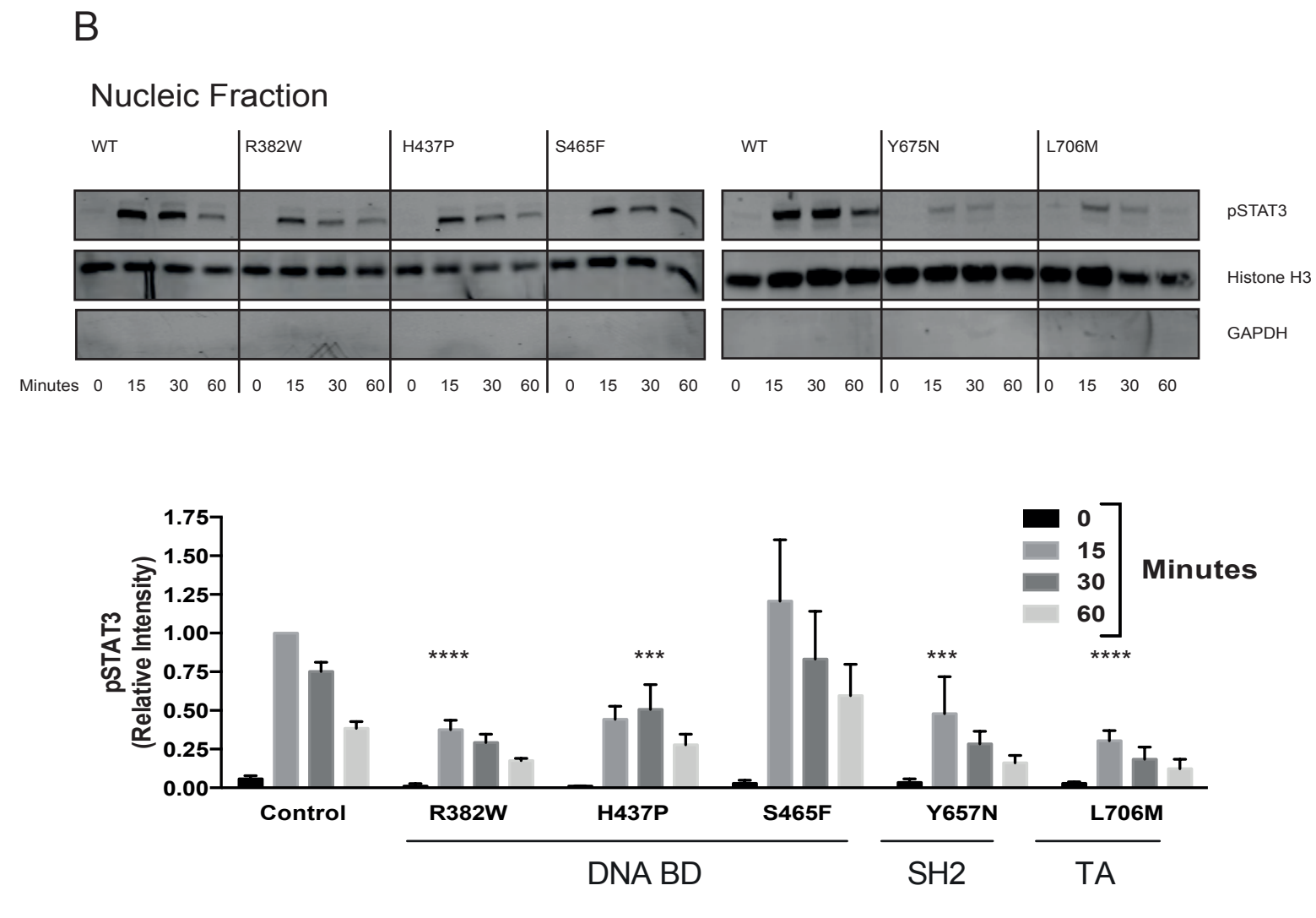
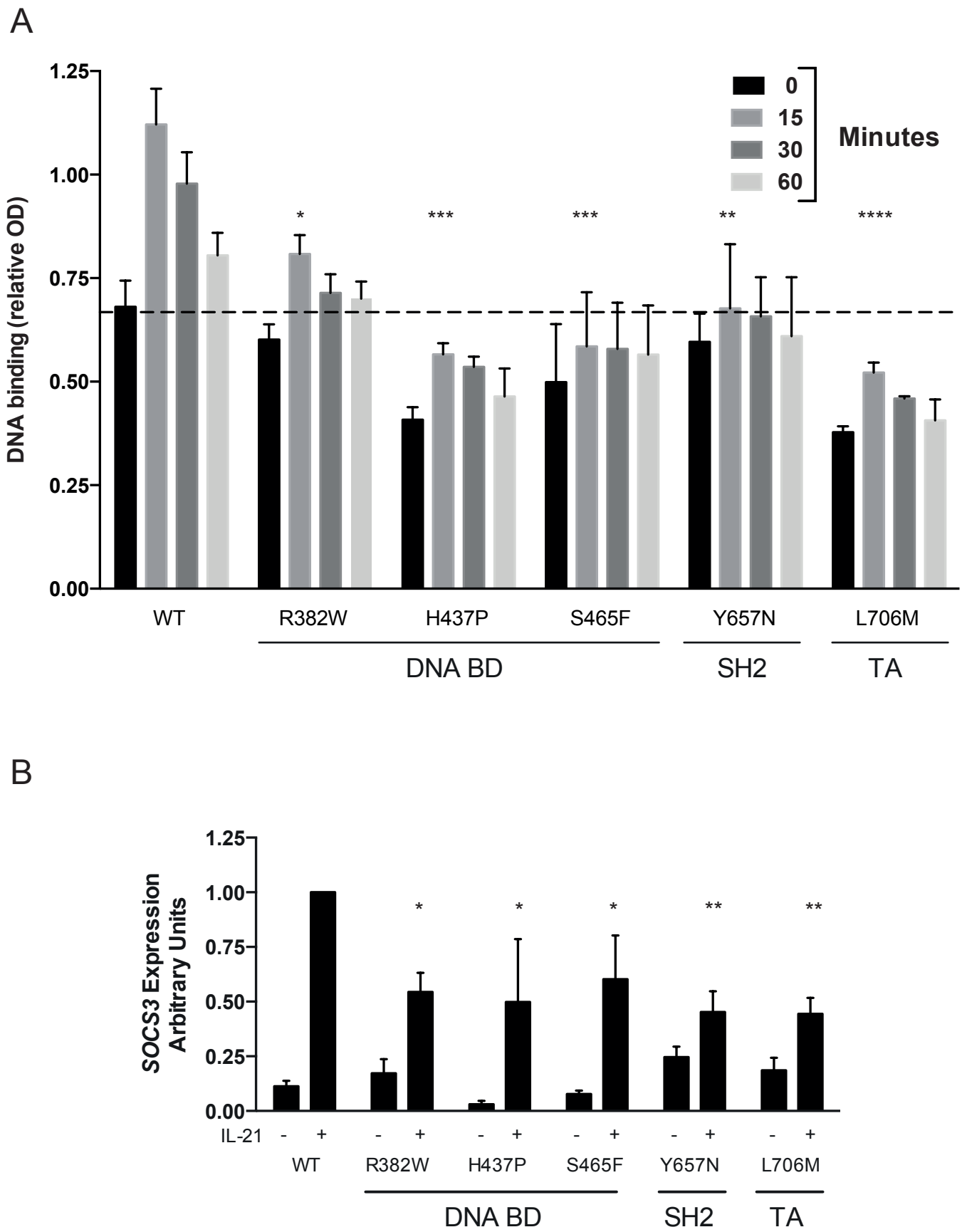

B
Nucleic Fraction
WT
R382W
H437P
S465F
WT
Y675N
L706M
pSTAT3
Histone H3
GAPDH
Minutes 0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60 0 15 30 60
pSTAT3 (Relative Intensity)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
15
30
60
Minutes
****
***
***
****
Control
R382W
H437P
S465F
Y657N
L706M
DNA BD
SH2
TA

Figure 6


A
DNA binding (relative OD)
1.25
1.00
0.75
0.50
0.25
0.00
0
15
30
60
Minutes
*
***
***
**
****
WT
R382W
H437P
S465F
Y657N
L706M
DNA BD
SH2
TA
B
SOCS3 Expression Arbitrary Units
1.25
1.00
0.75
0.50
0.25
0.00
*
*
*
**
**
IL-21
- + - + - + - + - + - +
WT
R382W
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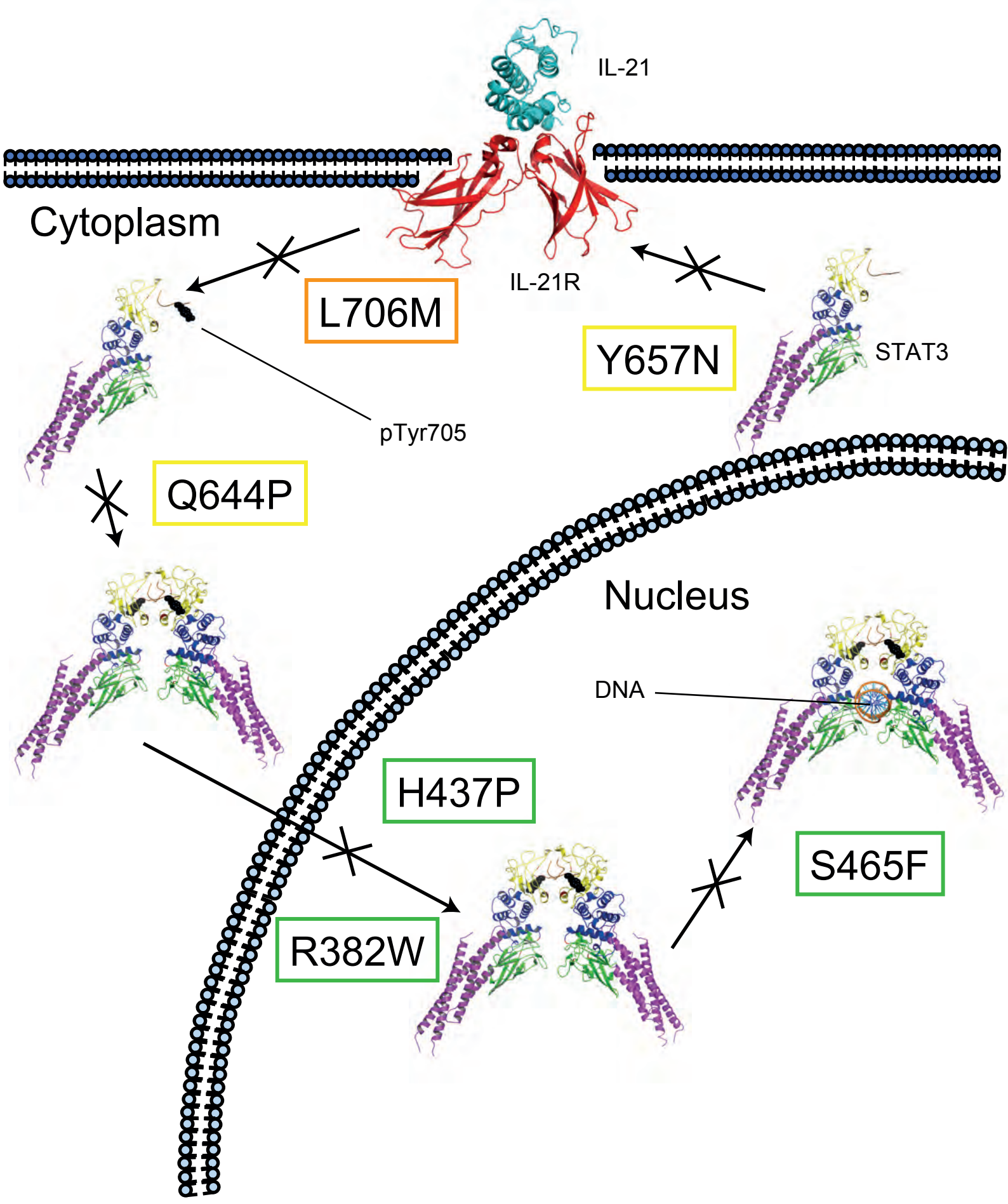
S465F
Y657N
L706M
DNA BD
SH2
TA

Figure 7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