A novel intramolecular negative regulation of mouse Jak3 activity by tyrosine 820

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Abstract
Jak3, a member of the Janus kinase family, is essential for the cytokine receptor common gamma (γ) chain-mediated signaling. During activation of Jak3, tyrosine residues are phosphorylated and potentially regulate its kinase activity. We identified a novel tyrosine phosphorylation site within mouse Jak3, Y820, which is conserved in human Jak3, Y824. IL-2-induced tyrosine phosphorylation of Jak3 Y824 in human T cell line HuT78 cells was detected by using a phosphospecific, pY824, antibody. Mutation of mouse Jak3 Y820 to alanine (Y820A) showed increased autophosphorylation of Jak3 and enhanced STAT5 tyrosine phosphorylation and transcriptional activation. Stably expressed Jak3 Y820A in F7 cells, an IL-2 responsive mouse pro-B cell line Ba/F3, exhibited enhanced IL-2-dependent cell growth. Mechanistic studies demonstrated that interaction between Jak3 and STAT5 increased in Jak3 Y820A compared to Jak3 WT. These data suggest that Jak3 Y820 plays a role in negative regulation of Jak3-mediated STAT5 signaling cascade upon IL-2-stimulation. We speculate that this occurs through an interaction promoted by the tyrosine phosphorylated Y820 or a conformational change by Y820 mutation with either the STAT directly or with the recruitment of molecules such as phosphatases via a SH2 interaction. Additional studies will focus on these interactions as Jak3 plays a crucial role in disease and health.

Introduction
Tyrosine kinases play crucial roles in regulating cellular signal transductions and are targeted as a therapeutic drug discovery for several diseases(1). In cytokine signaling pathways, the Janus family tyrosine kinase (Jak) constitutively associates with the cytokine receptors. Upon activation by cytokine stimulation the kinase is activated and essential for conveying cytokine signals to downstream molecules, such as the signal transducers and activators of transcriptions (STATs)(2,3). The Jak/STAT signal pathway has crucial roles in biological processes, such as embryogenesis, organogenesis, haemopoiesis, innate and adaptive immunity, inflammatory response, and others(2,4-7). The Jak Family members, Jak1, Jak2, Jak3 and Tyk2, contain a conserved tyrosine kinase domain (JH1) and six-homology domains (JH2-JH7). Jak1, Jak2 and Tyk2 are ubiquitously expressed in mammalian cells and tissues, whereas Jak3 is predominantly expressed in hematopoietic and immune cells(8,9). Upon cytokine-binding to the receptor, the Jaks are activated through reciprocal interaction and trans-phosphorylation of the JH1 domain. The pseudo-kinase domain (JH2) has an important role for both positive and negative regulation of kinase domain(10) which is not fully understood. The cytokine receptors are tyrosine phosphorylated by Jaks, providing binding sites for Src homology 2 (SH2) domain-containing proteins ie., STATs. Subsequently, recruited STAT proteins are tyrosine phosphorylated by Jaks, then STATs translocate to the nucleus to induce cytokine responsive target gene transcription(11,12).

Jak3 specifically associates with the common cytokine receptor gamma chain (γc), a common subunit of the interleukin-2 receptor family (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21(13,14). Defect in Jak3 or γc results in severe immunodeficiency (SCID) in human and rodent(5,14-16). Jak3 mutations can also result a number of other pleiotrophic phenotypes, constitutive STAT phosphorylation has been found in various leukemia patients(10). Several tyrosine phosphorylation sites in Jak3 have been identified and examined for their functions in cytokine signaling. Two adjacent tyrosines Y980 and Y981 in human Jak3 kinase domain are phosphorylated, and positively or negatively regulate catalytic activity(17). In addition, both Y904 and Y939 in human Jak3 are phosphorylated in response to IL-2, conserved substitution of these residues attenuate its catalytic activity, suggesting phosphorylation of these tyrosines positively regulates Jak3(18). Tyrosine 785 of human Jak3 is also known as a site for autophosphorylation by IL-2-treatment, that is a binding target for the SH2 domain-containing adaptor protein, SH2-Bβ (19). The ATP binding site is required for Jak3 kinase activity, therefore replacement or mutation of the ATP coordinating lysine (K855A in human, K851A in mouse) results in the kinase-dead Jak3(17,20).
We identified a novel phosphorylation site Y820 of mouse Jak3, which corresponding to the human Jak3 Y824. Amino acid substitution of this tyrosine of Jak3 to alanine resulted in elevated Jak3 activity to associate with STAT5 and to induce STAT5 phosphorylation. Thus, phosphorylation of Jak3 Y820 or a conformational change of Jak3 caused by Y820A mutation in mouse as well as Jak3 Y824 in human leads to down-regulate Jak3 activity. We here propose a new intramolecular regulation of Jak3 activity.

Materials and methods

in vitro phosphorylation

Sf9 cells infected with indicated Jak3 virus or HEK293T cells transfected with Myc-Tagged Jak3 were harvested and lysed (50 mM Tris–HCl,pH 7.4, 0.15 M NaCl, containing 1% Triton X-100, 10% glycerol,1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonylfluoride, and 10 ng/ml each of aprotinin, pepstatin, and leupeptin). Jak3 proteins were immunoprecipitated from cell lysates using Jak3 antiserum or anti-Myc antibody and protein A–Sepharose (Sigma). In vitro kinase reactions were performed as described(21,22), briefly immune complexes of Jak2 were washed in kinase buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 0.1 mM sodium orthovanadate, 5 mM $MnCl_2$, and 5 mM $MgCl_2$) and mixed with 5 μCi/μl [γ-^{32}P] ATP at 25 ℃ for 30 min. The products of these reactions were separated by SDS-PAGE.

Phosphopeptide mapping

Analysis of phosphopeptide mapping was performed with some modification as previously described(23). Briefly, ^{32}P in vitro kinase labeled Jak2 proteins were resolved by SDS-PAGE, visualized by autoradiography, and in-gel digestion was performed with TPCK-treated trypsin (Sigma) and lysilendopeptidase (Lys-C) (Wako). The addition of Lys-C in the digestion decreased the number of ^{32}P-labeled spots on the map in preliminary experiments by increasing complete digestion with trypsin. The proteolytic peptide mixture was separated in two dimensions on thin layer cellulose (2DTLC) plates either in the presence or absence of unlabeled synthetic peptides as noted. ^{32}P-labeled peptides were visualized by autoradiography and unlabeled peptide position was determined by ninhydrin staining.

Reagents, expression plasmids and antibodies

Recombinant human IL-2 Sigma-Aldrich (St. Louis, MO, USA) Expression vectors for FLAG-tagged STAT5a and STAT5b(24), STAT5a Y694F (25)as well as plasmids for STAT5-LUC(26), were kindly provided by Dr. D. Wang (The Blood Res. Inst., Milwaukee, WI), Dr. J. N. Ihle (St. Jude CRH, Memphis, TN) and Dr. H. Wakao (Helix Res. Inst., Chiba, Japan), respectively. Expression vectors for Myc-tagged Jak3 mutants were generated by PCR methods and sequenced (primer sequences are available upon request). Anti-pSTAT5a (phospho STAT5a Y694) and anti-Myc (9B11) antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). Anti-pJak3 (phosphoJak3 Y980), anti-Jak3 and anti-STAT5a/b antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin, anti-FLAG, anti-Myc (9E10) and anti-phosphotyrosine (PY20) antibodies were from Sigma-Aldrich (St. Louis, MO, USA). The anti-phospho Jak3 Tyr820 (anti-pJak3 Y820) rabbit polyclonal antibody was generated by immunizing with the peptide EERHLKpYISLLGKG, corresponding to residues 814 to 827 of murine Jak3, conjugated to keyhole limpet hemocyanin and boosted three times at monthly intervals, and immune serum was obtained.

Cell culture, transfection and luciferase assays

Cell culture, transfection, luciferase assays and cell proliferation assays F7, a Ba/F3-derived transformant clone expressing the wild-type human IL-2R β was kindly provided by Dr. T. Taniguchi (Univ. of Tokyo)(27). F7 was maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), G418 (0.5 mg/ml) and 10% conditioned medium from WEHI-3B cells as a source of IL-3. A stable F7 transformant expressing Myc-Jak3 WT and Y820A were established as described previously (17) and maintained in the above medium in the presence of Hygromycin B (0.2mg/ml). Human T cell lymphoma, HuT78 were maintained in RPMI1640 medium supplemented with 10% FBS. Human embryonic kidney carcinoma cell line 293T was maintained in DMEM containing 10% FBS and transfected by the standard calcium precipitation protocol(28). Luciferase assay was performed as described(29). At 36 h after transfection, the cells were lysed in 50 μl of Reporter Lysis Buffer (Promega, Madison, WI) and assayed for luciferase activities according to the manufacturer's instructions. Luciferase activities were normalized to the β-galactosidase activities. Three or more independent experiments were carried out for each assay.

Cell proliferation assay

Cell proliferation was determined by Cell Counting Kit-8 (Wako Chemicals, Tokyo, Japan) according to the manufacturer's instructions. F7 ($5x10^3$/well) cells were cultured in 96-well plate with the increasing amounts

of IL-2 or WEHI-3B supernatant. The cells were cultured for 60 hrs and then were pulsed for 1 h with WST-8. The absorbance was measured at a test wavelength of 450 nm (OD450) and a reference wavelength of 595 nm (OD595) using a microplate reader (Bio-Rad Laboratories).

Immunoprecipitation and immunoblotting

The immunoprecipitation and immunoblotting assays were performed as described previously(29). Briefly, cells were harvested and lysed in a lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 20,000 x g for 20 min at 4°C. Antibody and protein G-sepharose mixture were added to lysates and incubated for 2h at 4°C with gentle rotation. The beads were washed three times and the immune complexes were then resolved by SDS-PAGE. After transfer, the PVDF membranes (PerkinElmer; Boston, MA) were incubated in the skimmed milk blocking buffer for 1 h at RT and immunoblotted with the appropriate primary antibodies. Following primary antibody incubation, secondary antibodies were applied for 1 h at RT and immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA).

Statistics

Statistical comparisons included one-way ANOVA and Student's t test as specified in the Figure legends using Excel and Prism software. Statistical significance was set at $P < 0.05$. All data are mean ± S.E.M. No statistical methods were used to calculate sample size estimates.

Results

Jak3 synthetic peptide phosphorylation, tryptic digestion, and 2D-TLC resolution

Baculorovirus produced Jak3 in vitro phosphorylation system will be used to identify the autophosphorylation sites, similar to previous work on Jak2(21). The mouse Jak3 JH1 with linker region, named D4, was cloned and expressed in Sf9 cells (Fig. 1A and B). Jak3 D4 immunocomplexes were subjected to in vitro kinase assay in the presence of ^{32}P-ATP, resolved on SDS-PAGE, excised, digested with trypsin and Lys-C, and resolved by 2D-TLC. Eight spots with incorporated with ^{32}P were observed. These spots were assigned to synthetic tyrosine-phosphorylated peptides in Jak3 D4 region (Fig. 2A) by their identical migration with the ^{32}P tryptic peptides. No unassigned peptides were observed, supporting the function of JH1 as being exclusively a tyrosine kinase. Resolution on 2D-TLC allowed the assignment of each peptide to its corresponding phosphopeptide from in vitro phosphorylated Jak3 D4 (Fig. 2B). According to the 2D-TLC mapping, 4 out of 9 peptides were transphosphorylated at known tyrosine phosphorylation sites, Y780, Y934, and Y975/976. The unique spot 6 is faint but reproducible (Fig. 2A), and the respective peptide gave a strong spot (Fig. 2B), although the spot 6 may not be fully phosphorylated. Thus, the spot 6 corresponding to tyrosine at 820 was transphosphorylated in vitro kinase reaction, suggesting that Y820 is a novel target for Jak3 autophosphorylation. Some additional spots in 2D-TLC mapping were observed reproducibly, and they seem to be due to partial digestion or aggregation.

Human Jak3 Tyr824 is phosphorylated on IL-2 stimulation in HuT78 cells

To confirm and analyze the phosphorylation of Y820 residue on Jak3 a rabbit sera was generated utilizing the phospho-Jak3 Y820 peptide. The peptide sequence is conserved between mouse and human Jak3 (Supplementary Figure 1). To confirm the specificity of the anti-phospho-Jak3 Y820 peptide sera, IL-2 stimulated human T cell lymphoma cell line HuT78(30) were probed for phosphorylated Jak3. Following the IL2 activation the phosphorylation was examined with anti-PY, pY820, and pY980 antibodies (Fig. 3A). Tyrosine phosphorylation was evident with all antibodies confirming the Y820 is indeed phosphorylated in response to Jak3 activation.

Mouse Jak3 Tyr820 is autophosphorylated in HEK293T cells

To examine the role of Jak3 Y820 a series of Myc-tagged expression plasmid for mouse Jak3 WT were constructed. Jak3 WT, Y820A, Y820D, Y820F or K851A, a kinase-dead construct was transfected in HEK293T cells. The cell lysates were then immunoprecipitated with anti-Myc antibody and immunoblotted

with anti-PY, pY980, pY820, or Myc antibody (Fig. 3B). Auto-tyrosine phosphorylation of Jak3 was observed in Jak3 WT and Y820A, but not in Jak3 K851A transfected cells. Comparing the phosphorylation signals with anti-pTyr antibodies and pY980 residue, it is observed that Jak3 Y820A appears to have more tyrosine phosphorylation than Jak3 WT. While, Jak3 Y820D showed significantly decreased tyrosine phosphorylation compared with Jak3 WT, and Y820F looks slightly increased phosphorylation but not significant. Phosphorylation of Jak3 Y820 was diminished in Jak3 Y820A, Y820D and Y820F while Y820 phosphorylation was detected in Jak3 WT and K851A. These results suggest that Y820 of mouse Jak3 is an autophosphorylation site, and phosphorylation of the activation domain of Jak3, Y980, is increased in Jak3 Y820A mutant, perhaps Y820 governs the signaling cascade of IL-2 activation.

Jak3 Y820A mutation enhances phosphorylation and activation of STAT5 in HEK293T cells

STAT5 is known as the major substrate of Jak3, and phosphorylation of STAT5 is essential for its nuclear translocation and transcriptional activation(16) of cytokine regulation. Therefore, we examined the phosphorylation of STAT5a in Jak3 WT, Y820A, Y820D, Y820F or K851A transfected HEK293T cells. STAT5a is phosphorylated by Jak3 WT, and the extent of phosphorylation was significantly enhanced when Jak3 Y820A was expressed (Fig. 4A and 4B), phosphorylation of STAT5a was not detected when Jak3 K851A was expressed. Expression of Jak3 Y820D caused decreased phosphorylation of STAT5a, and Jak3 Y820F has similar extent of STAT5a phosphorylation compared to Jak3 WT. STAT5a transcriptional activity was measured by reporter gene assay using STAT5-responsive element DNA-conjugated luciferase gene (31). STAT5 reporter gene induction was observed in transiently expressed Jak3 WT in HEK293T cells. Interestingly, Jak3 Y820A-transfected cells showed significantly higher reporter gene induction compared to Jak3 WT-transfected cells, while Jak3 Y820D-transfection caused lower reporter induction than WT. The STAT5 transcriptional activation was comparable in Jak3 WT and Y820F transfected cells and K851A-transfection failed to induce STAT5 reporter gene expression (Fig. 4C). Similar amounts of the transient ectopic Jak3 expression were confirmed by western blotting (Fig. 4D). STAT5a isoform STAT5b was also tested and showed increased phosphorylation and enhanced transcriptional activation when Jak3 Y820A was expressed (Supplementary Figure 2A and B). Thus, Jak3 Y820A mutant positively regulates STAT5 signaling or in contrast the phosphorylation of Y820 promotes a reduction in STAT5 phosphorylation.

Jak3 Y820A enhances phosphorylation of STAT5 in response to IL-2 and increases IL-2-dependent cell growth in F7 cells

Because over expression of Jak3 Y820A augmented STAT5 activation in HEK293T cells, the role of Jak3 Y820A in response to IL-2-stimulation was examined. To evaluate the Jak3 functions in IL-2 signaling, we employed a Ba/F3-derived transformant, F7 cell that is IL-3 dependent with a stable transfected wild-type human IL-2R β . Jak3 constructs representing WT and Y820A were then transfected to examine IL-2 response. IL-2 stimulation showed phosphorylation of endogenous STAT5 in all stable cell lines F7/Jak3 WT and F7/Jak3 Y820A (Fig. 5A). Phosphorylation of STAT5 at 15 and 30 min after IL-2 stimulation was enhanced in Jak3 Y820A-expressing F7 cells compared to Jak3 WT-expressing F7 cells. Similarly, the ratio of phosphorylation of STAT5 (pSTAT5/STAT5) was significantly increased in F7/Jak3 Y820A than F7/Jak3 WT at 15 and 30 min after IL-2-stimulation (Fig. 5B).

While IL-3 is necessary for Ba/F3 cell proliferation, IL-2R β expressed F7 is able to propagate in an IL-2/STAT5 dependent manner even in the absence of IL-3(27). To confirm the functional effect of Jak3 Y820A mutant on the IL-2 signaling cascade, we performed IL-2 dependent cell proliferation assay using the series of F7 stable cell lines. Consistent with the observation of the STAT5 phosphorylation, IL-2-dose dependent cell growth was significantly increased in F7/Jak3 Y820A cells compared to F7/Jak3 WT cells (Fig. 5C). On the other hand, IL-3 dependent cell growth was comparable among the stable F7 cell lines (Fig. 5D). Together, Jak3 Y820A mutation positively regulates downstream STAT5 signaling in response to IL-2-stimulation in F7 cells and correlates with enhanced proliferation.

Interaction between STAT5 and Jak3 is increased in F7/Jak3 Y820A cells

To investigate the mechanism of how Jak3 Y820A enhances STAT5 signaling, we examined the autophosphorylation abilities of Jak3 WT, Y820A and K851A by in vitro kinase assay (Figure 6A and B). HEK293T cells were transfected with Myc-Jak3 WT, Y820A or K851A, then lysed and immunoprecipitated

with Myc-antibody. The immunoprecipitates were subjected to in vitro kinase assay described in experimental procedures. Although autophosphorylation of Jak3 was strongly detected in Jak3 WT, as expected with fewer autophosphorylation sites in the Y820A mutant, the level of perceived autophosphorylation was reduced by more than 50% in Y820A and 90% in K851A compared to WT. This suggests that enhanced STAT5 activation by Jak3 Y820A is not correlated with autophosphorylation.

Examination of the interaction of STAT5a with mouse Jak3 was undertaken. FLAG-tagged STAT5a was transfected with Myc-tagged Jak3 WT or Y820A in HEK293T cells, then the cells were lysed and immunoprecipitated with anti-Myc antibody (Fig. 6C). Compared to Jak3 WT, Jak3 Y820A strongly interacted with STAT5a in over expressed HEK293T cells. Consistently, the increased interaction between STAT5b and Jak3 Y820A was also observed (Supplementary Figure 4). Phosphorylation of tyrosine 694 in STAT5a is important for its activation. We examined whether unphosphorylated STAT5a (Y694F) can bind to Jak3 Y820A. FLAG-tagged STAT5a WT or Y694F was transfected with Myc-Jak3 Y820A in HEK293T cells, then the cells were lysed and immunoprecipitated with anti-FLAG antibody (Fig. 6D). The interaction of Myc-Jak3 Y820A with either STAT5a WT or Y694F was comparable. Furthermore, we assessed the interaction between endogenously expressed STAT5 and stably overexpressed Jak3 in F7 cells. F7 cells treated without or with IL-2 for 10 min were lysed and immunoprecipitated with anti-Myc antibody, then immunoprecipitates were immunoblotted with anti-STAT5a antibody (Fig. 6E). Interaction between STAT5a and Jak3 WT and Y820A was observed in IL-2 stimulated F7 cells, and this association was significantly enhanced in Jak3 Y820A compared to WT (Fig. 6F). These data suggest that enhancement of STAT5 activity occurs with increased association of Jak3 Y820A with STAT5.

Discussion

These studies have identified a novel tyrosine phosphorylation site in Jak3, Y820, and evaluated the functional role of its post-translational modification in response to IL-2. Mutation of Y820 to alanine of mouse Jak3 but not phenylalanine leads to enhanced STAT5 phosphorylation and increased STAT5 transcriptional activity. In contrast, mutation of Y820 to aspartic acid shows decreased STAT5 phosphorylation and transcriptional activity. Stably expressed Jak3 Y820A in F7 cells results in enhanced STAT5 phosphorylation and increased cell growth by IL-2 treatment. Furthermore, interaction between STAT5 and Jak3 is augmented in Y820A mutant compared to WT. Thus, Jak3 Y820A mutant enhances STAT5 activity by facilitation of Jak3-STAT5 association. Since Y820F did not show the same increase in activity as Y820A, we could not exclude the possibility that the Y820A mutation, rather than phosphorylation, induces a conformational change that increases the activity of Jak3. This phenomenon reminiscent the activating mutation of JAK2, V617F, which is well known in polycythemia vera (31). These are likely to propose an interesting hypothesis that Jak3 Y820 is involved in Jak3 functions through its phosphorylation and/or the regulation of Jak3 conformation.

While Jak3 kinase activity is reduced in Jak3 Y820A mutant, phosphorylation of STAT5 appears to increase in Jak3 Y820A mutant compare to WT. The outcome of the Y820A of Jak proteins appears to result in increased phosphorylation of STAT5, which is confirmed by the transcriptional increase in reporter constructs requiring STAT5 phosphorylation. This occurs despite the apparent decrease in autophosphorylation of the Jak3 in in vitro kinase activity. These studies further demonstrate that association of Jak3 with STAT5 appears to be increased with the Y820A mutation. This is a paradox, because the effects on STAT5 phosphorylation are different between substitution of Y820 to alanine and aspartic acid, but our results suggest perhaps Jak3 Y820 attenuates the response of STAT5 phosphorylation and its downstream transcriptional regulation.

In response to IL-2 in HuT78 cells, phosphorylation of Jak3 detected by anti-PY antibody, and pY980 was similar at 15 and 30 min after stimulation. The peak of phosphorylation of Y820 was observed to increase in the later time period as demonstrated by the pY820 sera. Further, phosphorylation of pan-tyrosine and Y980 were diminished in Jak3 K851A transfected HEK293T cells, although phosphorylation of Y820 was sustained in Jak3 K851A. These data correlate with the phosphorylation of STAT5 and therefore support the hypothesis that phosphorylation of Jak3 Y820 has different control mechanisms from the other tyrosine phosphorylation of Jak3.

These results also support recruitment of a Jak3 kinase protein phosphatases, including CD45 such T-cell protein tyrosine phosphatase (TC-PTP), PTP1B and SHP2(33-36). Phosphorylated Y820 of Jak3 or a conformational change of Jak3 might be targeted to recruit these phosphatases for inactivating Jak3.

Suppressor of cytokine signaling (SOCS) family members is also responsible for attenuating Jak family kinases activities(37,38). SOCS family protein contains SH2 domain, which bind to a phosphorylated tyrosine residue, suggesting the possibility that phosphorylated Y820 of Jak3 or a conformational change of Jak3 may provide a binding site for SH2 domain of SOCS or phosphatases. Our data is likely to propose a novel negative regulation of Jak3 via intramolecular mechanisms and is the focus of future research.

A large number of Jak3 mutations have been identified and reported in SCID patients(39,40), while there is no report on Y824 mutation in SCID. However, several Jak3 mutations are identified in the half of the cases of a hematological disease, T-cell prolymphocytic leukemia (T-PLL)(41). Among them, point mutation at the tyrosine 824 residue substituted to aspartic acid (Y824D) in Jak3 is identified in T-PLL cases, whose malignant cells reveal constitutive phosphorylation of STAT1 and STAT3, but not STAT5(41). Thus, Jak3 Y824 mutation in human T-PLL might be oncogenic to promote malignant transformation by STAT1 and STAT3 activation. Our studies demonstrate Y820A mutation in mouse Jak3 results in hyper activation of STAT5, while Y820D mutation suppressed STAT5 activation. This could also be the case for other STATs recruitment and regulation such as observed in T-PLL Jak3 Y824D. Taken together with our data, disruption of intracellular inhibition by human Jak3 Y824 may induce excess of Jak3 activity, leading to malignant transformation.

We here identified a novel phosphorylation site at Y820 of mouse Jak3, corresponding to Y824 of human Jak3. Jak3 Y820A positively, and Jak3 Y820D negatively, regulates the Jak-STAT signaling through intramolecular mechanisms. Jak3 Y820 is likely to play an important role in Jak3-STAT5 signals of Jak3 via its phosphorylation and/or a conformational change of Jak3. Our finding will provide a new approach to understand how Jak3 activity is controlled.

Author contributions

Y.S., K.K., and T.M. conducted the experiments. J.K., R.M., Y.K., and M.F. analyzed data. Y.S., K.O., B.A.W., and T.M. conceived the study, analyzed data and wrote the manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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Figure Legends

Fig. 1. Identification of novel phosphorylated tyrosine residues in mouse Jak3. (A) Schematic view of mouse Jak3 is shown. D4 domain is described as a black bar. (B) Sf9 cells infected without or with Jak3 virus were immunoprecipitated with anti-Jak3 serum and immunoblotted with anti-Jak3 serum. (C) Peptides synthesized with flanking trypsin recognition site. The predicted trypsin digest phosphopeptide products were also synthesized and designate by the corresponding tyrosine number from the sequence.

Fig. 2. Autophosphorylation of tryptic digested Jak3 synthetic peptide. (A) Baculovirus produced Jak3 was immunoprecipitated, subjected to in vitro kinase reaction, and resolved by SDS-PAGE. The excised band containing Jak3 was treated by in-gel tryptic digestion, peptides were extracted, and the resulting lyophilized product was resolved by 2D-TLC and visualized by autoradiograph (Left panel). Schematic view of the assignment of the tryptic peptides resolved on 2D-TLC (Right panel). (B) Synthetic peptides were ^{32}P labeled with Jak3 immunoprecipitated complexes, separated on SDS-PAGE and subjected to in-gel tryptic digestion. Samples were resolved on 2D-TLC with the predicted tryptic phosphopeptide. Dashed circles represent the migration of ninhydrin stained phosphopeptide and ^{32}P-labeled protein is visualized by autoradiograph. Migration is determined in relationship to WT jak3.

Fig. 3. Phosphorylation of Tyr820 residue on Jak3. (A) HuT78 cells were stimulated with recombinant human IL-2 (2000 U/ml) for the indicated periods, then lysed and immunoprecipitated with anti-Jak3 antibody. An equal aliquot of immunoprecipitant was immunoblotted with anti-PY, anti-Jak3pY820, or anti-Jak3pY980 and anti-Jak3 antibodies. The graphs show the quantification of phospho-Jak3 levels normalized to Jak3. Independent experiments from 5 replicates are summarized and presented as mean ± SEM. **p<0.01 one-way ANOVA with Dunnett's multiple comparisons test. (B) HEK293T cells were transfected with a series of Myc-tagged Jak3 mutants (10 μg). Thirty-six hrs after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody and immunoblotted with anti-PY, anti-Jak3pY820, anti-Jak3pY980 and anti-Myc antibodies. Independent experiments from 3-4 replicates are summarized and presented as mean ± SEM. n.s., not significant, *p<0.05, **p<0.01, ***p<0.005, one-way ANOVA with Dunnett's multiple comparisons test.

Figure 4. Jak3 Y820A increases phosphorylation and transcriptional activity of STAT5a. (A) HEK293T cells in a 12-well plate were transfected with FLAG-tagged STAT5a (0.5 μg) and increasing amounts of Myc-tagged Jak3 WT, Y820A, Y820D, Y820F or K851A (0, 0.3 and 0.6 μg). Thirty-six hours after transfection the cells were lysed and immunoblotted with anti-pSTAT5 (upper panel), anti-FLAG (middle panel) and anti-Myc (lower panel) antibodies. (B) The graph shows the quantification of phospho-STAT5 levels normalized to total STAT5a proteins. Independent experiments from 3 replicates are summarized and presented as mean ± SEM. n.s., not significant, *p<0.05, ***p<0.005, one-way ANOVA with Tukey's multiple comparisons test. (C) HEK293T cells in a 24-well plate were transfected with STAT5-LUC (0.1 μg) and FLAG-tagged STAT5a (0.1 μg), and increasing amount of Myc-tagged Jak3 (0, 0.003 and 0.03 μg). Thirty-six hours after transfection, the cells were harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the SEM. n.s., not significant, ***p<0.005, Student's t test. (D) Total cellular protein collected from parallel cultures was analyzed by immunoblot with anti-Myc, anti-FLAG and anti-Actin antibodies.

Fig. 5. Jak3 Y820A increases IL-2 mediated proliferation and STAT5 phosphorylation in F7 cells. (A) F7/Jak3 WT and YA cells were stimulated with recombinant human IL-2 (10 U/ml) for the indicated periods. The cells were then lysed and total cell lysate was analyzed by immunoblotted with anti-pSTAT5, anti-STAT5a, anti-Myc and anti-actin antibodies. (B) The graph shows the quantification of phospho-STAT5 levels normalized to total STAT5a proteins. Independent experiments from 3 replicates are summarized and presented as mean ± SEM. *p<0.05, ***p<0.005, one-way ANOVA with Tukey's multiple comparisons test. (C, D) F7/Jak3 WT and YA cells ($5x10^3$/well) cells were cultured in 96-well plate with indicated concentration of IL-2 (C) or WEHI-3B supernatant (D) 60 hrs. The cell viability was determined by WST assay as described in Materials and methods. Data are the means of triplicate experiments ± SE. n.s., not significant, ***p<0.005, one-way ANOVA with Tukey's multiple comparisons test.

Fig. 6. Jak3 Y820A increases the interaction with STAT5. (A) HEK293T cells in a 10 cm dish were transfected with Myc-Jak3 WT, Y820A or K851A (10 μg). Thirty-six hours after transfection the cells were lysed and immunoprecipitated with anti-Myc antibody. The immunoprecipitates were incubated with [γ-32P]-ATP at

30 °C for 30 min. The products of these reactions were separated by SDS-PAGE (upper panel). Aliquots of immunoprecipitates and total cell lysate were immunoblotted with anti-Myc antibody (middle and lower panels). (B) The graph shows the quantification of each phosphorylation signal normalized to anti-Myc blot in the immunoprecipitates from 3 independent experiments. Mean ± SEM. *$p<0.05$, **$p<0.01$ one-way ANOVA with Dunnett's multiple comparisons test. (C) HEK293T cells were transfected with FLAG-tagged STAT5a and Myc-tagged Jak3 WT or YA mutants (5 μg). Thirty-six hrs after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody and then, immunoblotted with anti-FLAG and anti-Myc antibodies. An aliquot of each total cell lysate was blotted with anti-FLAG and anti-Myc antibodies. (D) HEK293T cells were transfected with Myc-tagged Jak3 YA (3 μg) and FLAG-tagged STAT5 WT or YF mutant (2 μg). Thirty-six hrs after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc and anti-FLAG antibodies. An aliquot of each total cell lysate was blotted with anti-Myc and anti-FLAG antibodies. (E) F7 cells stimulated without or with IL-2 (10 U/ml) for 10 min were lysed and immunoprecipitated with anti-Myc antibody, then, immunoblotted with anti-STAT5a and anti-Myc antibodies. (F) The graph shows the quantification of each proteinlevels in the immunoprecipitates normalized to total cell lysate from 4 independent experiments. Mean ± SEM. n.s., not significant, *$p<0.05$, one-way ANOVA with Tukey's multiple comparisons test.

Supplementary Data

A novel intramolecular regulation of mouse Jak3 activity by phosphorylation of a tyrosine 820

Yuichi Sekine, Kazuna Kikkawa, Bruce A. Witthuhn, Jun-ichi Kashiwakura, Ryuta Muromoto, Yuichi Kitai, Masahiro Fujimuro, Kenji Oritani and Tadashi Matsuda

Material included:
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3

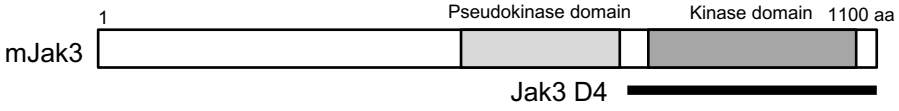
Supplementary Figure 1. Comparison of human and mouse Jak3 protein sequence around novel tyrosine phosphorylation site. Human Jak3 aa 818 to 833 and mouse Jak3 aa 814 to 827 were shown. Tyrosine phosphorylation sites were highlighted.

Supplementary Figure 2. Jak3 Y820A increases phosphorylation and transcriptional activity of STAT5b. (A) HEK293T cells in a 12-well plate were transfected with FLAG-tagged STAT5b (0.5 μg) and increasing amounts of Myc-tagged Jak3 WT, Y820A, Y820D, Y820F or K851A (0, 0.3 and 0.6 μg). Thirty-six hours after transfection the cells were lysed and immunoblotted with anti-pSTAT5 (upper panel), anti-FLAG (middle panel) and anti-Myc (lower panel) antibodies. (B) HEK293T cells in a 24-well plate were transfected with STAT5-LUC (0.1 μg) and FLAG-tagged STAT5b (0.1 μg), and increasing amount of Myc-tagged Jak3 (0, 0.003 and 0.03 μg). Thirty-six hours after transfection, the cells were harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the SEM. ***$p<0.005$, Student's *t* test.

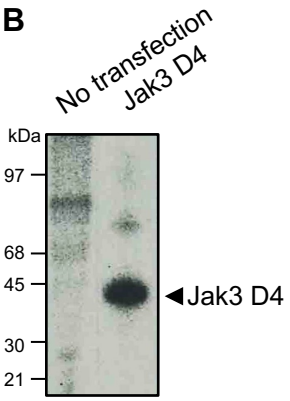
Supplementary Figure 3. Interaction between STAT5b and Jak3. HEK293T cells were transfected with FLAG-tagged STAT5b and Myc-tagged Jak3 WT or YA mutants (5 μg). Thirty-six hrs after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody and then, immunoblotted with anti-FLAG and anti-Myc antibodies. An aliquot of each total cell lysate was blotted with anti-FLAG and anti-Myc antibodies.

Figure 1

A



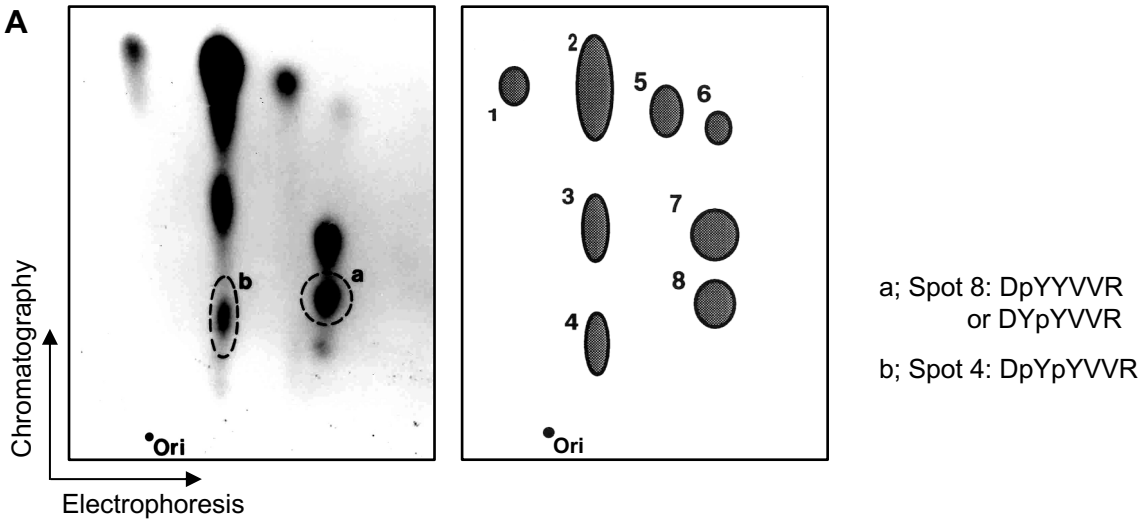
B



C

Synthetic peptides	Predicted tryptic peptides	Assignment on 2D map in Fig. 2A
Linker domain		
Y780: LITSDYELLSDPTPGIPSPR	LITSDYELLSDPTPGIPSPR	1, 2, 5
Y805: DELCGGAQLYACQDPAIFEER	DELCGGAQLYACQDPAIFEER	
Y820: HLKYISLLGK	YISLLGK	2, 6
Kinase domain		
Y837: LCRYDPLGDNTGPLVAVK	YDPLGDNTGPLVAVK	
Y883/887: VKYRGVSYGPGRQ	YRGVSYGPGRQ	
Y900: LVMEYLPSGCLR	LVMEYLPSGCLR	
Y934: DRLLLFAWQICKGMEYLGARRCVHRDLAA	GMEYLGAR	3, 7
Y975/976: DFGLAKLLPLGKDYYVVREPGQSPIF	DYYVVR	4, 8
Y969: EPGQSPIFWYAPESLSDNIFSR	EPGQSPIFWYAPESLSDNIFSR	

Figure 2


A
b
a
Ori
1
2
3
4
5
6
7
8
Ori
Chromatography
Electrophoresis
a; Spot 8: DpYYVVR
or DYpYVVR
b; Spot 4: DpYpYVVR

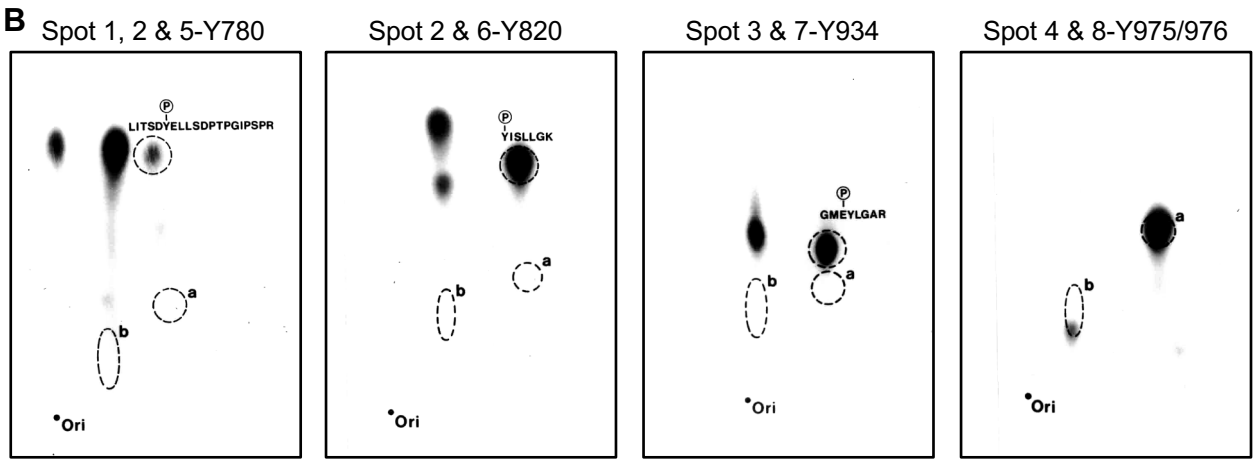

B
Spot 1, 2 & 5-Y780
Spot 2 & 6-Y820
Spot 3 & 7-Y934
Spot 4 & 8-Y975/976
LITSDYELLSDPTPGIPSPR
YISLLGK
GMEYLGAR
a
b
Ori

Figure 3

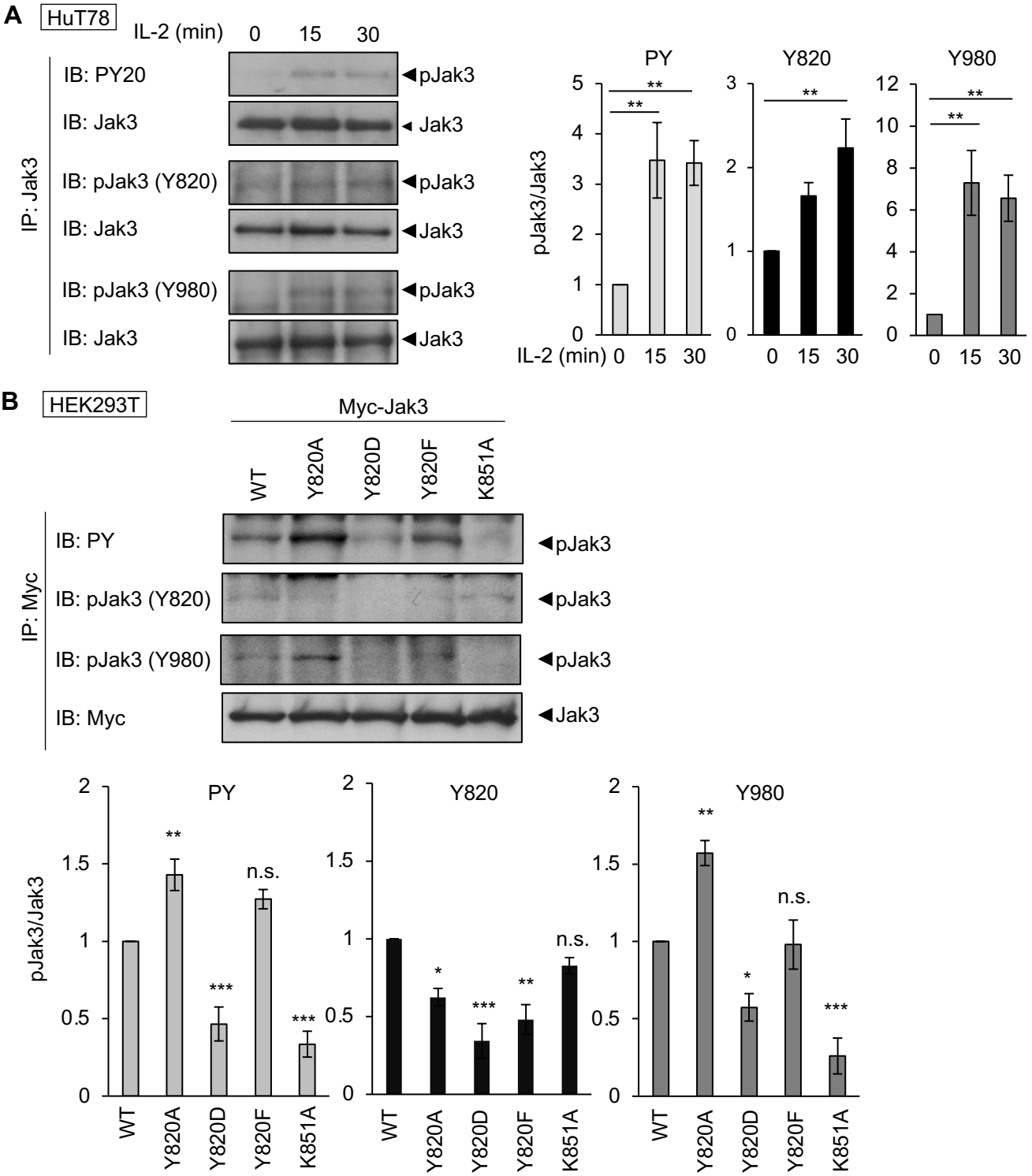

A
HuT78
IL-2 (min) 0 15 30
IP: Jak3
IB: PY20
IB: Jak3
IB: pJak3 (Y820)
IB: Jak3
IB: pJak3 (Y980)
IB: Jak3
pJak3
Jak3
PY
Y820
Y980
pJak3/Jak3
IL-2 (min) 0 15 30
B
HEK293T
Myc-Jak3
WT
Y820A
Y820D
Y820F
K851A
IP: Myc
IB: PY
IB: pJak3 (Y820)
IB: pJak3 (Y980)
IB: Myc
pJak3
Jak3
PY
Y820
Y980
pJak3/Jak3
n.s.

Figure 4

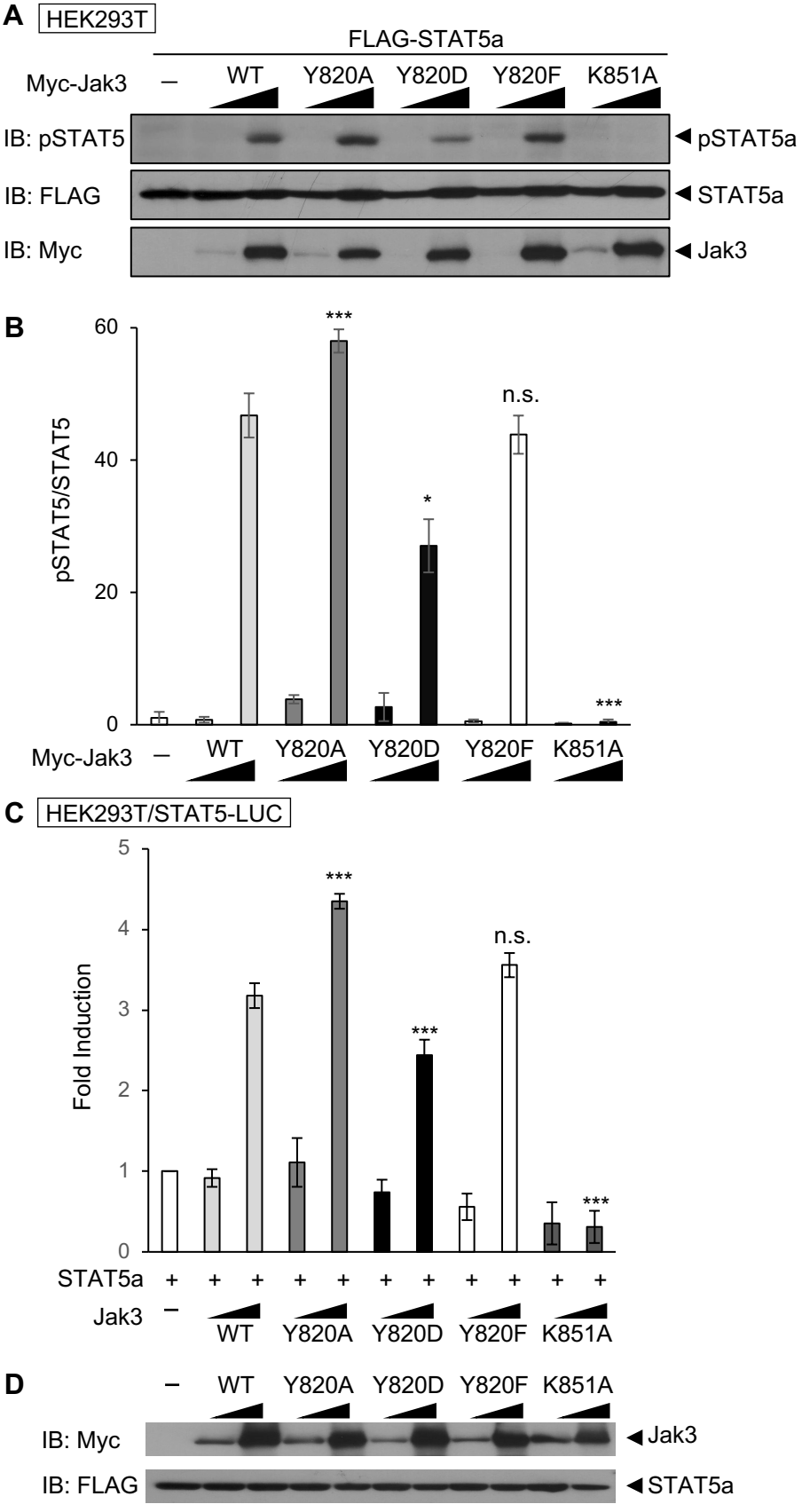

A
HEK293T
FLAG-STAT5a
Myc-Jak3
–
WT
Y820A
Y820D
Y820F
K851A
IB: pSTAT5
pSTAT5a
IB: FLAG
STAT5a
IB: Myc
Jak3
B
pSTAT5/STAT5
60
40
20
0
***
n.s.
*
***
Myc-Jak3
–
WT
Y820A
Y820D
Y820F
K851A
C
HEK293T/STAT5-LUC
Fold Induction
5
4
3
2
1
0
***
n.s.
***
***
STAT5a
+
Jak3
–
WT
Y820A
Y820D
Y820F
K851A
D
–
WT
Y820A
Y820D
Y820F
K851A
IB: Myc
Jak3
IB: FLAG
STAT5a

Figure 5

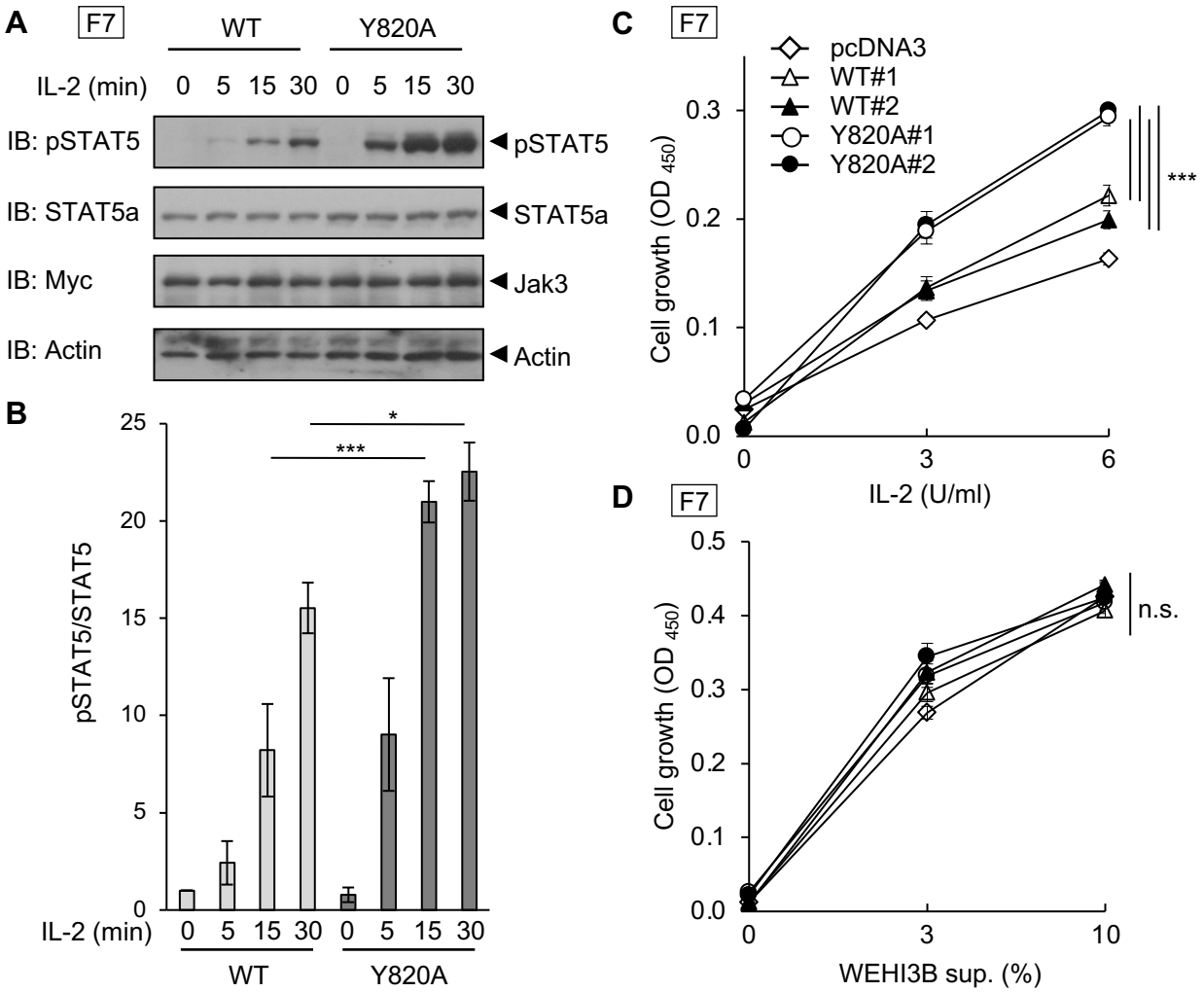
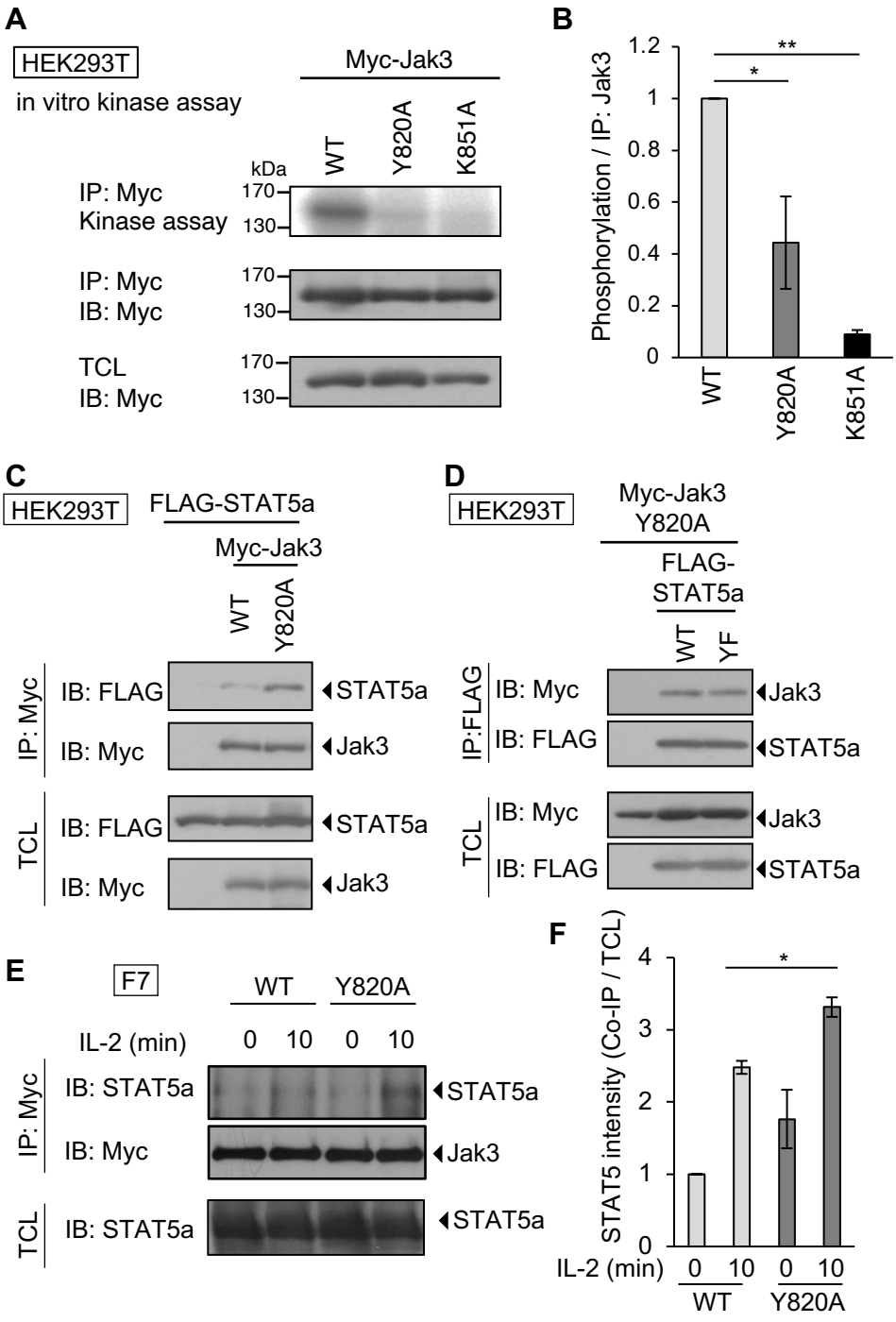

A
F7
WT
Y820A
IL-2 (min) 0 5 15 30 0 5 15 30
IB: pSTAT5
pSTAT5
IB: STAT5a
STAT5a
IB: Myc
Jak3
IB: Actin
Actin
B
pSTAT5/STAT5
IL-2 (min) 0 5 15 30 0 5 15 30
WT
Y820A
C
F7
pcDNA3
WT#1
WT#2
Y820A#1
Y820A#2
Cell growth (OD 450)
IL-2 (U/ml)
***
D
F7
Cell growth (OD 450)
WEHI3B sup. (%)
n.s.

Figure 6

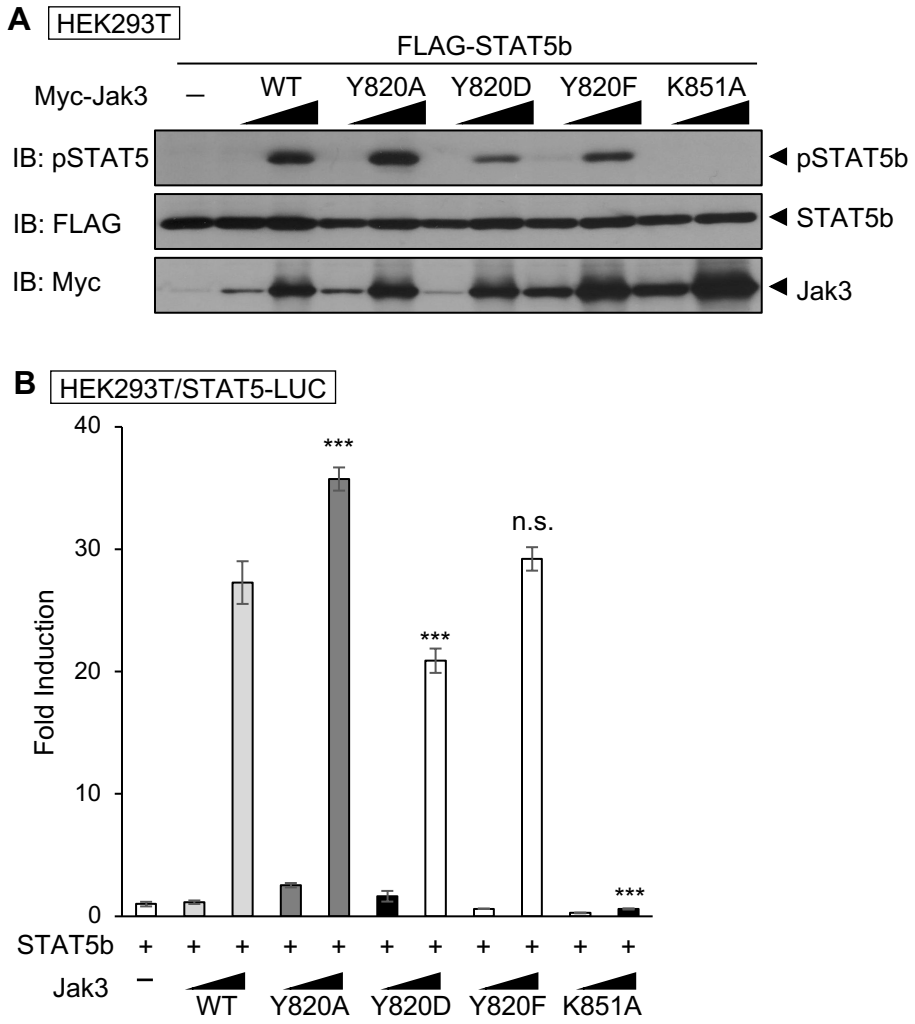

A
HEK293T
in vitro kinase assay
Myc-Jak3
WT
Y820A
K851A
kDa
IP: Myc
Kinase assay
170
130
IP: Myc
IB: Myc
TCL
IB: Myc
B
Phosphorylation / IP: Jak3
1.2
1
0.8
0.6
0.4
0.2
0
**
*
WT
Y820A
K851A
C
HEK293T
FLAG-STAT5a
Myc-Jak3
WT
Y820A
IP: Myc
IB: FLAG
STAT5a
IB: Myc
Jak3
TCL
IB: FLAG
STAT5a
IB: Myc
Jak3
D
HEK293T
Myc-Jak3
Y820A
FLAG-
STAT5a
WT
YF
IP:FLAG
IB: Myc
Jak3
IB: FLAG
STAT5a
TCL
IB: Myc
Jak3
IB: FLAG
STAT5a
E
F7
WT
Y820A
IL-2 (min)
0
10
0
10
IP: Myc
IB: STAT5a
STAT5a
IB: Myc
Jak3
TCL
IB: STAT5a
STAT5a
F
STAT5 intensity (Co-IP / TCL)
4
3
2
1
0
*
IL-2 (min)
0
10
0
10
WT
Y820A

Supplementary Data

Supplementary Figure 1

human 818 EERHLKYISQLGKG 833
mouse 814 EERHLKYISLLGKG 827

Supplementary Figure 2



Supplementary Figure 3