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Suppression of STAT3 Activity by Duplin, Which Is a Negative Regulator of the Wnt Signal

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Duplin was originally isolated as a negative regulator of β-catenin–dependent T-cell factor (Tcf) transcriptional activity in the Wnt signaling pathway. However, Duplin knockout mice exhibit embryonic lethality at 5.5-em day, suggesting that Duplin has important roles other than as a negative regulator of the Wnt signal. To identify new roles of Duplin, the Duplin-binding proteins were screened. PIAS3, which is a SUMO E3 ligase and acts as an inhibitor of signal transducer and activator of transcription (STAT3), was identified as a Duplin-binding protein. Duplin was sumoylated, but PIAS3 affected neither the sumoylation of Duplin nor its ability to inhibit Tcf-4 activity. Like PIAS3, Duplin suppressed the leukemia-inhibitory factor (LIF)–induced STAT3 transcriptional activity. Duplin did not affect the LIF-dependent tyrosine phosphorylation or nuclear localization of STAT3 but inhibited the formation of complex between STAT3 and DNA. Although STAT3 is not modified with SUMO, PIAS3 inhibited the STAT3 activity in a manner partially depending on its SUMO E3 ligase activity. Duplin suppressed the LIF-dependent STAT3 activity independently of sumoylation. These results demonstrate that Duplin inhibits not only Tcf-4 but also STAT3, suggesting that Duplin may act as a repressor for multiple transcriptional factors.

Key words: Duplin, PIAS3, signal transduction, STAT3, transcription.

Abbreviations: Ab, antibody; DMEM, Dulbecco's modified Eagle's medium; GSK-3β, glycogen synthase kinase-3β; IB, immunoblot; IL, interleukin; IP, immunoprecipitation; LIF, leukemia inhibitory factor; NLS, nuclear localization signal; PI3K, phosphatidylinositol 3 kinase; SAP, scaffold attachment factor A/B, acinus, PIAS; STAT, signal transducer and activator of transcription; Tcf, T-cell factor; WT, wild type.

Duplin has been shown to act as a negative regulator for the Wnt signaling pathway by binding to β-catenin in the nucleus (*1*). β-Catenin not only regulates cell-to-cell adhesion by interacting with cadherin, but also functions as a component of the Wnt signaling pathway (*2*). The Wnt signaling pathway is conserved evolutionally and regulates cellular proliferation and differentiation (*3, 4*). Wnt stabilizes cytoplasmic β-catenin, and the accumulated β-catenin is translocated to the nucleus, where it binds to the transcription factor T-cell factor (Tcf)/lymphoid enhancer factor and thereby stimulates the expression of various genes (*5*).

Duplin resides in the nucleus by binding to importin α and competes with Tcf-4 for the binding to β-catenin, thereby inhibiting Wnt-3a– and β-catenin–dependent Tcf-4 activation in mammalian cells (*1*, *6*). Duplin also inhibits the expression of *siamois*, a Wnt-responsive gene (*7*, *8*), induces ventralization, and suppresses Wnt-8– and β-catenin–induced axis duplication in *Xenopus* embryos. Nuclear localization of Duplin is necessary for its inhibition of Wnt-dependent activation of Tcf-4 in mammalian cells and ventralization in *Xenopus* embryos. Furthermore, a Duplin mutant that does not bind to β-catenin also induces ventralization and inhibits siamois-dependent axis duplication in *Xenopus* embryos (*6*). These results suggest that Duplin acts downstream of siamois via some mechanism other than inhibition of the interaction between β-catenin and Tcf-4 at least in *Xenopus* embryos.

Duplin is expressed ubiquitously predominantly from early- to mid-stage mouse embryogenesis (*9*). The development of Duplin-deficient mice is arrested at gastrulation with massive apoptosis (*9*). In these embryos, increased expression of known β-catenin/Tcf target genes including *Axin2* and *cyclin D1* is not observed, suggesting that the lack of Duplin does not result in the constitutive activation of the Wnt signal. These results indicate that Duplin is indispensable for normal mouse development, probably as a result of its functions that are independent of inhibition of the Wnt signaling pathway. These findings together with the observations in *Xenopus* embryos indicate that Duplin may act on transcriptional factors other than Tcf and thereby regulate gene expression.

The signal transducer and activator of transcription 3 (STAT3) protein is activated by the interleukin-6 (IL-6) family of cytokines, epidermal growth factor, and leptin (*10, 11*). IL-6 type cytokines, including IL-6, IL-11, and leukemia inhibitory factor (LIF), are an important family of mediators involved in the regulation of the acute-phase response to injury and infections (*12*). Besides their

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functions in inflammation and the immune response, these cytokines play critical roles in haematopoiesis, liver and neuronal regeneration, embryonal development, and fertility. The cytokines induce the tyrosine-phosphorylation of STAT3 by activating Janus kinase. Phosphorylated STAT3 then forms a dimer and translocates into the nucleus to activate specific genes (*12*). The STAT3 transcriptional activity is regulated in several ways. PIAS3 has been identified as a negative regulator of STAT3 (*13*). PIAS3 has a RING domain and acts as a SUMO E3 ligase (*14*). Modification of target proteins with SUMO, one of the ubiquitin-like proteins, changes their subcellular localization, transcriptional activity, and protein stability. For instance, Tcf-4 and androgen receptor are modified with SUMO, and sumoylation enhances or represses their transcriptional activity (*15, 16*). However, whether the STAT3 activity is regulated by sumoylation is not clear.

Here we show that Duplin binds to PIAS3. PIAS3 neither induces the sumoylation of Duplin nor affects the ability of Duplin to inhibit the β-catenin/Tcf pathway. However, interestingly, Duplin binds to STAT3 and suppresses LIF-dependent activation of STAT3 as well as PIAS3. Therefore, Duplin may regulate gene expression by inhibiting multiple transcriptional factors.

MATERIALS AND METHODS

Materials and Chemicals—pCMV5-Flag/PIAS3, pUC/EF-1α/β-catenin^{SA}, and TOP-*fos*-Luc were supplied by Drs. K. Shuai (University of California, San Francisco), A. Nagafuchi (Kumamoto University, Kumamoto, Japan), and H. Clevers (University Hospital, Utrecht, The Netherlands), respectively. COS, HEK-293, and HeLaS3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and 10% fetal calf serum, respectively. HEK-293T cells were cultured in DMEM/Ham's F12 containing 10% fetal calf serum. Anti-Myc antibody was prepared from 9E10 cells. Anti-Duplin antibody was prepared as described (*1*). Anti-Flag (M2) antibody, anti–GMP-1 (SUMO-1), anti-STAT3, anti-phospho STAT3 (Tyr^{705}), anti-HA, anti-UBC9, and anti–glycogen synthase kinase-3β (GSK-3β) antibodies were purchased from Sigma-Aldrich (Steinheim, Germany), Zymed Laboratories Inc. (San Francisco, CA), Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling Technology (Beverly, MA), Covance Laboratories (Richmond, CA), and Transduction Laboratories (Lexington, KY), respectively. LIF was from Chemicon Inc. (Temecula, CA). Other materials were purchased from commercial sources.

Plasmid Construction—pCGN/Duplin, pCGN/Duplin-(1-482), pCGN/Duplin-(482-749), pCGN/Duplin-(1-668), pCGN/Duplin-ΔNLS (Δ500-584), pBJ-Myc/Duplin, pBTM116HA/Duplin, pBTM116HA/Duplin-(482-749), pCGN/SUMO-1, pCGN/SUMO-1 (ΔGG), 5xAPREspLuc, pCAG/STAT3, and pCAG-HA/STAT1 were constructed as described (*1*, *6*, *15*, *17*, *18*). Standard recombinant DNA techniques were used to construct the following plasmids: pBJ-Myc/Duplin^{K457R}, pBJ-Myc/Duplin^{K609R}, pBJ-Myc/Duplin$^{K457/512/609/654R}$, pCGN/Duplin$^{K457/512/609/654R}$, pBTM116HA/Duplin-(482-668), pBTM116HA/Duplin-(668-749), pBTM116HA/Duplin-(482-565), pBTM116HA/Duplin-(565-668), pCGN/UBC9, pCGN/UBC9$^{C93R/L97A}$, pGAD/PIAS3, pCMV5-Flag/PIAS3-(1-223), pCMV5-Flag/PIAS3-(224-584), and pCMV5-Flag/PIAS3$^{C299A/S304A}$. Some of the constructs in these plasmids were made by digesting the original plasmids with restriction enzymes and inserting the fragments of interest into the vectors. The other constructs were made by inserting fragments generated using the Expand High Fidelity PCR system (Roche Diagnostics GmbH, Manheim, Germany) into the vectors.

Sumoylation Assay—To examine the sumoylation of Duplin in intact cells, COS cells (in 35-mm-diameter dishes) were transfected with pBJ-Myc– and pCGN-derived plasmids. At 48 h after transfection, the cells were lysed in 200 μl of RIPA buffer (10 mM sodium-phosphate buffer pH 7.2, 150 mM NaCl, 1% sodium-deoxycholate, 1% Triton X-100, and 0.1% SDS) containing 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 0.4 mM sodium-orthovanadate, and 10 mM *N*-ethylmaleimide as described (*15*, *17*). The lysates (200 μg of protein) were immunoprecipitated with anti-Myc antibody, and then the precipitates were probed with anti–SUMO-1 and anti-Duplin antibodies.

Assay of STAT3 Activity—To examine the transcriptional activity of STAT3, the indicated amounts of pCGN- and pCMV5-Flag–derived plasmids were transfected into HEK-293 or 293T cells (in 35-mm-diameter dishes) with 5xAPREspLuc (0.5 μg), pME18S/lacZ (0.5 μg), and pCAG/STAT3 (1 μg) as described (*19*). The total DNA concentration in each transfection experiment was kept constant by adding vector plasmid DNA. Twenty four hours after transfection, cells were either left untreated or were treated with LIF (20 ng/ml) for 6 h. The cells were lysed and the luciferase activity was measured using a PicaGene (Toyo B-NET Co., Ltd., Tokyo, Japan). To standardize the transfection efficiency, pME18S/lacZ carrying the SRα promoter linked to the coding sequence of the β-galactosidase gene was used as an internal control. All the experiments were performed at least three times, and the results shown are means ± the standard error.

Electrophoretic Mobility Shift Assay—A double-stranded 25-mer oligonucleotide (5′-GTC GAC ATT TCC CGT AAA TCG TCG A-3′) containing high affinity STAT3-binding site (underlined) was used as the STAT3 probe as described (*10*). The double-stranded oligonucleotide was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions were done for 20 min at room temperature by incubating 8 μg of nuclear extracts and 20 fmol of labeled oligonucleotide in 24 μl of binding buffer [10 mM Tris-HCl pH 7.5, 10 mM HEPES-KOH pH 7.9, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 1 μg of poly(dI-dC), and 10 μg of BSA]. When necessary, a large excess (2 pmol) of unlabeled probe or the anti-STAT3 antibody (0.5 μg) was added. Samples were electrophoresed on a 5% polyacrylamide gel, and the gel was then dried and the bands were visualized by autoradiography.

Others—Immunoblotting, immunoprecipitation, immunohistochemical analyses, and RNA interference analyses were performed as described (*15*, *17*, *20*). The transcriptional activity of Tcf-4 was measured as described (*1*).

RESULTS

Identification of PIAS3 as a Duplin-Binding Protein—To identify protein(s) that are involved in the functions of Duplin, we screened a mouse brain cDNA library with the yeast two-hybrid method using the C-terminal half of Duplin [Duplin-(482-749)] as bait. Among 2.6×10^5 clones, 4 clones were found to confer both His^+ and $LacZ^+$ phenotypes, and one of them was PIAS3 (Fig. 1A). PIAS3 acts as a negative regulator of STAT3 and plays a role in stimulating sumoylation as a SUMO E3 ligase (*14*). In the yeast two-hybrid assay, PIAS3 bound to Duplin, Duplin-(482-749), and Dulin-(482-668), but not to Duplin-(668-749) (Fig. 1B). Since Duplin-(668-749) binds to β-catenin (*1*), Duplin interacts with PIAS3 via sites different from the β-catenin–binding region. Since neither Duplin-(482-565) nor Duplin-(565-668) bound to PIAS3, the whole structure of Duplin-(482-668) may be required for the interaction with PIAS3. When Myc-Duplin alone was expressed, it showed a diffuse nuclear distribution, whereas Flag-PIAS3 was localized predominantly to punctate structures in the nucleus (Fig. 1C). Coexpression with Flag-PIAS3 resulted in a remarkable distribution of Myc-Duplin into punctate structures, which colocalized with Flag-PIAS3 (Fig. 1C). Biochemical immunoprecipitation assay indicated that HA-Duplin-(482-749) indeed formed a complex with Flag-PIAS3 when both proteins were coexpressed in COS cells (Fig. 1D). Flag-PIAS3-(224-584) localized to the nucleus, while Flag-PIAS3-(1-223) was located in the cytoplasm (Fig. 1C). Consistent with these findings, Flag-PIAS3-(224-584), but not Flag-PIAS3-(1-223), bound to HA-Duplin-(482-749) (Fig. 1D). These results indicate that Duplin interacts with PIAS3 in the nucleus.

Modification of Duplin with SUMO—To examine whether PIAS3 is involved in the sumoylation of Duplin, Duplin was coexpressed with SUMO-1. In COS cells expressing Myc-Duplin and HA-SUMO-1, two slowly migrating forms of Myc-Duplin were observed (Fig. 2A). The SUMO-1 precursor is processed by a C-terminal hydrolase to produce the mature form, and exposure of the C-terminal Gly-Gly residues is required for the conjugation of SUMO-1 to substrates (*21*, *22*). To examine whether these slowly migrating bands of Myc-Duplin are due to modification with SUMO-1, we expressed HA-SUMO-1 (ΔGG). This is a SUMO-1 mutant in which the C-terminal six amino acids, including Gly-Gly, are deleted, and the substrates are not modified with this SUMO-1 mutant. The higher-molecular mass Myc-Duplin bands were not observed (Fig. 2A). Although PIAS3 was coexpressed with Myc-Duplin and HA-SUMO-1, it enhanced the sumoylation of Myc-Duplin only very slightly, if at all (Fig. 2B), suggesting that PIAS3 does not act as a SUMO E3 ligase for Duplin.

It has been proposed that ψKXD/E (ψ is a hydrophobic residue and X is any amino acid) is a consensus sequence for sumoylation (*23*–*25*). Duplin contains three typical consensus sequences for sumoylation and one similar sequence: $KK^{457}QE$, $LK^{512}EE$, $IK^{609}PE$, and $VK^{654}KE$ (Fig. 1A). We mutated the lysine residues at amino acid positions 457, 512, 609, and 654 to arginine, and expressed these lysine mutants of Myc-Duplin with HA-SUMO-1 in COS cells. One of the slowly migrating bands of Duplin, corresponding to SUMO-modified Duplin, disappeared when Lys^{609} was mutated (Fig. 2C). Mutations of Lys^{457}, Lys^{512}, and Lys^{654} did not affect the sumoylation of Duplin (Fig. 2C and data not shown). When all four of the lysine residues were mutated (KR4), one sumoylation band was still detected (Fig. 2C). These results imply that at least Lys^{609} is a sumoylation site of Duplin and suggest that a lysine residue that is not located in the consensus sequence is also sumoylated. The KR mutant of Duplin (Duplin-KR4) inhibited β-catenin–dependent Tcf-4 activity to a similar extent to wild-type Duplin (Fig. 2D). Furthermore, coexpression with SUMO-1 and PIAS3 did not affect the ability of Duplin to inhibit Tcf-4 transcriptional activity (Fig. 2D). Therefore, sumoylation of Duplin is not involved in its ability to inhibit the Wnt signaling.

Inhibition of STAT3 Activity by Duplin—Since PIAS3 was originally identified as an inhibitor of STAT3-mediated gene activation by blocking the DNA binding activity of STAT3 (*13*), we asked whether Duplin affects the transcriptional activity of STAT3. LIF activated STAT3 in HEK-293 cells (Fig. 3A). Expression of HA-Duplin inhibited the LIF-dependent activation of STAT3 (Fig. 3A). Although HA-Duplin-KR4, HA-Duplin-(1-668), and HA-Duplin-(482-749) suppressed the LIF-dependent STAT3 activity as well as HA-Duplin did, HA-Duplin-(ΔNLS) and HA-Duplin-(1-482) did not (Fig. 3A). These results suggest that the inhibitory effect of Duplin on STAT3 requires the nuclear localization of Duplin but not the β-catenin–binding domain. Consistent with the previous observations (*26*), LIF induced the tyrosine phosphorylation (Tyr^{705}) of STAT3 and its nuclear localization (Fig. 3B). Expression of HA-Duplin did not inhibit LIF-dependent tyrosine phosphorylation and nuclear entry of STAT3 (Fig. 3B), suggesting that Duplin does not affect these steps. Instead, HA-Duplin formed a complex with STAT3 in intact cells, and it bound to LIF-dependent tyrosine-phosphorylated STAT3 in the nucleus (Fig. 3C). Therefore, we tested the effects of Duplin on the DNA-binding activity of STAT3. Nuclear extracts from HEK-293T cells were prepared and analyzed in the electrophoretic mobility shift assay with a STAT3-binding site as a probe. Treatment of the cells expressing STAT3 with LIF induced the binding of STAT3 to the probe (Fig. 3D). The complex of STAT3 and the probe was super-shifted by the addition of anti-STAT3 antibody, and it disappeared upon the addition of a large excess of cold probe (Fig. 3D). The expression of HA-Duplin but not HA-Duplin-(ΔNLS) suppressed the LIF-dependent binding of STAT3 to DNA (Fig. 3D). These results suggest that Duplin binds to STAT3 in the nucleus and inhibits its transcriptional activity by blocking the binding of STAT3 to DNA.

To examine whether STAT3, PIAS3, and Duplin interact functionally, these proteins were expressed in HEK-293T cells. Consistent with the findings that Flag-PIAS3-(224-584) was localized to the nucleus (see Fig. 1D), Flag-PIAS3-(224-584), but not Flag-PIAS3-(1-223), formed a complex with STAT3 (Fig. 4A). STAT3 and Flag-PIAS3-(224-584) were detected in the HA-Duplin-(482-749) immune complex, and STAT3 and HA-Duplin-(482-749) were observed in the Flag-PIAS3-(224-584) immune complex (Fig. 4A). Flag-PIAS3 inhibited the STAT3 transcriptional activity, but neither Flag-PIAS3-(1-223) nor Flag-PIAS3-(224-584) did (Fig. 4B), suggesting that the binding of PIAS3 to STAT3 is not sufficient for the inhibition of STAT3 by PIAS3. Flag-PIAS3 enhanced the Duplin-dependent

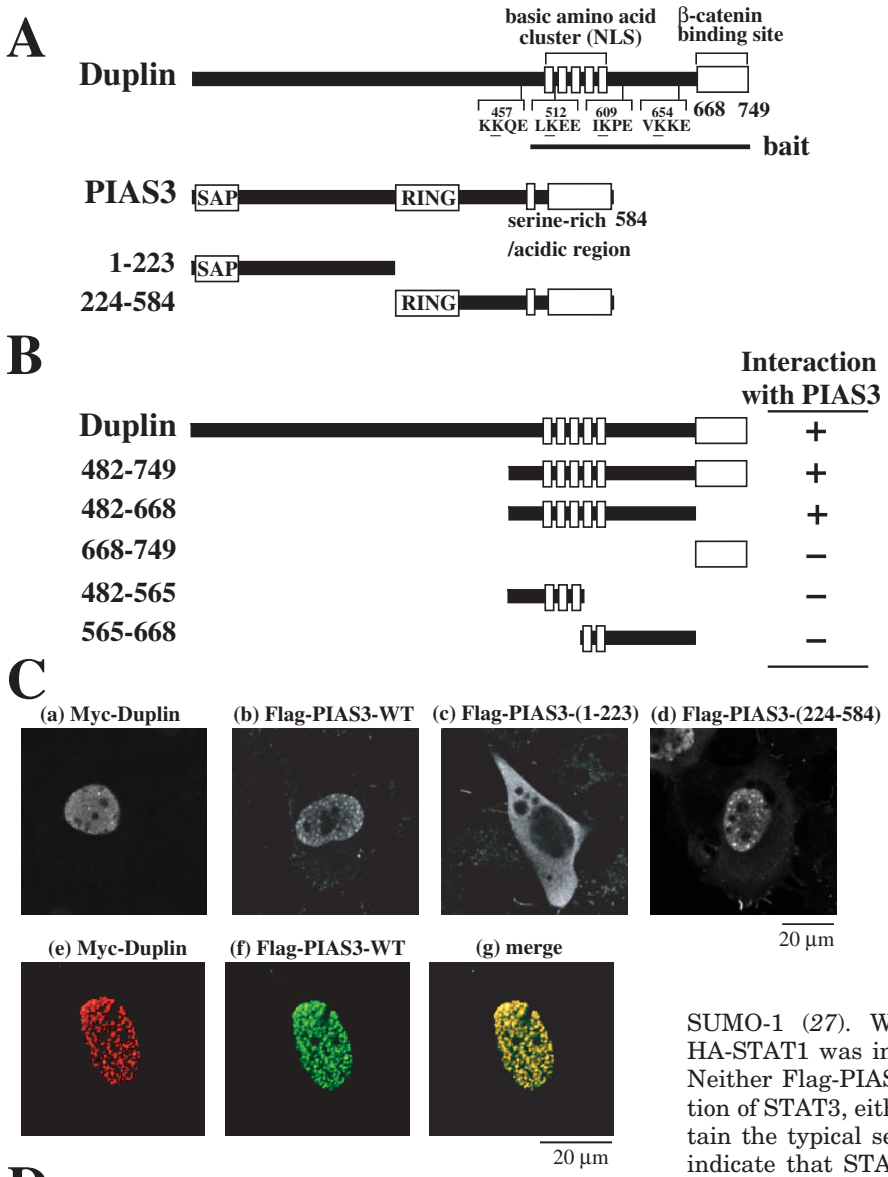

Fig. 1. **Identification of PIAS3 as a Duplin-binding protein.** (A) Schematic representations of Duplin and PIAS3. The deletion mutant of Duplin used as bait is underlined. NLS, nuclear localization signal; SAP, scaffold attachment factor A/B, acinus, PIAS. (B) Interaction of deletion mutants of Duplin with PIAS3 in the yeast two-hybrid assay. L40 cells were cotransformed with pBTM116HA-derived deletion mutants of Duplin and pGAD/PIAS3. To assay for β-galactosidase activity, cells were streaked on permissive (histidine-containing) medium and incubated for 3 days at 30°C, and then β-galactosidase activity was assayed. (C) Localization of Duplin and PIAS3. COS cells expressing Myc-Duplin (a), Flag-PIAS3 (b), Flag-PIAS3-(1-223) (c), Flag-PIAS3-(224-584) (d) or Myc-Duplin and Flag-PIAS3 (e and f) were stained with anti-Myc or anti-Flag antibody. The merged image (g) shows the colocalization of Myc-Duplin with Flag-PIAS3. WT, wild type. (D) Interaction of PIAS3 with Duplin in intact cells. The lysates (20 μg of protein) of COS cells expressing the indicated proteins were probed with anti-HA and anti-Flag antibodies. The lysates (180 μg of protein) were immunoprecipitated with anti-HA antibody and the immunoprecipitates were probed with anti-HA and anti-Flag antibodies. IP, immunoprecipitation; Ab, antibody.

inhibition of STAT3 only slightly, if at all (Fig. 4B). This might be because that Duplin and PIAS3 inhibit STAT3 by the similar mechanism.

Sumoylation-Dependent and Independent Inhibition of STAT3—STAT1 has been shown to be modified with SUMO-1 (*27*). When coexpressed with Myc-SUMO-1, HA-STAT1 was indeed sumoylated, but STAT3 was not. Neither Flag-PIAS3 nor HA-UBC9 induced the sumoylation of STAT3, either (Fig. 5A). Since STAT3 does not contain the typical sequences for sumoylation, these results indicate that STAT3 is not a substrate for sumoylation. PIAS3 has a RING domain, which is essential for SUMO E3 ligase activity (*14*). The PIAS3 RING mutant (PIAS3$^{C299A/S304A}$), in which Cys^{299} and Ser^{304} are changed to Ala, inhibited the STAT3 activity less efficiently than wild-type PIAS3 (Fig. 5B). Therefore, SUMO E3 ligase activity may be partially involved in the PIAS3-dependent inhibition of STAT3. We also tested whether UBC9, which is a SUMO-activating enzyme (E2) and essential for the sumoylation process (*28*), influences STAT3. The expression of wild-type UBC9 suppressed the LIF-dependent STAT3 activity, while a dominant-negative form of UBC9 (UBC9$^{C93R/L97A}$), in which Cys^{93} and Leu^{97} are changed to Arg and Ala, respectively, enhanced it (Fig. 5C). Furthermore, an siRNA for UBC9 enhanced LIF-dependent STAT3 activity (Fig. 5C). Taken together, these results suggest that sumoylation is involved in STAT3-dependent gene expression, although STAT3 is not modified with SUMO. Duplin and UBC9 additively inhibited the LIF-dependent activation of STAT3 (Fig. 5D). Furthermore, Duplin could inhibit the LIF-dependent STAT3 activity that was enhanced by the

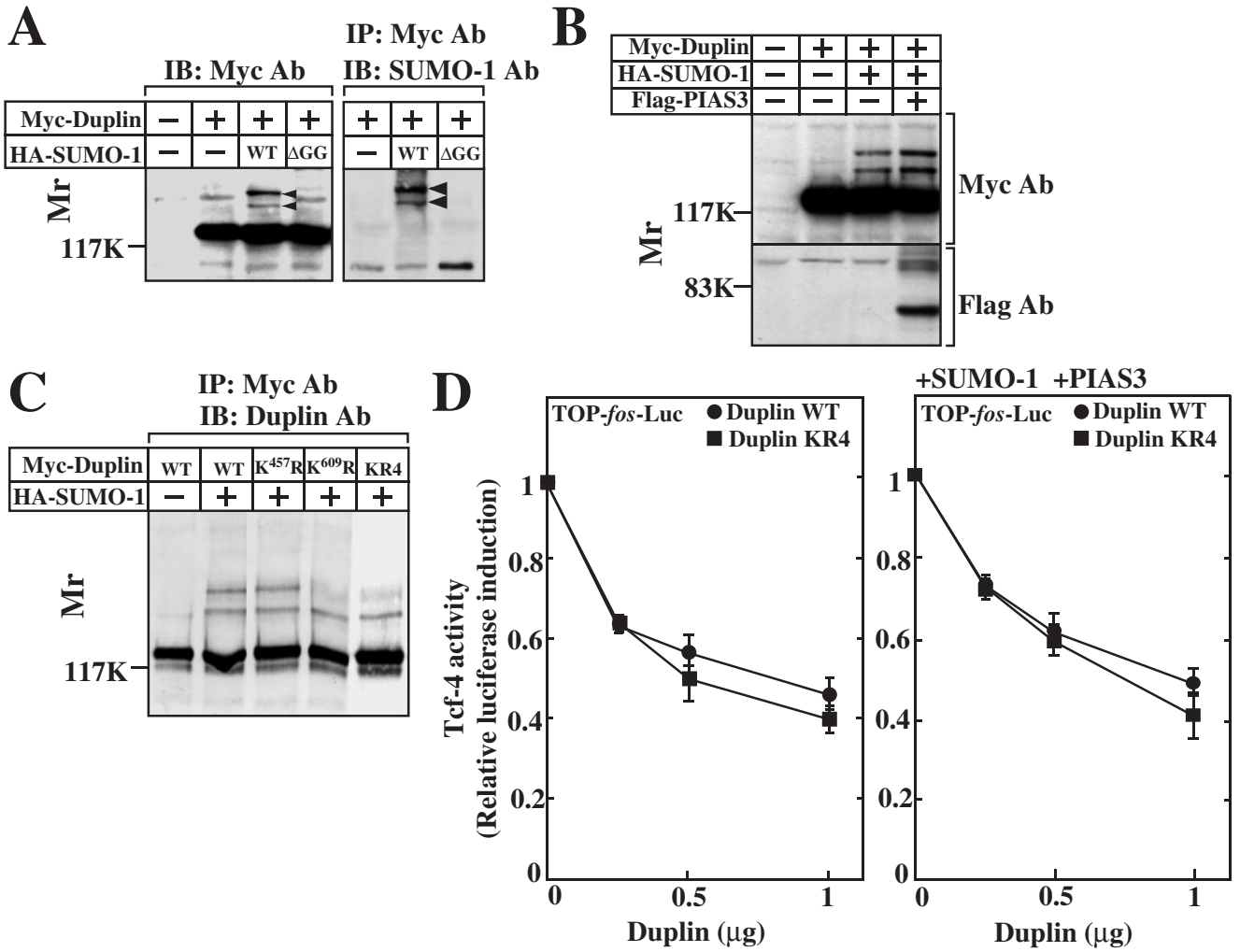

Fig. 2. **Effect of sumoylation of Duplin on its actions.** (A) Modification of Duplin with SUMO-1. The cell lysates (20 μg of protein) of COS cells expressing the indicated proteins were probed with anti-Myc antibody. The lysates (180 μg of protein) were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were probed with anti-SUMO-1 antibody. Arrowheads indicate slowly migrated bands of Myc-Duplin. IB, immunoblot; WT, wild type; ΔGG, SUMO-1-(1-95). (B) Effect of PIAS3 on the sumoylation of Duplin. The lysates (20 μg of protein) of COS cells expressing the indicated proteins were probed with anti-Myc and anti-Flag antibodies. (C) Identification of the sumoylation site of Duplin. The lysates (200 μg of protein) of COS cells expressing the indicated proteins were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were probed with anti-Duplin antibody. $K^{457}R$, a Duplin mutant in which Lys^{457} is changed to Arg; $K^{609}R$, a Duplin mutant in which Lys^{609} is changed to Arg; KR4, a Duplin mutant in which $Lys^{457,512,609,654}$ are changed to Arg. (D) Effect of the sumoylation of Duplin on β-catenin–dependent Tcf-4 activation. The indicated amount of pCGN/Duplin or its KR mutant, pEF-BOS/hTcf-4E (0.1 μg), pUC/EF-1α/β-$catenin^{SA}$ (0.1 μg), and TOP-*fos*-Luc (0.5 μg) were transfected into HEK-293 cells with or without pCGN/SUMO-1 and pCMV5-Flag/PIAS3. The luciferase activity was measured and expressed relative to the level in cells transfected without pCGN/Duplin.

siRNA for UBC9 or the dominant-negative form of UBC9 (Fig. 5, C and D). These results indicate that STAT3 activity is regulated by sumoylation indirectly and that Duplin inhibits the activity independently of sumoylation.

DISCUSSION

We originally identified Duplin as a negative regulator of Tcf-dependent transcription. To identify possible new roles of Duplin, we searched for Duplin-binding proteins, and identified PIAS3 as one of them. Duplin was modified with SUMO-1, but PIAS3 did not enhance the sumoylation of Duplin, although it has been reported to have the ability to enhance sumoylation of interferon regulatory factor-1 (*29*). Judging from the mobility shift on SDS-PAGE, Duplin has two sumoylation sites. We identified Lys^{609} as one of the sumoylation sites and found that sumoylation at Lys^{609} was not involved in the Duplin-dependent inhibition of Tcf-4. Although we can not exclude the possibility that another sumoylation site is involved in the inhibitory activity, it is unlikely, because coexpression with SUMO-1 and PIAS3 did not affect the inhibitory activity of Duplin. These results suggest that the sumoylation of Duplin does not regulate its inhibitory activity for Wnt signaling. We are currently investigating the physiological significance of the sumoylation of Duplin.

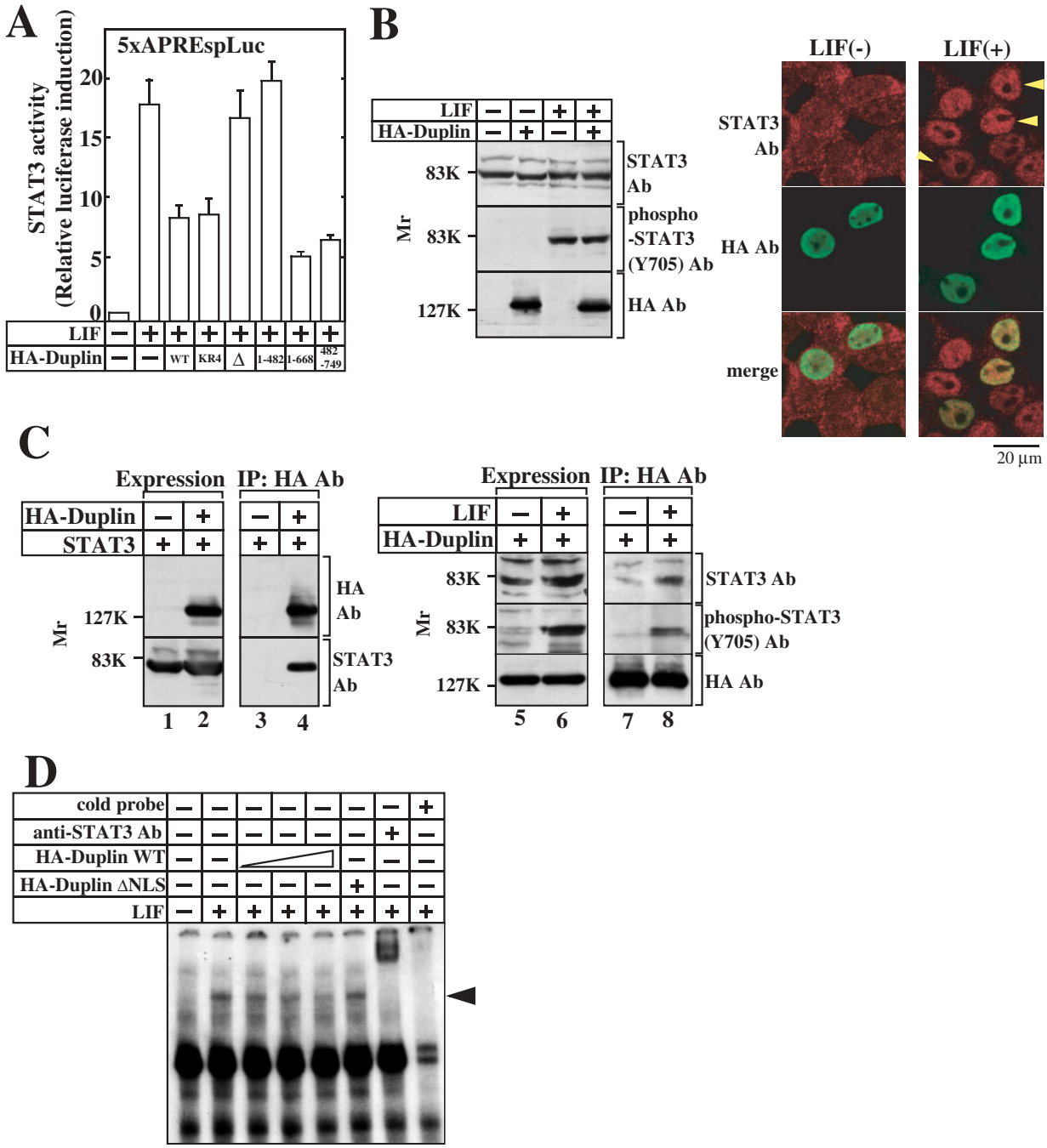

Fig. 3. **Inhibition of STAT3 activity by Duplin.** (A) Inhibition of STAT3-mediated gene expression by Duplin. Mutants of pCGN/Duplin (0.5 μg) and pCAG/STAT3 (1 μg) were transfected with 5xAPREspLuc (0.5 μg) into HEK-293 cells. The cells were either left untreated or were treated with LIF (20 ng/ml) for 6 h. The luciferase activity was measured and expressed relative to the level in cells not treated with LIF. Δ, Duplin-ΔNLS (Δ500-584). (B) Effects of Duplin on LIF-dependent tyrosine phosphorylation and nuclear localization of STAT3. HEK-293 cells expressing HA-Duplin were either left untreated or were treated with LIF (20 ng/ml) for 30 min. The cell lysates were probed with anti-STAT3, anti–phospho-STAT3 (Tyr^{705}), and anti-HA antibodies (left panel) or the cells were stained with anti-STAT3 and anti-HA antibodies (right panel). Arrowheads indicate the cells expressing HA-Duplin. (C) Interaction of STAT3 with Duplin in intact cells. The lysates (20 μg of protein) of HEK-293T cells expressing HA-Duplin and STAT3 were probed with anti-HA and anti-STAT3 antibodies (lanes 1 and 2). The lysates (180 μg of protein) were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were probed with the anti-HA and anti-STAT3 antibodies (lanes 3 and 4). After HEK-293T cells expressing HA-Duplin were untreated or treated with LIF (20 ng/ml) for 30 min, nuclear extracts were prepared. The nuclear extracts (20 μg of protein) were probed with anti-STAT3, anti-phospho-STAT3 (Tyr^{705}), and anti-HA antibodies (lanes 5 and 6). The extracts (180 μg of protein) were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were probed with anti-STAT3, anti–phospho-STAT3 (Tyr^{705}), and anti-HA antibodies (lanes 7 and 8). (D) Electrophoretic mobility shift assay. Nuclear extracts (8 μg of protein) from HEK-293T cells expressing STAT3 and HA-Duplin or HA-Duplin-ΔNLS were incubated with a ^{32}P-labeled oligonucleotide containing the STAT3-binding sequence in the presence or absence of anti-STAT3 antibody or 100-fold excess of cold probe. The arrowhead indicates the position of the STAT3-DNA complex.

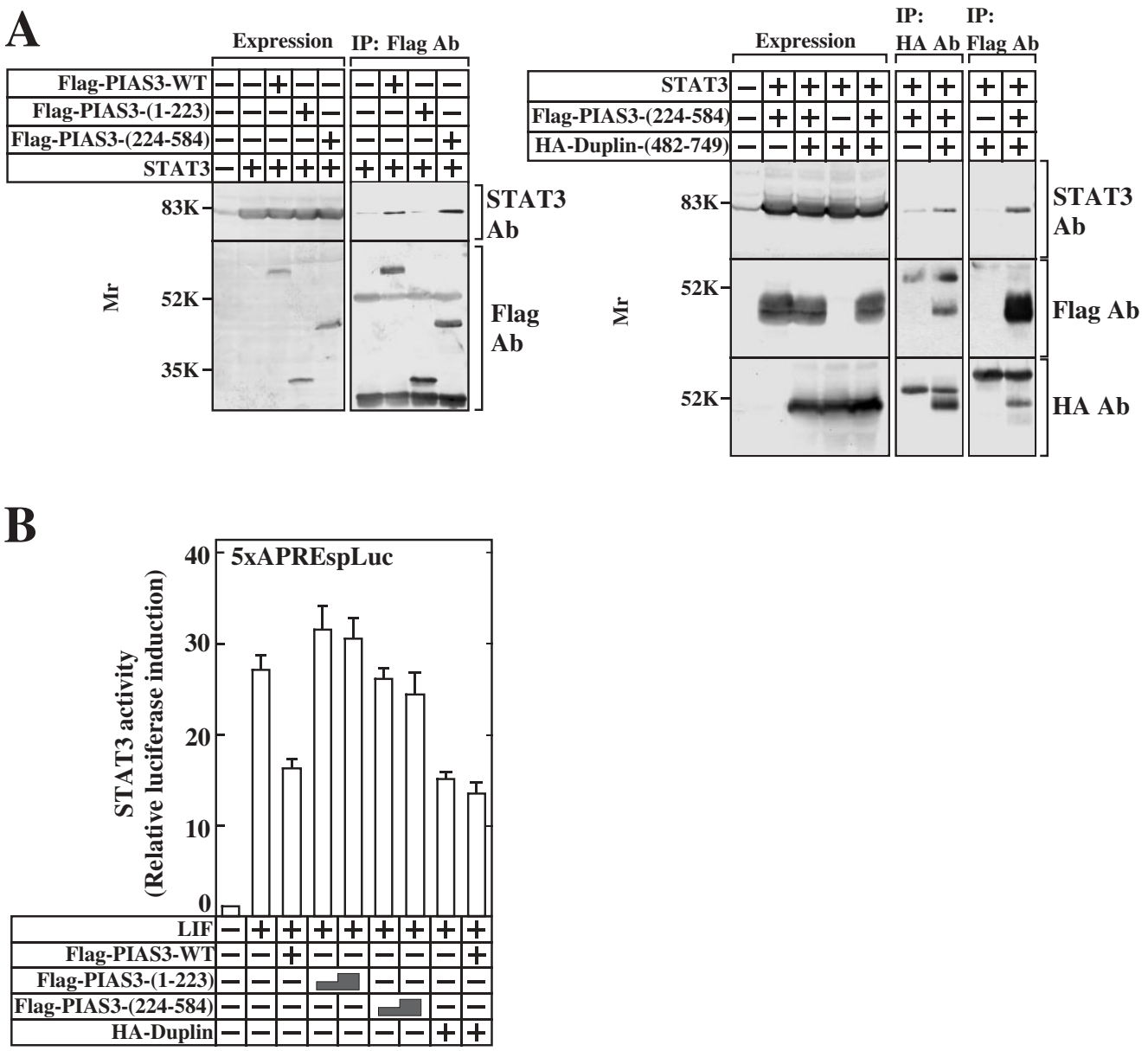

Fig. 4. **Inhibition of STAT3 activity by Duplin and PIAS3.** (A) Complex formation between STAT3, Duplin, and PIAS3. (Left panel) The lysates (20 μg of protein) of HEK-293T cells expressing the indicated proteins were probed with anti-STAT3 and anti-Flag antibodies. The lysates (180 μg of protein) were immunoprecipitated with anti-Flag antibody, and the immunoprecipitates were probed with anti-STAT3 and anti-Flag antibodies. (Right panel) The lysates (20 μg of protein) of HEK-293T cells expressing the indicated proteins were probed with anti-STAT3, anti-Flag, and anti-HA antibodies. The lysates (180 μg of protein) were immunoprecipitated with anti-HA or anti-Flag antibody and the immunoprecipitates were probed with anti-STAT3, anti-Flag, and anti-HA antibodies. (B) Inhibition of STAT3-mediated gene expression by PIAS3 and Duplin. pCMV5-Flag/PIAS3 WT (0.25 μg), pCMV5-Flag/PIAS3-(1-223) (0.25 μg or 0.5 μg), or pCMV5-Flag/PIAS3-(224-584) (0.25 μg or 0.5 μg), and pCGN/Duplin (0.5 μg) were transfected with 5xAPREspLuc (0.5 μg) into HEK-293T cells. Twenty-four hours after transfection, cells were either left untreated or were treated with LIF (20 ng/ml) for 6 h. The luciferase activity was measured and expressed relative to the level in cells not treated with LIF.

The activity of STAT3 has been shown to be regulated by interaction with other proteins. The C-terminal transactivation domain of STAT3 interacts with CBP/p300, which is a histone acetyltransferase (*30*). The forkhead transcriptional factor FKHR binds to STAT3 and enhances the activity of STAT3-dependent promoters (*31*). The nuclear zinc-finger protein EZI also interacts with STAT3 and enhances the transactivation of STAT3 by keeping it in the nucleus (*32*). In this study we found that Duplin has another function by which it inhibits the LIF-dependent STAT3 transcriptional activity. Although Duplin affected neither the LIF-induced tyrosine phosphorylation nor the nuclear localization of STAT3, it formed a complex with STAT3 and inhibited its DNA-binding activity. The inhibitory activity of Duplin toward STAT3 required its nuclear localization

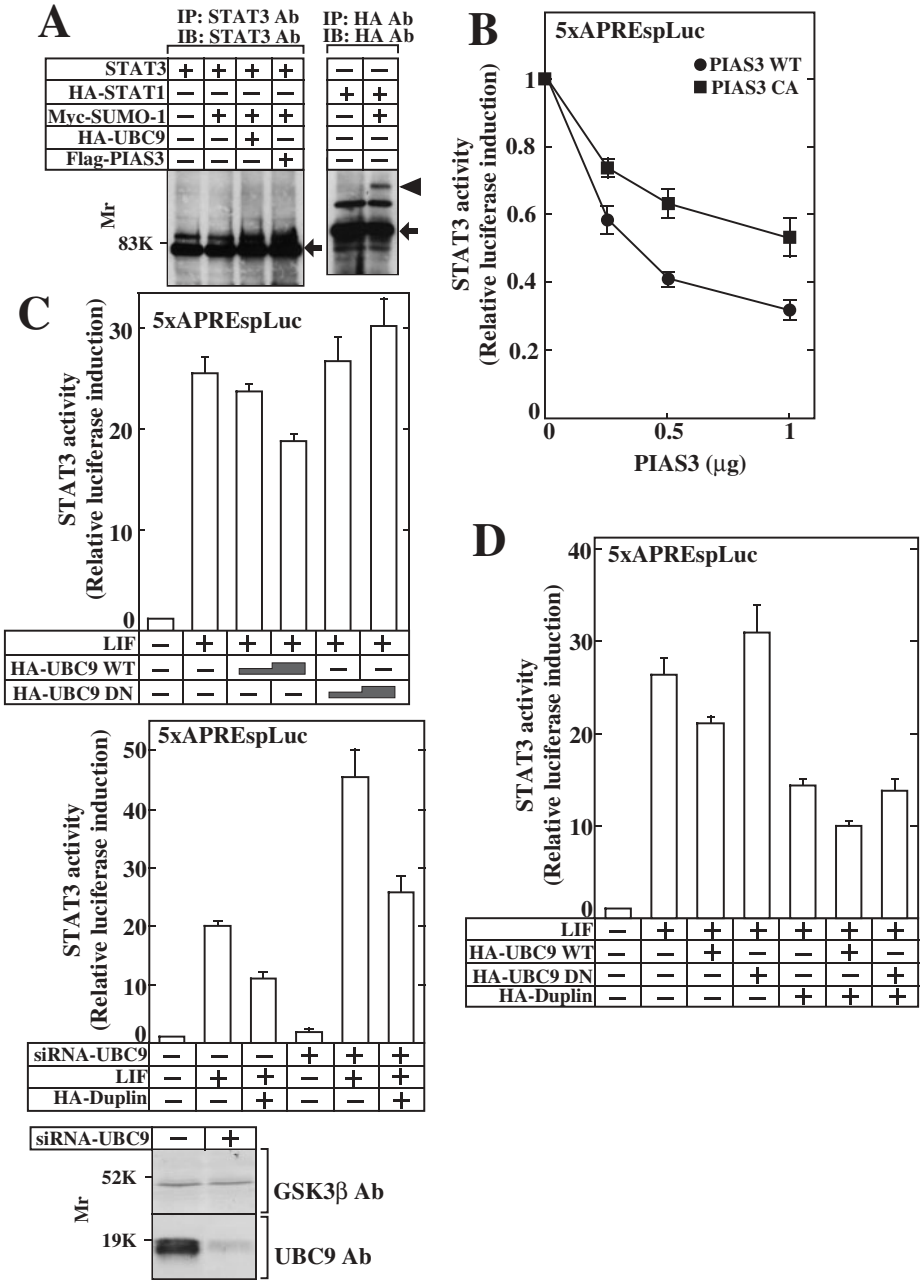

Fig. 5. **Inhibition of STAT3 activity through sumoylation.** (A) STAT3 is not modified with SUMO-1 in intact cells. pCAG/STAT3 (1 μg) or pCAG-HA/STAT1 (1 μg) was transfected into HEK-293T cells with or without pBJ-Myc/SUMO-1 (0.5 μg), pCMV5-Flag/PIAS3 (0.2 μg), or pCGN/UBC9 (0.5 μg). Cell lysates (200 μg of protein) prepared by extraction with RIPA buffer were immunoprecipitated with anti-STAT3 or anti-HA antibody, and the immunoprecipitates were probed with anti-STAT3 or anti-HA antibody. Arrows indicate the position of STAT3 (left panel) and HA-STAT1 (right panel). Arrowhead indicates the slowly migrating form of HA-STAT1. (B) E3 ligase activity of PIAS3 is partially involved in the inhibition of STAT3 activity. Various amounts of pCMV5-Flag/PIAS3 or pCMV5-Flag/PIAS3 CA and 5xAPREspLuc (0.5 μg) were transfected into HEK-293T cells. Twenty four hours after transfection, cells were either left untreated or were treated with LIF (20 ng/ml) for 6 h. The luciferase activity was measured and expressed relative to the level in cells transfected without pCMV5-Flag/PIAS3 or pCMV5-Flag/PIAS3 CA. (C) Effect of UBC9 on STAT3 activity. (Upper panel) pCGN/UBC9 WT or pCGN/UBC9$^{C93R/L97A}$ (0.5 μg or 1 μg) and 5xAPREspLuc (0.5 μg) were transfected into HEK-293T cells. The assay conditions were the same as those in (B). (Middle panel) HeLaS3 cells were transfected with or without double stranded RNA oligos for UBC9. Forty-eight hours after transfection, pCGN/Duplin (0.5 μg) and 5xAPREspLuc (0.5 μg) were transfected into the cells, and the cells were either left untreated or treated with LIF (20 ng/ml) for 6 h. The luciferase activity was measured and expressed relative to the level in the untreated cells. (Bottom panel) Reduction of the protein levels of UBC9 by RNAi. (D) Inhibition of STAT3 activity by Duplin and UBC9. pCGN/UBC9 WT or pCGN/UBC9$^{C93R/L97A}$ (1 μg) and/or pCGN/Duplin (0.5 μg) were transfected with 5xAPREspLuc (0.5 μg) into HEK-293T cells. The assay conditions were the same as those in (B).

signal but not the β-catenin–binding domain. Therefore, Duplin inhibits Tcf-4 and STAT3 by different mechanisms. The LIF-STAT3 signaling plays a role during embryogenesis. It has been reported that STAT3 induces the expression of p55α and p50α, which are regulatory subunits for a p110 catalytic subunit and inhibit phosphatidylinositol 3 kinase (PI3K) activity, and thereby downregulate PI3K-mediated survival signaling (*33*). The development of Duplin-deficient mice is arrested at gastrulation with massive apoptosis (*9*), suggesting that reduction of Duplin induces apoptosis. Therefore, Duplin and STAT3 may regulate apoptosis and survival cooperatively.

The C-terminal regions of Duplin and PIAS3 were necessary for their nuclear localization, and these proteins bound to STAT3 in the nucleus. Although Duplin, PIAS3, and STAT3 formed a complex, cooperative inhibitory action of Duplin and PIAS3 toward STAT3 was not observed. Since Duplin itself is able to inhibit the binding of STAT3 to DNA, the interaction of Duplin with PIAS3 may not function in the inhibition of STAT3. The functional significance of the complex between Duplin and PIAS3 is currently under investigation.

We showed that STAT3 is not modified with SUMO and that PIAS3 does not affect the sumoylation of STAT3, in accord with the findings in a recent report (*34*). Therefore, STAT3 is not regulated by sumoylation directly. However, mutations in the RING domain of PIAS3 partially suppressed the inhibition of STAT3 by PIAS3. Overexpression of UBC9 inhibited the STAT3 activity, and overexpression of a dominant negative form of UBC9 and siRNA for UBC9 enhanced the STAT3 activity. Therefore, it seems that SUMO regulates the STAT3 activity by modifying other proteins involved in the STAT3 action. Although we do not know a target protein of PIAS3 in the STAT3 pathway, it is generally known that SUMO modification of some transcriptional components allows them to repress transcription by recruiting histone deacetylases that in turn generate a transcriptionally repressive chromatin environment (*35*). Our preliminary results showed that Trichostatin A, which is an inhibitor of histone deacetylases, enhances LIF-dependent STAT3 activity (data not shown), suggesting that the acetylation of histone regulates the transcriptional activity of STAT3. However, it is unlikely that histone deacetylases are involved in the PIAS3-dependent inhibition of STAT3, because Trichostatin A did not affect the inhibition of STAT3 by PIAS3 (data not shown). A target protein of PIAS3 in the STAT3 pathway remains to be clarified. On the other hand, since Duplin still inhibited the STAT3 activity upregulated by the inactivation of UBC9, Duplin inhibits STAT3 by a different mechanism from sumoylation. Further studies will be necessary for understanding the whole picture of the inhibitory mechanism of gene expression by Duplin.

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