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Title: Regulation of STAT3-mediated signaling by LMW-DSP2

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Running title: Regulation of STAT3-mediated signaling by LMW-DSP2

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Abstract

Signal transducer and activator of transcription 3 (STAT3), which mediates biological actions in many physiological processes, is activated by cytokines and growth factors, and has been reported to be constitutively activated in numerous cancer cells. In this study, we examined whether low molecular weight-dual specificity phosphatase two (LMW-DSP2) is involved in the regulation of the interleukin 6 (IL-6)-/leukemia inhibitory factor (LIF)-/STAT3-mediated signaling pathway. IL-6/LIF induced LMW-DSP2 expression in murine testicular or hepatoma cell lines, while LMW-DSP2 overexpression in 293T cells suppressed IL-6-induced phosphorylation and activation of STAT3. Furthermore, LMW-DSP2 suppressed the expression of IL-6-induced endogenous genes. In contrast, small-interfering RNA-mediated reduction of LMW-DSP2 expression enhanced IL-6-induced STAT3-dependent transcription. In fact, LMW-DSP2 interacted with STAT3 in vivo and endogenous LMW-DSP2 bound to STAT3 in murine testicular GC-1 cells. These results strongly suggest that LMW-DSP2 acts as a negative

regulator of the IL-6/LIF/STAT3-mediated signaling pathway.

The signal transducer and activator of transcription (STAT) is known to mediate cell proliferation, differentiation and survival in immune responses, hematopoiesis, neurogenesis and other biological processes (Darnell et al.,1994; Ihle,1996; O'Shea,1997). For example, STAT3 is involved in the epithelial-mesenchymal transition during gastrulation, organogenesis, wound healing and cancer progression (Levy et al.,2002). Constitutive or dysregulated expression of STATs has been identified in cancer cells and oncogene-transfected cells and also shown to be involved in a wide range of other diseases, including autoimmune diseases (Levy et al.,2002;Bromberg et al.,2000). Therefore, STAT activation is tightly regulated by a variety of mechanisms. The protein inhibitor of activated STAT (PIAS) family of proteins decreases STAT-dependent transcription by blocking STAT-DNA binding in the nucleus (Shuai et al.,2003). Suppressor of cytokine signaling (SOCS) proteins are induced by STATs and play roles in the negative feedback of STAT activation (Yasukawa et al.,2000). Cytoplasmic tyrosine phosphatases, such as SH2-containing phosphatase 1 (SHP1), SHP2 and protein-tyrosine phosphatase 1B (PTP1B), also prevent further STAT activation in the cytoplasm (Shuai et al.,2003; Yasukawa et al.,2000). Nuclear tyrosine

phosphatases, such as TC45, dephosphorylate nuclear STATs, thereby allowing them to return to the cytoplasm (Shuai et al.,2003). We further reported that the nuclear isoform of TC-PTP was a potential negative regulator of interleukin 6 (IL-6)-mediated signaling, through STAT3 dephosphorylation and deactivation, as well as prolactin/STAT5-mediated signaling (Aoki et al.,2002;Yamamoto et al.,2002).

Dual specificity phosphatases (DSPs)/MAP kinase phosphatases (MKPs) are known to regulate MAP kinase-mediated signaling pathways, including ERK, JNK or p38 MAPK (Alonso et al.,2003). In previous studies, we cloned a distinct class of low molecular weight DSPs (LMW-DSPs) (Aoyama et al.,2001;Aoki et al.,2001) that contain a single catalytic domain, but lack a putative common docking site for MAPKs, designated the *cdc25* homology domain. The first LMW-DSP to be cloned was the VH1 protein from the *Vaccinia* virus (Guan et al.,1991). A related phosphatase was cloned in mammalian cells and designated VHR, for VH1-related (Ishibashi et al.,1992). Both VH1 and VHR differ from other DSPs in that they are much smaller (19 and 21 kDa, respectively). VH1 has been reported to dephosphorylate both MAP kinases and STAT1

(Najarro et al.,2001), while VHR appears to be specific for ERK and JNK (Denu et al.,1995;Alonso et al.,2001). LMW-DSP2 was found to dephosphorylate and deactivate p38 MAPK and JNK, but not ERK (Aoyama et al.,2001). LMW-DSP2 has also been referred to as VHX (Alonso et al.,2002), JSP1 (Shen et al.,2001) and JKAP (Chen et al.,2002). However, the physiological functions of LMW-DSPs have remained unclear, since they appear to be less efficient than many other MAPK-specific DSPs.

Dephosphorylation of activated STATs is one of the key regulatory mechanisms in cytokine signaling. In a previous study, we showed that the nuclear isoform of TC-PTP dephosphorylated PRL-activated STAT5a and STAT5b and IL-6/leukemia inhibitory factor (LIF)-activated STAT3 (Aoki et al.,2002;Yamamoto et al.,2002). As mentioned above, we also cloned a new class of DSPs (LMW-DSP-1, -DSP2, -DSP4, -DSP6, -DSP10 and -DSP11) from a mouse testis cDNA library and found that they were specifically and abundantly expressed in the testes (Aoyama et al.,2001;Aoki et al.,2001). Recently, LMW-DSP2 was found to belong to the subfamily of small DSPs related to the *Vaccinia virus* VH1 phosphatase (Aoyama et al.,2001;Guan et

al.,1991;Alonso et al.,2002;Shen et al.,2001;Chen et al.,2002). The finding that VHI phosphatase blocked interferon (IFN)- γ signaling by dephosphorylating STAT1 (Najarro et al.,2001) led us to examine whether LMW-DSP2 is involved in the regulation of the STAT3-mediated signaling pathway.

To investigate the involvement of LMW-DSP2 in STAT3-mediated signaling, we first examined whether LMW-DSP2 expression was regulated by the IL-6 family of cytokines in the mouse testicular cell line GC-1 and mouse hepatoma cell line Hepa 1-6 using RT-PCR. As shown in Figure 1a (left panel), immediate early induction of LMW-DSP2 mRNA expression (at 15 min) was observed in GC-1 cells after treatment with LIF. Furthermore, IL-6 stimulated LMW-DSP2 mRNA expression in Hepa 1-6 cells at an immediate early time point, similar to the case with LIF (Figure 1b; left panel). We could also observe the LIF/IL-6/STAT3-mediated SOCS3 mRNA expression (at 30 min) in these cells (Figure 1a and b; left panels). When we monitored STAT3 phosphorylation in aliquots of these cell extracts after similar treatments with LIF or IL-6, LIF and IL-6 each stimulated phosphorylation of Tyr705 in STAT3 at 15 min after

stimulation in both cell types (Figure 1a and b; right panels). However, neither LIF nor IL-6 treatment showed any significant induction of Ser727 phosphorylation in STAT3. We attempted to examine the changes of protein level of LMW-DSP2. Unfortunately, we could not detect the endogenous protein of LMW-DSP2 in the total lysates of these cells, because of the low detection sensitivity of anti-LMW-DSP2 antibody immunoblotting. We also tested whether very early induction of LMW-DSP2 by IL-6 or LIF is dependent on the ERK pathway using an ERK inhibitor, U0126. Treatment of U0126 in GC-1 cells resulted in a reduction of IL-6 or LIF-induced LMW-DSP2 expression (data not shown). These results indicate that LMW-DSP2 mRNA expression is induced by LIF or IL-6 in mouse testicular and hepatoma cells through the ERK pathway.

To examine whether LMW-DSP2 has any effects on STAT3-mediated transcriptional activation, we used transient transfection assays. The STAT3-mediated transcriptional responses were measured by using STAT3-LUC, in which the β 2-microglobulin promoter drives expression of a luciferase (LUC) reporter gene (Nakajima et al., 1996).

293T cells transfected with STAT3-LUC were treated with LIF, and the LUC activities were determined. When cells were co-transfected with LMW-DSP2, the transcriptional activation of STAT3-LUC decreased in a dose-dependent manner compared with that of mock vector-transfected cells (Figure 2a). Two amino acids in LMW-DSP2, namely Asp (D)-57 and Cys (C)-88, have previously been shown to participate in the catalytic mechanism of DSP activity (Aoyama et al., 2001). Wild-type (WT) as well as catalytically inactive Asp/Ala (D/A) and Cys/Ser (C/S) forms of LMW-DSP2 were also co-transfected into 293T cells. No decreases in STAT3 activation were observed when the cells were co-transfected with LMW-DSP D/A and C/S (Figure 2b), suggesting that the phosphatase activity of LMW-DSP2 is essential for STAT3 deactivation.

We further examined whether LMW-DSP2 acts as an inhibitor of IL-6/STAT3-mediated transcriptional activation. To examine the effect of LMW-DSP2 on IL-6-mediated transcriptional activation through STAT3, we performed transient transcription assays using Hep3B and HeLa cells. The IL-6-mediated STAT3 transcriptional responses were measured by STAT3-LUC, as described above. As shown in Figure 2c

and d, expression of LMW-DSP2 WT, but not LMW-DSP2 D/A, deactivated IL-6-induced transcriptional activation of STAT3-LUC in a dose-dependent manner in both Hep3B and HeLa cells. In HeLa cells, IL-6 treatment induced STAT3-mediated endogenous SOCS3 mRNA expression (Figure 2e). Next, we tested the effect of LMW-DSP2 on the endogenous SOCS3 mRNA expression induced by IL-6. As shown in Figure 2e, RT-PCR analyses revealed that IL-6-induced endogenous SOCS3 mRNA expression was decreased in HeLa cells transfected with LMW-DSP2, but not a mock vector. These results suggest that LMW-DSP2 acts as an inhibitor of IL-6-induced transcriptional activation of STAT3 in Hep3B and HeLa cells.

To further explore whether LMW-DSP2 regulates STAT3-mediated transcriptional activation, we used small interfering RNA (siRNA) to reduce the endogenous expression of LMW-DSP2 in HeLa cells. HeLa cells were transfected with a specific siRNA for LMW-DSP2 or a control siRNA, and aliquots of total RNA isolated from the transfected cells were subjected to RT-PCR analysis, which confirmed reductions in LMW-DSP2 mRNA expression. Next, we determined the effects of these siRNAs on

IL-6-induced STAT3-LUC activation in HeLa cells. As shown in Figure 2f and g, siRNA-mediated reduced expression of LMW-DSP2 resulted in a significant enhancement of IL-6-induced STAT3-LUC activation and IL-6-induced SOCS3 mRNA expression, strongly suggesting that LMW-DSP2 regulates STAT3-mediated transcriptional activation in HeLa cells. Similarly, we examined the effect of LMW-DSP2 siRNA on interferon- or erythropoietin-induced STAT activation in HeLa cells (data not shown). However, we could not observe any significant enhancement of these cytokine signaling, suggesting that LMW-DSP2 specifically acts STAT3-mediated signaling.

Next, we assessed the changes in STAT3 phosphorylation, which triggers its activation, in 293T cells. LMW-DSP2 WT or D/A was co-transfected with Myc-tagged STAT3 into 293T cells. Time course analyses of LIF-induced STAT3 phosphorylation demonstrated that, upon co-expression of LMW-DSP2 WT, ligand-induced tyrosine- or serine-phosphorylation of STAT3 was remarkably decreased, compared with the levels in mock vector- and LMW-DSP2 D/A-transfected cells (Figure 3a). We next examined

LMW-DSP2 siRNA on IL-6-induced phosphorylation of STAT3 in HeLa cell. As shown in Fig. 3b LMW-DSP2 siRNA treatment significantly enhanced IL-6-induced phosphorylation of STAT3. These results indicate that LMW-DSP2 dephosphorylates STAT3 and negatively regulates LIF-mediated STAT3 transcriptional activation in 293T cells. A STAT3 phosphatase, TC-PTP was also shown to dephosphorylate Jak kinases to regulate cytokine signaling (Simoncic et al.,2002). Indeed, we could observe dephosphorylation of tyrosine-phosphorylated Jak2 by overexpression of LMW-DSP2 in 293T cells (data not shown), suggesting that LMW-DSP2 regulates IL-6/LIF-mediated signaling through dephosphorylation of Jaks and STAT3.

We further assessed the effect of LMW-DSP2 on the nuclear translocation of STAT3. Expression vectors for FLAG-tagged STAT3 and/or Myc-tagged LMW-DSP-2 WT or D/A were transfected into Hep3B cells. At 48 h after the transfection, the cells were left untreated or treated with IL-6. As shown in Figure 3b (right panels), STAT3 translocated into the nucleus of Hep3B cells after 30 min of stimulation with IL-6. In a previous study, we used indirect immunofluorescence staining to show that LMW-DSP2 was

localized throughout the cytoplasm and nucleus in COS7 cells (Aoyama et al.,2001). In Hep3B cells, LMW-DSP2 was also localized throughout the cytoplasm and nucleus, but was predominantly present in the cytoplasm. Next, we examined the co-localization of LMW-DSP2 with STAT3 in Hep3B cells. As shown in Figure 3b, STAT3 failed to translocate into the nucleus when co-transfected with LMW-DSP2 WT, but not LMW-DSP2 D/A, although the staining pattern of the translocated STAT3 tended to be diffuse, even after co-transfection with LMW-DSP2 D/A. Therefore, overexpression of LMW-DSP2 inhibits the nuclear translocation of STAT3 mainly through dephosphorylation of STAT3. We also tested the effect of siRNA on STAT3 nuclear translocation. However, we could not detect a significant enhanced nuclear accumulation of STAT3 by siRNA treatment.

One of the mechanisms consistent with the above-described data is a direct interaction between STAT3 and LMW-DSP2, which triggers its deactivation. We first tested this possibility by co-immunoprecipitation experiments. Expression vectors encoding Myc-tagged LMW-DSP2 WT together with or without FLAG-tagged wild-

type STAT3 (STAT3 WT) or STAT3 YF, which has a substitution of Tyr(Y)-705 for Phe (F) (Nakajima et al.,1996) were transiently transfected into 293T cells. At 36 h after transfection, the transfected 293T cells were stimulated with or without LIF and then lysed, immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates obtained were analyzed by western blotting with an anti-Myc antibody. As shown in Figure 4a, LMW-DSP2 bound to STAT3WT but not to STAT3 YF, suggesting this interaction occurs in a phosphotyrosine-dependent manner. We next tested the interaction of STAT3 with a series of LMW-DSP2 mutants. Expression vectors encoding FLAG-tagged STAT3 and Myc-tagged LMW-DSP2 WT or its inactive mutants D/A or C/S were transiently transfected into 293T cells. As shown in Figure 4b, STAT3 interacted with each of LMW-DSP2 WT, D/A and C/S. Although LMW-DSP2 C/S showed a slightly stronger interaction with STAT3 than LMW-DSP2 WT, LMW-DSP2 D/A showed a weaker interaction with STAT3 than both LMW-DSP2 WT and C/S, suggesting that Asp-57 may be close to the binding site of STAT3 on LMW-DSP2.

To further confirm that endogenous LMW-DSP2 interacts with STAT3 in vivo, cell

extracts obtained from LIF-stimulated GC-1 cells were subjected to co-immunoprecipitation experiments. As shown in Figure 4c, anti-LMW-DSP2 immunoprecipitates of GC-1 cell extracts contained the STAT3 protein. This result suggests that endogenous LMW-DSP2 interacts and forms a complex with STAT3 in GC-1 cells.

In this study, for the first time, we have demonstrated that LMW-DSP2 is an important regulator of STAT3 functions in the downstream of IL-6/LIF signaling, and may thus play critical roles in the progression of IL-6-related diseases. More detailed understanding of the interaction between STAT3 and LMW-DSP2 is therefore important, since this new information may allow the development of novel therapeutic approaches for these conditions.

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References

Alonso A, Burkhalter S, Sasin J, Tautz L, Bogetz J, Huynh H, Bremer MC, Holsinger LJ,

Godzik A, and Mustelin T. (2004) *J. Biol. Chem.*, **279**, 35768-35774.

Alonso A, Merlo JJ, Na S, Kholod N, Williams S, Posada J, and Mustelin T. (2002) *J. Biol. Chem.*, **277**, 5524-5528

Alonso A, Narisawa S, Bogetz J, Tautz L, Hadzic R, Huynh H, Williams S, Alonso A,

Rojas A, Godzik A, and Mustelin T. (2003) *Top. Curr. Genet.*, **5**, 333-358

Alonso A, Saxena S, Williams S, and Mustelin T. (2001) *J. Biol. Chem.*, **276**, 4766-4771

Aoki N, and Matsuda T. (2002) *Mol. Endocrinol.*, **16**, 58-69.

Aoki N, Aoyama K, Nagata M, and Matsuda T. (2001) *J. Biochem.*, **130**, 133-140

Aoyama K, Nagata M, Oshima K, Matsuda T, and Aoki N. (2001) *J. Biol. Chem.*, **276**,
27575-27583,

Bromberg J, and Darnell Jr JE. (2000) *Oncogene*, **19**, 2468-2473

Chen AJ, Zhou G., Juan T, Colicos SM, Cannon JP, Cabrera-Hansen M, Meyer CF,

Jurecic R, Copeland NG., Gilbert DJ, Jenkins NA, Fletcher F, Tan TH, and Belmont JW.

(2002) *J. Biol. Chem.*, **277**, 36592-366601

Darnell Jr JE, Kerr IM, and Stark GR. (1994) *Science*, **264**, 1415-1421

Denu JM, and Dixon JE. (1995) *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 5910-5914

Guan KL, Bryoles SS, and Dixon JE. (1991) *Nature*, **350**, 359-362

Ihle JN. (1996) *Cell*, **84**, 331-334

Imoto S, Sugiyama K, Muromoto R, Sato N, Yamamoto T, and Matsuda T. (2003) *J.*

Biol. Chem., **278**, 34253-34258

Ishibashi T, Bottaro DP, Chan A, Kiki T, and Aaronson SA. (1992) *Proc. Natl. Acad. Sci.*

U.S.A., **89**, 12170-12174

Levy DE, Lee CK. (2002) *J. Clin. Invest.*, **109**, 1143-1148

Muromoto R, Sugiyama K, Takachi A, Imoto S, Sato N, Yamamoto T, Oritani K,

Shimoda K, and Matsuda T. (2004) *J. Immunol.*, **172**, 2985-2993

Najarro P, Traktman P, and Lewis JA. (2001) *J. Virol.*, **75**, 3185-3196

Nakajima K, Yamanaka Y, Nakae K, Kojima H, Ichiba M, Kiuchi N, Kitaoka T, Fukada

T, Hibi M, and Hirano T. (1996) *EMBO J.*, **15**, 3651–3658.

O'Shea JJ. (1997) *Immunity*, **7**, 1-11

Sekine Y, Yamamoto T, Yumioka T, Sugiyama K, Tsuji S, Oritani K, Shimoda K,

Minoguchi M, Yoshimura A, and Matsuda T. (2005) *J. Biol. Chem.*, **280**, 8188-8196

Shen Y, Luche R, Wei B, Gordon ML, Diltz CD, and Tonks NK. (2001) *Proc. Natl.*

Acad. Sci. U. S. A., **98**, 13613-13618

Shuai K, and Liu B. (2003) *Nat Rev. Immunol.*, **3**, 900-911

Simoncic PD, Lee-Loy A, Barber DL, Tremblay ML, and McGlade CJ. (2002) *Curr*

Biol., **12**, 446-453

Yamamoto T, Sekine Y, Kashima K, Kubota A, Sato N, Aoki N, and Matsuda T. (2002)

Biochem. Biophys. Res. Commun., **297**, 611-617

Yasukawa H, Sasaki A, and Yoshimura A. (2000) *Annu. Rev. Immunol.*, **18**, 143-164

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Figure 1. LIF and IL-6 induced mRNA expression of LMW-DSP2 in testicular and hepatoma cells

Murine testicular cell line, GC-1(a) and hepatoma cell line, Hepa 1-6 (b), were maintained in DMEM containing 10% fetal calf serum (FCS). Cells were treated or untreated with LIF (INTERGEN) (100 ng/ml) or IL-6 (a kind gift from Ajinomoto Co.) (50 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using LMW-DSP2, SOCS3 and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) primers (Aoyama et al.,2001). RT-PCR products were separated on a 1% agarose gel. After the similar treatment with LIF or IL-6, the cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μ M sodium orthovanadate and 1 μ M phenylmethylsulfonyl), and an aliquot of total extracts were examined with Western blot using anti-pSTAT3 (Tyr705), anti-pSTAT3 (Ser727) (Cell signaling Technologies) or anti-STAT3 antibody (Santa Cruz). This figure is representative of three separate experiments.

Figure 2. LMW-DSP2 deactivated LIF- or IL-6-induced STAT3-mediated transcriptional activation

a. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected in a 12-well plate were transfected with STAT3-LUC (Nakajima et al.,1996) (0.4 μ g) and/or indicated amounts of empty vector or expression vector for LMW-DSP2 by the standard calcium precipitation protocol. At 36 h after transfection, the cells were stimulated with LIF (100 ng/ml) for additional 8 h. The stimulated cells were harvested, and luciferase activities were measured (Sekine et al.,2005). b. 293T cells in a 12-well plate were transfected with STAT3-LUC (0.4 μ g) and/or indicated amounts of WT, D/A or C/S of LMW-DSP2 as described the above. c and d. Human hepatoma cell line Hep3B and human cervix carcinoma cell line, HeLa, were maintained in DMEM containing 10 % FCS (Imoto et al.,2003;Muromoto et al2004). Hep3B(c) and HeLa (d) cells in a 12-well plate were transfected with STAT3-LUC (0.5 μ g) and/or indicated amounts of WT or D/A of LMW-DSP2 using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were stimulated with IL-6

(50 ng/ml) for additional 6 h. The stimulated cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). At least three independent experiments were carried out for each assay. e. HeLa cells in a 6-well plate were transfected with empty vector or Myc-LMW-DSP2 WT (0.5 μ g). At 36 h after transfection, the cells were stimulated with or without IL-6 (50 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SOCS3, G3PDH. RT-PCR products were separated on a 1% agarose gel. LMW-DSP2 protein expression level was monitored by Western blot using anti-Myc antibody. This figure is representative of three separate experiments. f. HeLa cells in a 24-well plate were transfected with siRNA targeting human LMW-DSP2 using Lipofectamine2000 (Invitrogen). siRNAs targeting human LMW-DSP2 used in this study were as follows: 5'-CUCAAAACCUGACAAGACAUUUCA-3'. The cells were then transfected with STAT3-LUC using jetPEI (PolyPlus-transfection). At 36 h after transfection, the cells were treated with IL-6 (50 ng/ml) for an additional 6 h. Total RNAs from these cells which treated with IL-6 (50ng/ml) for 30 min were also analyzed by RT-PCR using LMW-DSP2 or G3PDH primers, verifying siRNA-mediated reduction

in endogenous LMW-DSP2. The cells were then 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 S.D. g. HeLa cells were treated with control siRNA or LMW-DSP2 siRNA as described the above and cells were stimulated with IL-6 (50 ng/ml) for 30 min. Total RNA samples isolated from these cells were subjected to RT-PCR analysis as described the above.

Figure 3. LMW-DSP2 inhibits LIF-induced tyrosine-phosphorylation and nuclear translocation of STAT3

a. 293T cells in a 6 well-plate were transfected with Myc-tagged STAT3 (2 μ g) together with empty vector, Myc-tagged LMW-DSP2 WT or D/A mutant (4 μ g). Forty-eight hours after transfection, cells were starved for 3 h, followed by treatment with or without LIF (100 ng/ml) for the indicated periods. The cells were lysed, and then immunoblotted with anti-pSTAT3 (Tyr705) (upper panel), anti-pSTAT3 (Ser727) (middle panel) or anti-Myc antibody (lower panel). b. HeLa cells were treated with control siRNA or LMW-DSP2 siRNA as described the above and cells were stimulated

with IL-6 (50 ng/ml) for 30 min. The cells were lysed, and then immunoblotted with anti-pSTAT3 (Tyr705) (upper panel) or anti-STAT3 antibody (lower panel). c. Hep3B cells were transfected with FLAG-tagged STAT3 (1 μ g). At 30 min after stimulation, the cells were fixed with a solution containing 4% paraformaldehyde and reacted with rabbit anti-FLAG antibody and/or mouse anti-Myc antibody. Hep3B cells were also transfected with FLAG-tagged STAT3 (1 μ g) together with Myc-tagged LMW-DSP2 WT or D/A mutant (1 μ g). At 36 h after transfection, cells were treated with or without IL-6 (50 ng/ml) for 30 min, and then fixed and reacted with anti-FLAG polyclonal antibody and anti-Myc monoclonal antibody, and visualized with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and/or rhodamine-conjugated anti-mouse IgG (Chemicon) and observed under a confocal laser fluorescent microscope (Sekine et al.,2005). At the same time, the nuclei in the cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Wako). Images were obtained by using a Zeiss LSM 510 laser scanning microscope with an Apochromat x63/1.4 oil immersion objective and x4 zoom.

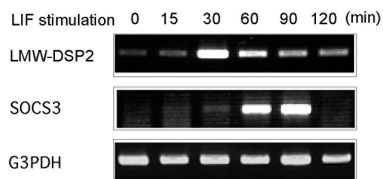
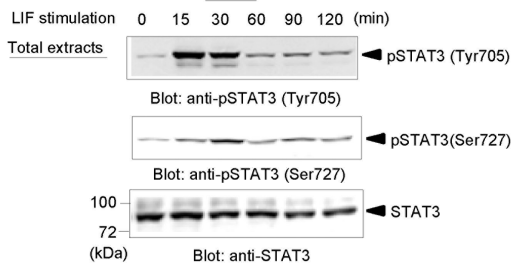
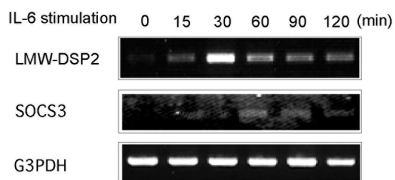
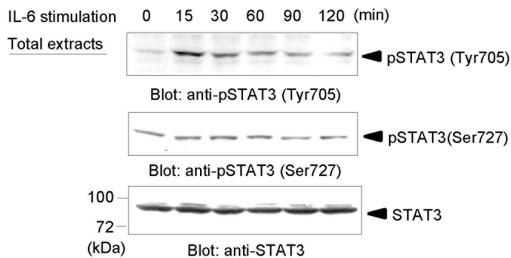
Figure 4. STAT3 and LMW-DSP2 physically interact in vivo

a. 293T cells (1×10^7 cells) were transfected with FLAG-tagged STAT3 WT (7.5 μ g) or STAT3 YF mutant (10 μ g) together with or without WT of LMW-DSP2 (10 μ g). At 36 h after transfection, the cells were stimulated with LIF (100 ng/ml) for additional 8 h. The cells were then lysed, and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-pSTAT3 (Tyr705) (lower panel) or anti-Myc antibody (bottom panel).

b. 293T cells (1×10^7 cells) were transfected with FLAG-tagged STAT3 (7.5 μ g) together with or without WT, D/A or C/S of LMW-DSP2 (10 μ g). At 48 h after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc (bottom panel).

c. Murine testicular GC-1 cells (2×10^7) were stimulated with LIF for 30min and the cells were lysed, and immunoprecipitated with control IgG or anti-LMW-DSP2 antibody and immunoblotted with anti-STAT3 antibody (upper panels) or anti-LMW-DSP2 antibody

(lower panels).

a**GC-1****GC-1****b****Hepa1-6****Hepa1-6****Fig. 1**

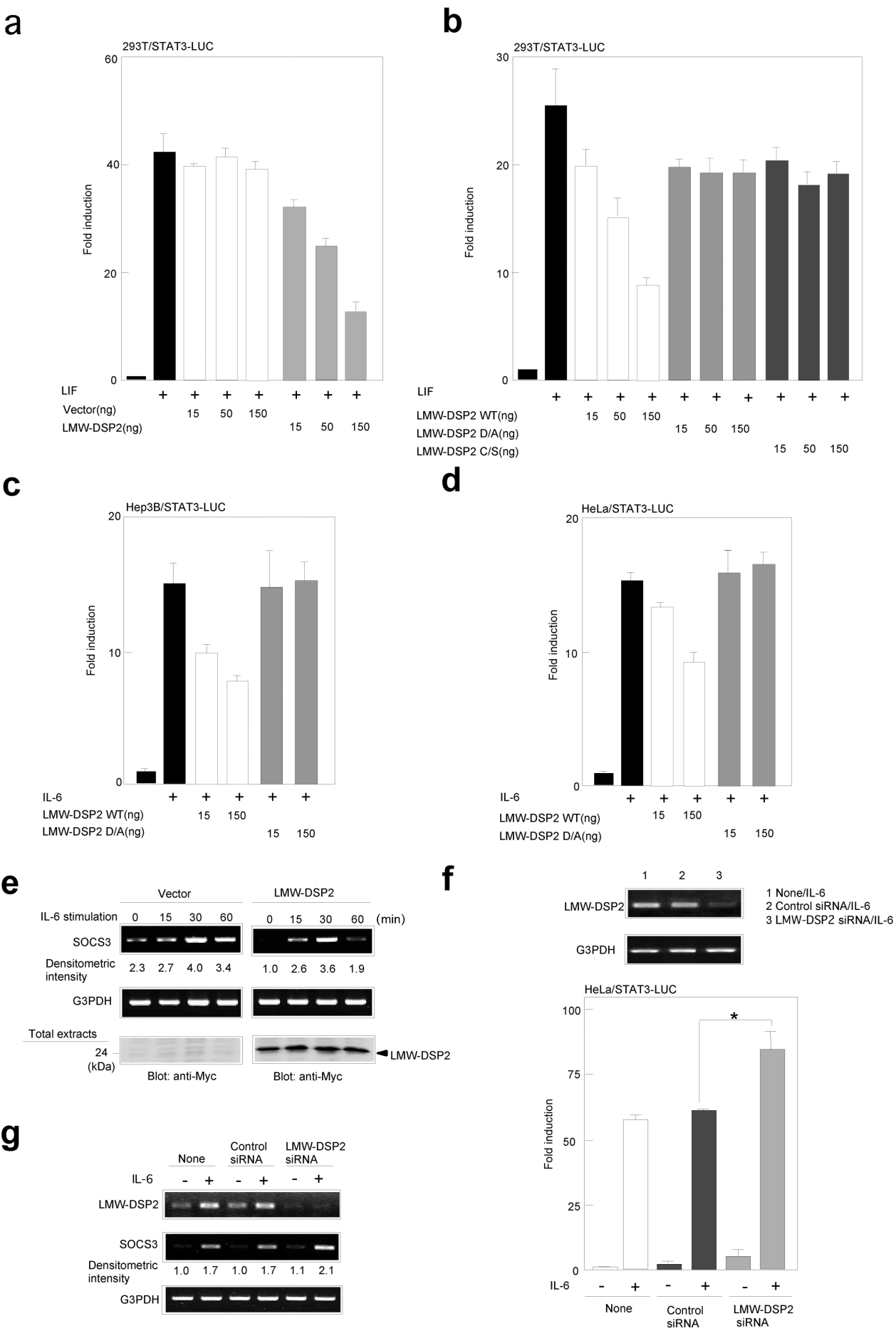
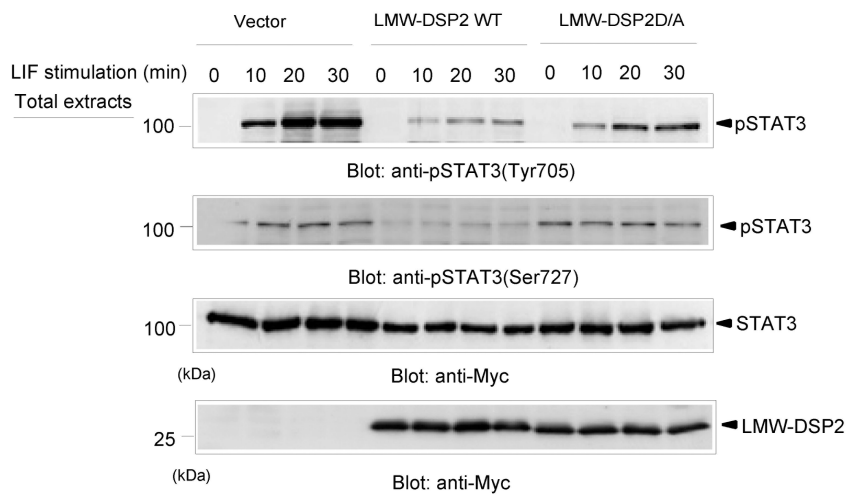
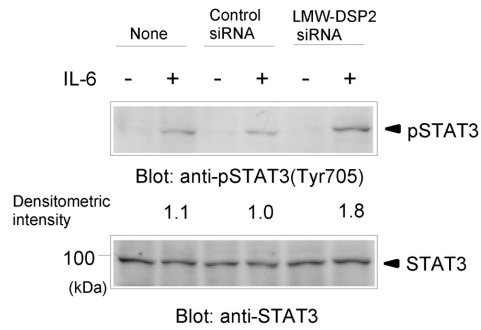
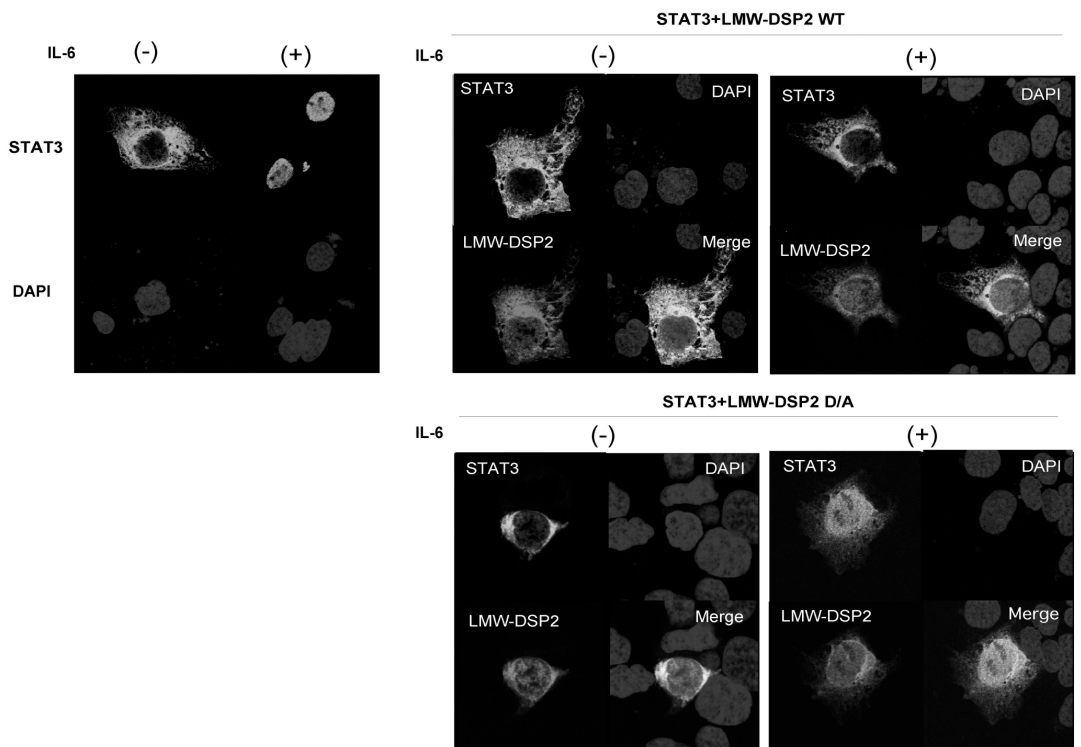
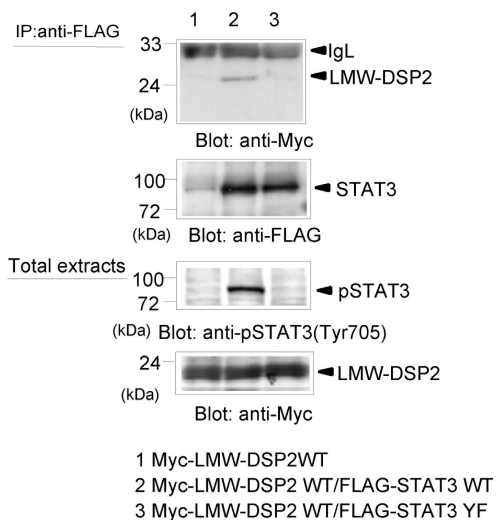
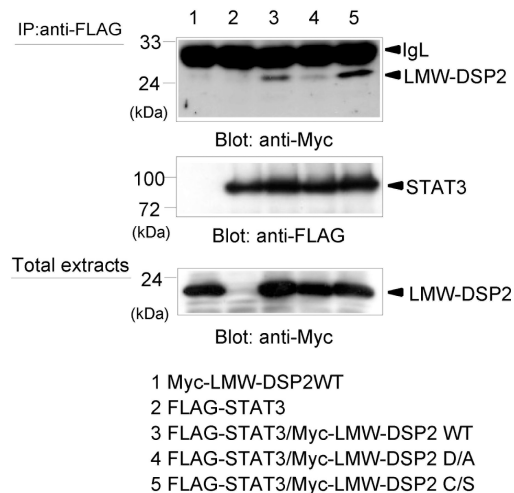
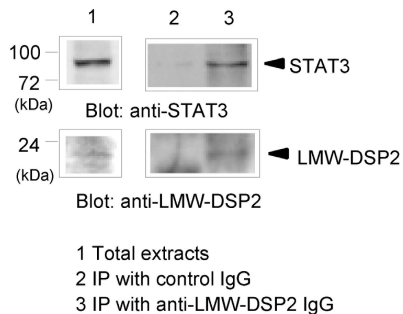


Fig. 2

a**b****c****Fig. 3**

a**b****c****Fig. 4**