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Do-Kyun Kim, PhD, Michael A. Beaven, PhD, Dean D. Metcalfe, MD, Ana Olivera, PhD



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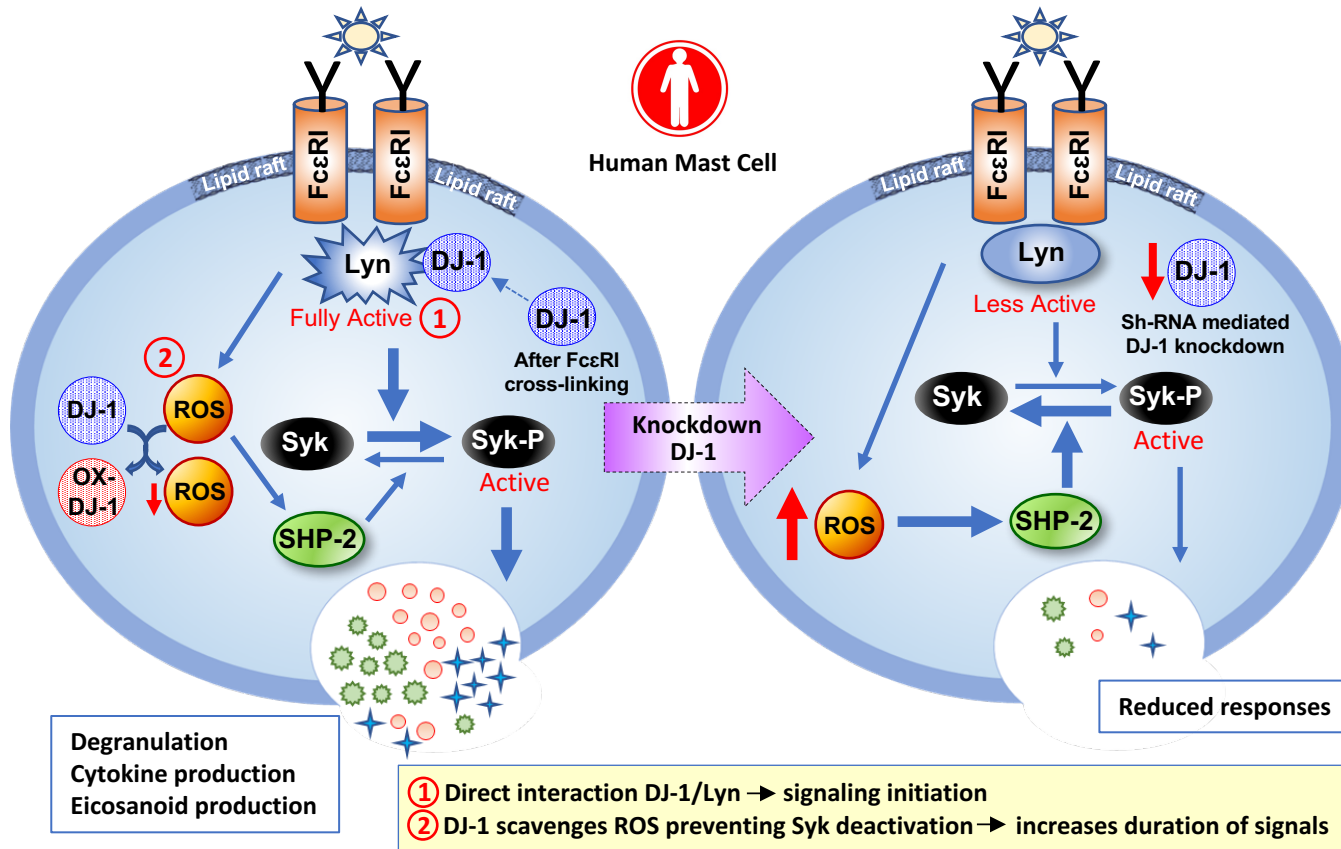
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## DJ-1 is Essential for Maximal IgE-Mediated Activation of Human Mast Cells



TITLE PAGE

Original Article

**Interaction of DJ-1 with Lyn is essential for IgE-mediated stimulation of human mast cells**

Do-Kyun Kim, PhD<sup>a</sup>, Michael A. Beaven, PhD<sup>b</sup>, Dean D. Metcalfe MD<sup>a</sup>, and Ana Olivera PhD<sup>a</sup>

<sup>a</sup> Mast Cell Biology Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

<sup>b</sup> Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

**Corresponding author**

Ana Olivera, PhD

Mast Cell Biology Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, NIAID-NIH, Building 10, Room 11N240, 10 Center Dr., Bethesda, MD, 20892. Fax: 301-480-8384, E-mail: ana.olivera@nih.gov

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**ABSTRACT**

**Background:** DJ-1 is a redox-sensitive protein with multiple roles in cell homeostasis whose levels are altered in mast cell (MC)-related disorders. However, whether DJ-1 can regulate human MC function is unknown.

**Objective:** We sought to investigate the potential role of DJ-1 in the responses of human MCs to antigen stimulation.

**Methods:** DJ-1 was silenced in human CD34<sup>+</sup>-derived and LAD2 MCs using lentiviral sh-RNA constructs. Release of  $\beta$ -hexosaminidase, PGD<sub>2</sub> and GM-CSF and changes in reactive oxygen species (ROS) levels were measured after Fc $\epsilon$ RI engagement. Enzymatic assays, sucrose density gradient centrifugation, immunoprecipitations, dot and Western blots, and confocal imaging were performed for signaling, cellular localization and co-association studies.

**Results:** DJ-1 knockdown substantially reduced mediator release as well as Lyn and Syk kinase activation and signaling by mechanisms which appeared largely unrelated to DJ-1 antioxidant activity. Upon Fc $\epsilon$ RI activation, non-oxidized rather than oxidized DJ-1 translocated to lipid rafts where it associated with Lyn, an interaction that appeared critical for maximal Lyn activation and initiation of signaling. Using purified recombinant proteins, we demonstrated that DJ-1 bound to Lyn directly but no other Src kinases, and this interaction was specific for human but not mouse proteins. In addition, DJ-1 reduced SHP-2 phosphatase activity by scavenging ROS thus preventing Syk dephosphorylation and perpetuating MC signaling.

**Conclusion:** We demonstrate a novel role for DJ-1 in the early activation of Lyn by Fc $\epsilon$ RI that is essential for human MC responses and which provides the basis for an alternative target in allergic diseases therapy.

**CAPSULE SUMMARY:**

We demonstrate a previously unrecognized interaction between DJ-1 and the Src kinase Lyn in antigen-activated human mast cells that is essential for maximal Lyn activation and the release of bioactive allergic and inflammatory mediators.

**KEY MESSAGES:**

- Ligation of FcεRI in human mast cells induces the early translocation of non-oxidized DJ-1 to lipid rafts where it interacts with Lyn enabling its full activation, downstream signaling and mast cell responses
- Later after FcεRI activation, the ability of DJ-1 to become oxidized quenches ROS levels and reduces the activity of the phosphatase SHP-2 which permits further Syk-mediated signaling.
- Further characterization of the interaction DJ-1 with Lyn could form the basis for alternative treatments in allergy.

**KEY WORDS:** Human mast cells, IgE receptor, Lyn activation, DJ-1, signaling, reactive oxygen species, Syk

**ABREVIATIONS**

Cbp - Csk binding protein

Csk - C-terminal Src kinase

FcεRI - High affinity receptor for IgE

GM-CSF - Granulocyte-macrophage colony-stimulating factor

GPCR- G-protein coupled receptors

HuMCs - CD34<sup>+</sup>-derived human mast cells

MC - Mast cells

mBMMC – Murine bone marrow mast cells

PGD<sub>2</sub> - Prostaglandin D<sub>2</sub>

ROS- Reactive Oxygen Species

SA - Streptavidin

SFK - Src family kinase

Syk - Spleen tyrosine kinase

## INTRODUCTION

Antigen-dependent activation of mast cells (MCs) occurs primarily through FcεRI, the high-affinity receptor for IgE, which causes the release and generation of vasoactive and inflammatory mediators of allergic inflammation<sup>1,2</sup>. Understanding the mechanisms that control IgE dependent MC activation is critical to the development of small molecular weight inhibitors of MC responses and to understanding aberrant regulation. It is currently understood that FcεRI aggregated by antigen coalesce within specialized microdomains of the plasma membrane (lipid rafts), and this causes the activation of Src family tyrosine kinases (SFKs), among which Lyn is the most critical for the initial stages of signaling elicitation<sup>3</sup>. Activated Lyn then phosphorylates the β and γ chains of the FcεRI, which respectively leads to recruitment of Lyn and recruitment/activation of spleen tyrosine kinase (Syk), an obligatory step for further propagation and divergence of signaling cascades leading to the release of bioactive mediators<sup>3-5</sup>. Targeting of these receptor-proximal signaling events is preferable since they are common to all antigen-induced MC responses.

We have recently demonstrated altered serum levels of DJ-1, an evolutionary conserved protein reported to protect cells against oxidative damage, in patients with atopic dermatitis patients; and a biphasic regulation in mastocytosis where DJ-1 was induced by IL-6 and implicated in the expansion of neoplastic MCs<sup>6,7</sup>. Although DJ-1 is abundantly expressed in human MC lines<sup>6,7</sup>, it is unknown whether DJ-1 can influence FcεRI-mediated activation of human MCs.

As will be shown, we find that absence of DJ-1 in human MCs (HuMCs) results in marked

103 inhibition of FcεRI-induced degranulation, cytokine and eicosanoid production by mechanisms  
104 which appear independent of its antioxidant activity. We further show a direct interaction of DJ-1  
105 with Lyn in lipid rafts and that this association is required for Lyn activation, initiation of human  
106 MC signaling and functional responses. The interaction with Lyn is specific for non-oxidized  
107 rather than oxidized DJ-1 and unique to the human homologs, since such interaction was not  
108 observed between murine DJ-1 and Lyn proteins. Our data thus details a critical here-to-fore  
109 unknown function for DJ-1 in human MC responsiveness as a novel activator of Lyn and which  
110 may be of therapeutic interest in developing strategies to target the MC compartment.

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## METHODS

### Human Mast Cell Cultures

Primary HuMCs were derived from CD34<sup>+</sup> peripheral blood progenitors isolated from healthy volunteers after informed consent under a protocol (NCT00001756) approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases (NIH). HuMCs and human LAD2 MCs were cultured as described<sup>8,9</sup>. For activation, HuMCs and LAD2 cells were sensitized overnight with biotinylated IgE (0.1 µg/ml), washed and the IgE-bound FcεRI crosslinked with 100 ng/ml streptavidin (SA).

**Knockdown of DJ-1 by sh-RNA.** Viral particles containing non-target (SHC002) or DJ-1-targeted short hairpin RNAs (sh-RNAs) (TRCN0000004921) in a pLKO1 vector were prepared as described<sup>10</sup> and used to transduce HuMC or LAD2. Cells were selected in media containing 0.2 µg/ml puromycin for 5 days<sup>10</sup>.

**Measurement of ROS and DJ-1.** ROS levels were measured with OxiSelect™ Intracellular ROS Assay kit and DJ-1 in supernatants of stimulated cells were measured by ELISA as described<sup>7</sup>. Alternatively, DJ-1 and oxidized DJ-1 were detected by Western blot<sup>7</sup>.

**SHP-1 and SHP-2 phosphatase and in vitro kinase assays.** Phosphatase activities of SHP-1 and SHP-2 in immunoprecipitates from SA-stimulated LAD2 cells were measured by a Duoset IC Phosphatase Assay. Kinase activities in immunoprecipitates of Lyn, Fyn, Fgr or Syk from cell lysates were determined by using an ELISA-based Universal Tyrosine Kinase Assay Kit.

**Isolation of lipid rafts.** LAD2 cells ( $2.0 \times 10^7$ ) were lysed in 2 ml of lysis buffer containing 0.5% Brij 58<sup>11</sup> for 30 min on ice. Lysates were mixed with an equal volume of 80% (w/v) sucrose in buffer. A sucrose gradient was prepared and processed by ultracentrifugation at 270,000g for 18 h at 4°C. Fractions were collected from the top to the bottom of the gradient for Western blot analysis.

**Confocal microscopy.** Non-stimulated and SA-stimulated HuMC or LAD2 cells were seeded into 8-well Lab-Tek chamber slides and centrifuged at 600 rpm for 5 min. After washing, cells were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 for 10 min. Cells were then incubated 1 h with 1% BSA before staining. Confocal images were obtained with a Leica SP5 X confocal laser scanning microscope.

**Statistical analysis.** Data were expressed as mean  $\pm$  SEM of values from 3 or more independent experiments. 1-way ANOVA was used to determine statistically significant differences between groups using Prism 6 software (Graph Pad Software, San Diego, CA, USA). Statistical significance was indicated as follows: \*P <0.05 and \*\*P <0.01.

A detailed description of the methods used in this study can be found in Supplementary Methods section.

## RESULTS

### Non-oxidized DJ-1 translocates to the plasma membrane early after FcεRI activation

In examining the role of DJ-1 in HuMCs, we first verified that activation via FcεRI resulted in a rapid increase in intracellular production of ROS which was maximal by 30 min, slowly declining thereafter (Fig. 1A), as reported<sup>12</sup>. Total intracellular levels of DJ-1 did not change but DJ-1 was visibly oxidized by 30 min at Cys106, the major residue responsible for its antioxidant function (Fig. 1B, upper panels). Intracellular DJ-1 oxidation plateaued by 1 h, coinciding with both DJ-1 secretion into the media and the decline in intracellular ROS levels (Fig 1A and Fig 1B, lower panels). Therefore, a significant antioxidant effect of DJ-1 was only apparent 30 min to 1 h after FcεRI activation.

Additionally, within 10 min after activation, a portion of DJ-1 localized to the plasma membrane and this translocation was independent of any significant oxidation in Cys106 as determined by Western blot analysis and confocal microscopy (Fig. 1B and C). In fact, oxidized DJ-1 appeared distinctly excluded from the plasma membrane at this time point. The redistribution of non-oxidized DJ-1 into the membrane was transient since after 1 h most of the total cellular DJ-1 had been oxidized and appeared in the cytosol or associated with organelles (Fig. 1B and C). By 4 h, oxidized DJ-1 moved to the plasma membrane coinciding with the secretion of DJ-1 into the extracellular media (Fig. 1A). Thus, DJ-1 secretion appears to be dissociated from degranulation which is completed within minutes of activation. Since the oxidized form of DJ-1 is preferentially secreted<sup>13</sup>, the late appearing membrane-localized and now oxidized DJ-1 may well represent molecules in transit to the extracellular space. Of note, the amounts of DJ-1

secreted into the media were only a fraction of the cellular DJ-1 content as indicated in the legend to Fig.1. The temporal changes in ROS as well as DJ-1 oxidation and localization were reproduced in the LAD2 MC line (Supplementary Fig. 1). Overall, these data indicate that the distinct rapid and transient translocation of the non-oxidized DJ-1 to the plasma membrane is unrelated to its scavenger function and may have functional consequences.

**DJ-1 is essential for HuMC responses by mechanisms greatly independent of its antioxidant activity,**

After demonstrating early translocation of DJ-1 to the plasma membrane, we explored whether loss of DJ-1 by shRNA knockdown would have an effect on FcεRI-induced responses. We found that DJ-1 silencing with lentivirus-mediated shRNA resulted in a >75% reduction in DJ-1 (Fig. 2A and Supplementary Fig. 2A and B) without affecting the expression of KIT and FcεRI (Supplementary Fig. 2C). DJ-1 knockdown markedly inhibited FcεRI-mediated degranulation in HuMCs both in the absence (Fig. 2B, blue line) or presence (Fig. 2B, red lines) of stem cell factor (SCF) which enhances FcεRI dependent degranulation<sup>14</sup>. Degranulation induced by thapsigargin, which causes cytosolic Ca<sup>2+</sup> increases by inhibiting Ca<sup>2+</sup> uptake into intracellular stores or by GPCR agonists (Figure 2C) was not affected by the loss of DJ-1 (Fig. 2C), consistent with specificity on FcεRI signaling. FcεRI-mediated release of prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) and GM-CSF (Fig. 2D and F), but not thapsigargin-induced PGD<sub>2</sub> release (Fig. 2E), were also substantially reduced with DJ-1-knockdown compared with non-target shRNAs. DJ-1 knockdown had similar effects on FcεRI dependent responses in LAD2 MCs (Supplementary Fig. 3A-D).

Pretreatment of HuMCs with the general ROS scavenger 2,2,6,6-tetramethylpiperidin- 1-oxyl (TEMPO) prior to MC stimulation reversed the augmented intracellular ROS levels in DJ-1 knockdown cells (Fig. 3A) but did not restore the inhibition of degranulation (Fig. 3B), or PGD<sub>2</sub> (Fig. 3C) and GM-CSF (Fig. 3D) secretion by DJ-1 shRNA silencing. Similarly, the antioxidant TEMPO did not restore FcεRI-dependent responses in LAD2 cells (Supplementary Fig. 3E). Higher concentrations of TEMPO (300 μmol/L) did not have significant additional effects on ROS levels over 100 μmol/L TEMPO (Fig. 3A and Supplementary Fig. 3F) but induced 20% cell death and thus this concentration was not used in the study. The results using concentrations of TEMPO that markedly reduce ROS levels (albeit not totally), are consistent with the conclusion that the defect in FcεRI-induced mediator release in DJ-1 knockdown HuMCs, is largely (about 40%) unrelated to increased intracellular ROS levels due to the loss of DJ-1 antioxidant activity.

#### **Elevated ROS caused by knockdown of DJ-1 result in enhanced activity of SHP-2 but not SHP-1**

DJ-1 has been reported to alter the function of the tyrosine Src homology 2 domain-containing phosphatase 1 (SHP-1) and SHP-2 in mouse bone marrow-derived MCs (mBMMCs), and negatively regulating FcεRI-induced activation<sup>6</sup>. In marked contrast, in human LAD2 cells, FcεRI induced activation of SHP-1 was not affected by DJ-1 knockdown (Fig. 4A). Further, in LAD2 cells, pretreatment with TEMPO (Fig. 4B), or incubation with H<sub>2</sub>O<sub>2</sub> in vitro did not alter activation of SHP-1 (Fig. 4C). This contrasts with mBMMCs, where inhibition of SHP-1

activity by DJ-1 deficiency was reversed by the antioxidant TEMPO and was concluded to be the main mechanism involved in the hypereactive phenotype found in DJ-1 deficient mBMMC<sup>6</sup>.

SHP-2 activation was, in contrast, enhanced in DJ-1 knockdown human LAD2 MCs (Fig. 4D), an effect shared with DJ-1 deficient BMMCs<sup>6</sup>. The enhanced activation of SHP-2 was fully restored by treatment with TEMPO (Fig. 4E), indicating this effect is ROS-mediated. Further confirming this conclusion, H<sub>2</sub>O<sub>2</sub> induced a dose-dependent increase in SHP-2 activity in immunoprecipitates after FcεRI ligation (Fig. 4F). These results indicate that while the role of DJ-1 as a negative regulator of SHP-2 activation is conserved in human and mouse MCs, DJ-1 plays no role in the regulation of SHP-1 in human MCs. Therefore, the antioxidant component of DJ-1 function in human MCs is restricted to the control of SHP-2 and this, together with the lack of effect on SHP-1, may help explain the opposing mediator release effects between human and mouse MCs.

#### **Knockdown of DJ-1 inhibits FcεRI-induced Lyn activation mostly independently of its effects on ROS**

Since non-oxidized DJ-1 translocated to lipid rafts early after ligation of FcεRI and the activation of SFKs including Lyn, Fyn, and Fgr are the most receptor-proximal events<sup>3, 4, 15-17</sup>, we explored the possibility that reduced DJ-1 expression affects their activity. The activation of Lyn kinase was significantly impaired in DJ-1-silenced LAD2 cells (Fig. 5A, left panel). However, the activities of Fyn, and Fgr (Fig. 5A, middle and right panels) were not affected. The inhibition of Lyn by DJ-1 knockdown was mostly independent of its regulation of ROS levels since it was

only partially reversed by treatment with TEMPO (Fig. 5B). In agreement, addition of H<sub>2</sub>O<sub>2</sub> to immunoprecipitated Lyn only mildly reduced its Lyn tyrosine kinase activity (Fig. 5C). As in LAD2 cells, DJ-1 knockdown also impaired the activation of Lyn in primary HuMCs and showed little dependency on ROS levels (Supplementary Fig. 4). These results also contrasted with the effect of DJ-1 deficiency or knockdown in mBMMC<sup>6</sup>, where Lyn was not found to be affected, and suggest that DJ-1 is an important contributor to Lyn activation in HuMCs but not mBMBCs, an effect that is in general unrelated to DJ-1 antioxidant activity.

#### **DJ-1 is critical for activation of Syk and Syk-dependent phosphorylation events independently of its effects on ROS**

Syk activation by Lyn is critical for early signaling events mediated by FcεRI<sup>18</sup>. Consistent with the effect on Lyn, Syk activity in immunoprecipitates was significantly reduced by DJ-1 knockdown in LAD2 MCs after FcεRI stimulation (Fig. 6A) and this effect was only partially restored by treatment with TEMPO (Fig. 6B). In agreement with the reduction in Syk activity, we observed reduced phosphorylation of Syk in tyrosines 525/526 and 352 (Fig. 6C, upper panel) that was minimally reversed with TEMPO (Fig. 6C, lower panel). Furthermore, knockdown of DJ-1 also substantially reduced phosphorylation of Syk-dependent targets including the adaptor linker for activation of T cells (LAT) and the downstream phosphorylation of PLCγ1, JNK and ERK (Fig. 6D). However, in agreement with the lack of effect on Fyn activity, Akt phosphorylation, which is downstream of Fyn activation<sup>16</sup>, was not significantly affected. Of note, only a small fraction of Lyn is needed to initiate signaling early after FcεRI engagement<sup>19-21</sup> and thus increased Lyn activity may not be readily detectable by immunoprecipitations and in

vitro kinase assays until later times as receptor clusters and signaling complexes enriched in Lyn become enlarged<sup>6, 19-22</sup> (Fig.5A).

Collectively, the data are consistent with an essential role for DJ-1 in the propagation of FcεRI-mediated Lyn-Syk signaling and human MC functions by mechanisms that fundamentally differ from its effects on mBMMC.

### **Non-oxidized DJ-1 is required for proper phosphorylation and activation of Lyn in lipid rafts**

Since DJ-1 was rapidly translocated to the plasma membrane where Lyn triggers signaling after FcεRI engagement, we investigated whether Lyn and DJ-1 colocalize and associate after stimulation. We detected DJ-1 in immunocomplexes with Lyn within 3 min of FcεRI crosslinking in both primary HuMCs (Fig. 7A) and LAD2 MCs (Fig. 7B), consistent with the finding that DJ-1 is critical for degranulation, which occurs within 2-3 min. However, association of DJ-1 and Lyn was maximal at 7 min and remained so for up to 15 min (Fig. 7A and B, upper panels). The persistence of the association DJ-1/Lyn after degranulation is ending suggests roles for DJ-1 in responses additional to degranulation, such as cytokine and PGD<sub>2</sub> release which are late MC responses. Confocal microscopic analysis confirmed the redistribution of a portion of DJ-1 to the plasma membrane and colocalization with Lyn early after stimulation (Fig. 7A and B, lower panels).

Since Lyn is enriched in lipid rafts after MC stimulation, an environment important for Lyn-mediated phosphorylation of FcεRI<sup>3</sup>, we suspected that DJ-1 may also re-localize to these rafts,

as was described in astrocytes after LPS activation<sup>23</sup>. Fractionation studies did indicate that a substantial portion of DJ-1 translocated from the cytoplasm fractions (fractions 8-11) into lipid rafts (fractions 4-6; as defined by the marker protein flotillin-1 and Lyn), thus colocalizing with Lyn following stimulation (Fig. 7C). Consistent with the confocal images shown in Fig. 1C, the majority of DJ-1 that translocated into lipid raft fractions was not oxidized (Fig. 7C). Furthermore, DJ-1 was detected in immunocomplexes with Lyn only in detergent-insoluble fractions (lipid rafts) after MC stimulation but not in detergent-soluble fractions (Fig. 7D) and the immunoprecipitated DJ-1 associating with Lyn in these fractions was not oxidized in Cys106 (Fig. 7D).

The association of Lyn with DJ-1 in lipid rafts after FcεRI engagement did not appear to be required for the translocation of Lyn into these domains since silencing of DJ-1 did not prevent enrichment of Lyn in lipid rafts (Fig. 7E). However, it did markedly inhibit FcεRI-mediated Lyn phosphorylation (Fig. 7E), indicating that DJ-1 facilitates Lyn activation.

### **Human non-oxidized DJ-1 directly interacts with human Lyn**

Because DJ-1 is reported to directly alter the activity of kinases and tyrosine kinases<sup>24-26</sup>, we next determined whether recombinant DJ-1 could induce Lyn activation *in vitro*. As shown in Fig. 8A, recombinant DJ-1 induced a dose-dependent increase in Lyn activity in Lyn-immunoprecipitates from resting LAD2 cells (Fig. 8A, black bars). However, this induction was not observed when DJ-1 was previously oxidized with H<sub>2</sub>O<sub>2</sub> (Fig. 8A, red bars), which together with the observation that oxidized DJ-1 was not present in the plasma membrane or lipid rafts

early after FcεRI engagement, indicates that the effect of DJ-1 on Lyn activity is independent of its antioxidant activity.

To test whether Lyn and DJ-1 interact directly, we performed dot blots with recombinant DJ-1 and recombinant tyrosine kinases. Increasing concentrations of either purified recombinant Lyn (Fig. 8B) or DJ-1 (Fig. 8C, left panel) immobilized in nitrocellulose showed immunoreactive dots after incubation with DJ-1 (Fig. 8B) or Lyn (Fig. 8C, left panel). In contrast, no immunoreactive dots were observed when either recombinant Fyn or Syk was employed (Fig. 8B and C, left panel). Spotted recombinant proteins immunoreacting to their corresponding antibodies are shown in Fig. 8C (right panel) as positive controls.

Furthermore, human recombinant oxidized DJ-1 did not show positive interactions, indicating that only non-oxidized DJ-1 is able to interact with Lyn (Fig. 8D). Dot blot assays also indicated that mouse recombinant DJ-1 did not interact with mouse Lyn (Supplementary Fig. 5A) in agreement with the lack of effect of DJ-1 on Lyn activity in mBMMC. Furthermore, this interaction appears to be specific between the human homologs because no immunoreactive dots were observed between mouse DJ-1/human Lyn or human DJ-1/mouse Lyn (Supplementary Fig. 5B and C). Collectively, these results demonstrate a direct interaction between Lyn and non-oxidized DJ-1 that enhances the Lyn activity and appears unique to the human proteins.

## DISCUSSION

MCs play a critical role in allergic disorders mainly through the activation of the high-affinity IgE receptor (FcεRI) expressed on the MC surface. When the IgE-FcεRI complex is aggregated by multivalent antigen, the open, active conformation of the Src family tyrosine kinase (SFK) Lyn is favored, causing autophosphorylation of Lyn and transphosphorylation of tyrosine residues in the  $\beta$  and  $\gamma$  subunits of adjacent receptors. The latter phosphorylation creates docking sites for the recruitment of the tyrosine kinase Syk. Syk phosphorylation by Lyn and consequent activation amplifies signaling cascades that result ultimately in the release of vasoactive and inflammatory mediators from the MC<sup>4,3,5</sup>. However, how exactly Lyn initiates these events in human MCs is not entirely clear. Here we demonstrate that direct binding of Lyn to DJ-1 in the plasma membrane is a required step for full Lyn kinase activation, transphosphorylation of the  $\gamma$  subunits of FcεRI and consequent responses in human MCs. This is a first report showing that DJ-1, by mechanisms partially independent of its action as a ROS scavenger, is critical for the organization of early events in human MC signaling.

Interest in understanding the biochemical and functional properties of DJ-1 (also known as PARK7) has been fueled by the association of DJ-1 mutations with familial early onset Parkinson's disease<sup>27</sup> and by altered levels in diseases such as cancer, stroke, dermatitis and mastocytosis<sup>6, 7, 28, 29</sup>. A well-established function of DJ-1 is to protect cells from oxidative damage partly by undergoing self-oxidation thus reducing ROS levels<sup>30-32</sup>, although other functions for DJ-1 independent of this feature have also been described<sup>24, 25, 33, 34</sup>. We confirmed that FcεRI engagement induced ROS generation in HuMCs<sup>12</sup> and found that self-oxidation of

DJ-1 30 min after activation occurred concomitantly with a decline in ROS levels, consistent with the antioxidant role for DJ-1. Our study further reveals a requirement of DJ-1 for degranulation, cytokine and eicosanoid responses in HuMCs that appeared to be, however, greatly independent of its antioxidant activity since treatment with an antioxidant only restored by 20% the diminished responses caused by DJ-1 silencing. This is consistent with reports indicating that ROS generation has little effect on FcεRI-mediated MC responses<sup>35</sup>. Thus, the data supports ROS-dependent and ROS-independent roles for DJ-1 in HuMC responses. We provide evidence that a consequence of DJ-1 antioxidant activity is to restrict SHP-2 activation by removing excess ROS levels which normally enhances SHP-2 phosphatase activity, as was observed in mBMMCs<sup>6</sup>. However, unlike in mBMMCs, DJ-1 had no effect on SHP-1 activity. Restricted SHP-2 activity would result in diminished Syk dephosphorylation and thus sustained Syk activation.

In addition, silencing of DJ-1 expression markedly reduced Lyn activity in HuMCs by a mechanism unrelated to its ROS scavenger activity, as was true for mediator release, and involved a direct, specific association with Lyn. DJ-1 did not similarly associate with Syk nor the SFK Fyn, and Akt signaling downstream of Fyn was unaffected by DJ-1 absence. DJ-1 has been reported to alter the activity of certain receptors and signaling proteins in part by direct association<sup>36, 37</sup>, in some instances by mechanisms not yet well understood<sup>24, 25, 33, 38</sup>. Based on our findings we propose a unique dual function for DJ-1 in FcεRI-activated human MCs sequentially regulating Lyn and Syk pathways: initially, when DJ-1 is not oxidized, by directly facilitating Lyn activation; and later, as it becomes oxidized, by reducing ROS levels and SHP-2 activity thus potentially alleviating SHP-2 repression of Syk activity (Fig. 8E).

We provide insights on how and where DJ-1 exerts its regulation of Lyn activation. Similar to other cells<sup>39, 40</sup>, in non-stimulated HuMCs, DJ-1 appeared localized intracellularly but shortly and transiently after activation, a fraction of non-oxidized DJ-1 translocated to lipid rafts. Lipid rafts are plasma membrane microdomains enriched in cholesterol, glycosphingolipid, and sphingolipids<sup>41, 42</sup>, believed to generally function as focal points for the consolidation of FcεRI signaling components. Lyn and DJ-1 not only colocalized but formed distinct Lyn/DJ-1 immune complexes in these domains in response to FcεRI ligation. Formation of such complexes did not require adaptor proteins since purified recombinant DJ-1 directly bound recombinant Lyn *in vitro*, and resulted in enhanced Lyn activity. Although the precise mechanisms for the effect on Lyn activity are not entirely clear they appear to be partly independent of its antioxidant function. It is unlikely that DJ-1 prevents interactions with Cbp/Csk complexes, a feed-back loop that keeps Lyn in an inactive state through phosphorylation of Lyn in Tyr507<sup>43</sup>, since DJ-1 deficiency did not alter Lyn phosphorylation in Tyr507 or the phosphorylation and levels of Cbp that co-immunoprecipitated with Lyn (Supplementary Fig. 6A). Nevertheless, DJ-1 knockdown blunted total phosphorylation of Lyn and its association with Fcγ subunits (Supplementary Fig. 6A) and recombinant DJ-1 was sufficient by itself to enhance Lyn kinase activity in Lyn immunoprecipitates from unstimulated MCs and of recombinant Lyn *in vitro* (Fig. 8A and Supplementary Fig. 6B) to suggest that a consequence of Lyn/DJ-1 interaction may be to promote the open, active conformation of Lyn.

The active conformation of Lyn is usually favored by binding of Lyn SH2 and SH3 domains to phosphotyrosine-containing sequences and proline-rich motifs, respectively<sup>44, 45</sup>. DJ-1 does not

contain proline-rich motifs, but it was phosphorylated on tyrosine after antigen stimulation (Supplementary Fig. 6C). The fact that purified recombinant DJ-1 and Lyn interacted in the absence of ATP in our studies suggests that DJ-1 phosphorylation is not necessary for binding, but may stabilize the active conformation of Lyn. However, it appears that a non-oxidized Cys106 is required for the interaction and enhancement of Lyn activity. The interaction between human Lyn and DJ-1 is also species-specific in that it was not observed between the two murine proteins or between human and murine homologs, which suggests an acquired function for DJ-1 in humans despite the overall sequence homology between species. This adds a cautionary note in translating mouse data to humans with respect to MC activation mechanisms.

Collectively, our results shed new light on the regulation of human MC responses after IgE receptor engagement by identifying DJ-1 as an unsuspected player in Lyn activation. This function of DJ-1 requires non-oxidized cysteine residues as well as translocation of DJ-1 to lipid rafts and direct interaction with Lyn that facilitates its kinase activity and initiation of signaling cascades. Thus, further characterization of DJ-1/Lyn interaction may open new avenues of therapeutic modalities in MC-derived diseases. The finding of DJ-1 as an essential regulator of Lyn may also be relevant for other immunoglobulin receptors and/or human immune cells where Lyn function is critical.

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**FIGURE LEGENDS**

**Figure 1. Non-oxidized DJ-1 localizes to the plasma membrane early after mast cell activation.** (A) Changes in intracellular ROS and release of DJ-1 into the extracellular media following stimulation with 100 ng/ml streptavidin (SA) of HuMCs sensitized with biotinylated IgE measured by ELISA. (B) Immunoblots showing changes in total DJ-1 and oxidized DJ-1 (at Cys106) inside cells or secreted to the media, as indicated. Quantification of blots is shown as bar graphs representing the average of 3 independent experiments. Loaded amounts of lysates (intracellular DJ-1) and of supernatants (extracellular) are not equivalent in “per cell” basis, being the latter 5 times more concentrated than those for the intracellular (see methods). (C) Confocal microscopic analysis of the intracellular localization of DJ-1 (green) and oxidized DJ-1 (red) in non-stimulated or SA-stimulated HuMCs for 0, 10, 60, and 240 min. Data are from a representative experiment of three independent experiments.

**Figure 2. DJ-1 expression is required for normal FcεRI-induced human mast cell responses.** (A) Reduced DJ-1 protein (left panel) and mRNA expression levels (right panel) in HuMCs transduced with either DJ-1 sh-RNA or non-targeted sh-RNA constructs. (B, C) Effect of DJ-1 knockdown (empty circles) in HuMC degranulation induced by SA (B) or the indicated concentrations of thapsigargin, complement component 3a (C3a) and Compound 48/80 (C). In B, IgE-sensitized HuMCs were stimulated with various concentrations of SA in media with (red lines) or without (blue line) 100 ng/ml SCF. Degranulation was measured as β-hexosaminidase activity released into the extracellular medium and expressed as percentage of the total cellular

$\beta$ -hexosaminidase activity. (D, E) Effect of DJ-1 knockdown in the production of PGD<sub>2</sub> secreted into the culture medium induced by the indicated concentrations of SA (D) or by thapsigargin (E) six hours after stimulation. PGD<sub>2</sub> was determined by ELISA. (F) Effect of DJ-1 knockdown in the production of the cytokine GM-CSF induced by SA. HuMCs were stimulated for 6 h and the amounts of GM-CSF released into the media determined by ELISA. All values are means  $\pm$  SEM from 3 independent experiments. \*P < 0.05, \*\*P < 0.01.

**Figure 3. The effect of DJ-1 knockdown in Fc $\epsilon$ RI -induced human mast cell responses is largely independent of its effect on ROS.** (A) Confocal microscopy images showing intracellular ROS levels in HuMCs transduced with either lentiviral DJ-1 shRNA or non-target (NT) shRNA and pretreated or not with TEMPO for 10 min prior to SA (100 ng/ml) stimulation for 30 min. NS, non-stimulated. Right panels show determinations of ROS levels in HuMC after pretreatment with 30, 100 or 300  $\mu$ mol/L TEMPO 10 min before stimulation with the indicated concentrations of SA. (B-D) Effect of the antioxidant TEMPO at the indicated concentrations on SA-induced degranulation (B), PGD<sub>2</sub> (C) and GM-CSF (D) secretion in HuMCs transduced with either lentiviral DJ-1 shRNA or non-target shRNA control. IgE-sensitized cells were pretreated with TEMPO for 10 min and then stimulated with SA. Degranulation (B) was measured as percentage of  $\beta$ -hexosaminidase released into the media 30 min after stimulation, and the amounts of PGD<sub>2</sub> (C) and GM-CSF (D) secreted into the culture medium were determined 6 hours after activation. All values are means  $\pm$  SEM from 3 independent experiments. \*P < 0.05, \*\*P < 0.01.

**Figure 4. The effect of DJ-1 knockdown on SHP-2 activation is mediated through increased ROS levels.** LAD2 cells transduced with lentiviral DJ-1 shRNA or non-target shRNA were

sensitized with IgE and then stimulated with 100 ng/ml SA for 15 min. SHP-1 (A) and SHP-2 (D) were immunoprecipitated from the cell lysates and their phosphatase activities measured as the amount of free phosphate released from a synthetic phosphopeptide in 30 min. (B,E) Effect of ROS removal by TEMPO in the changes in SHP-1 (B) and SHP-2 (E) activity by DJ-1 knockdown. LAD2 cells were pretreated with 100  $\mu$ mol/L TEMPO for 10 min before SA stimulation and the phosphatase activities determined as in A and D. (C,F) Effect of incubation with H<sub>2</sub>O<sub>2</sub> for 20 min on SHP-1 (C) and SHP-2 (F) activities from non-stimulated (NS) and SA-stimulated (SA) LAD2 cells. All values are means  $\pm$  SEM from 3 independent experiments. \*P < 0.05. SA, streptavidin stimulated; NS, non-stimulated.

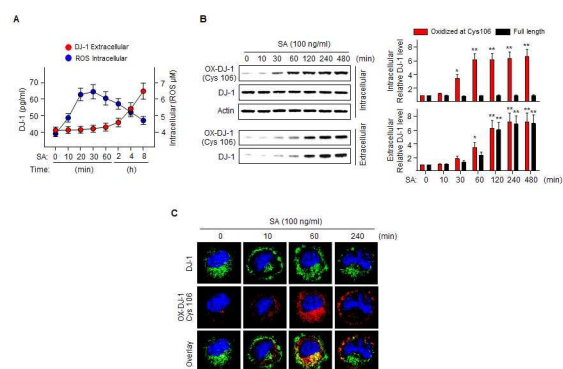
**Figure 5. DJ-1 knockdown suppresses Lyn activation mostly independently of its effects on ROS.** (A) Effect of DJ-1 knockdown in the activity of Src family kinases Lyn, Fyn and Fgr after SA stimulation of MCs. LAD2 cells transduced with lentiviral DJ-1 shRNA or non-target shRNA were sensitized with IgE and then stimulated with 100 ng/ml SA for the indicated times. Lyn, Fyn, and Fgr were immunoprecipitated and their kinase activities measured. (B) Effect of ROS removal by TEMPO in the loss of Lyn activation by DJ-1 knockdown. LAD2 cells were pretreated with 100  $\mu$ mol/L TEMPO for 10 min before SA stimulation. (C) Dose response effect of H<sub>2</sub>O<sub>2</sub> on the activity of immunoprecipitated Lyn from non-stimulated and SA-stimulated LAD2 cells. Lyn activity from immunoprecipitated Lyn samples was assayed *in vitro* in the presence of different concentrations of H<sub>2</sub>O<sub>2</sub> for 20 min. All values are means  $\pm$  SEM from 3 independent experiments. \*P < 0.05, \*\*P < 0.01. SA, streptavidin-stimulated; NS, non-stimulated.

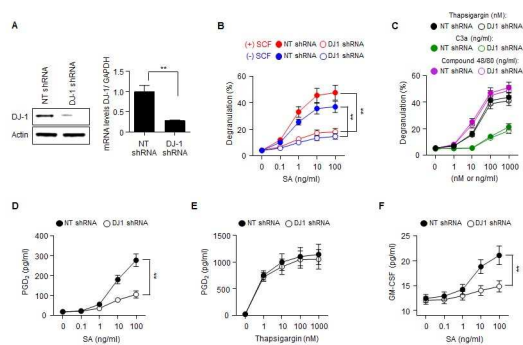
**Figure 6. DJ-1 knockdown suppresses Syk activation and downstream signals.** (A) Effect of DJ-1 knockdown in SA-induced activation of Syk. LAD2 cells transduced with lentiviral DJ-1 shRNA or non-target shRNA were sensitized with IgE and then stimulated with 100 ng/ml SA for the indicated times. Syk was immunoprecipitated and its activity in the immunoprecipitates measured using the ELISA-based Tyrosine Kinase Assay Kit. (B) Involvement of ROS in DJ-1 knockdown-induced effect on Syk activity. Cells were treated with TEMPO (100  $\mu$ mol/L) for 10 min prior to SA stimulation and Syk activity from the indicated lysates was determined as in A. Values are means  $\pm$  SEM from 3 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ . (C,D) Effect of DJ-1 knockdown on SA-induced signaling. Phosphorylation of Syk, LAT, PLC $\gamma$ 1, Akt, Jnk and Erk1/2 on LAD2 cells treated as in A was assessed by Western blotting using specific antibodies for the indicated proteins. Blots are representative of three experiments.

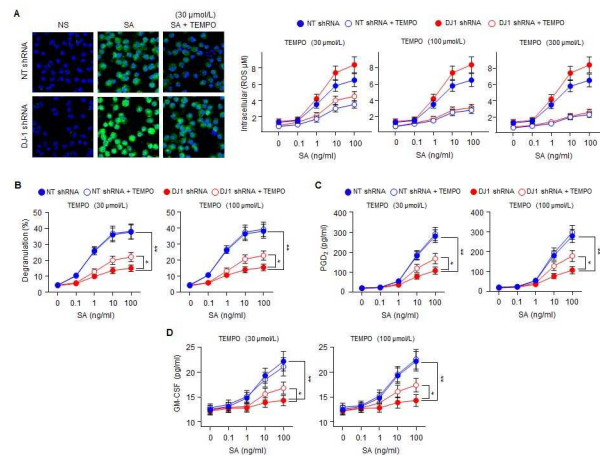
**Figure 7. After Fc $\epsilon$ RI crosslinking, DJ-1 interacts with Lyn in lipid rafts and enables Lyn phosphorylation.** (HuMCs (A) and (B) LAD2 cells were stimulated with 100 ng/ml SA for 0, 3, 7, and 15 min and Lyn was immunoprecipitated (upper panels). Presence of DJ-1 in the immunoprecipitates was demonstrated by Western blotting. Confocal images show the localization of DJ-1 (in green) and Lyn (in red) before and after SA stimulation of HuMCs (A) and (B) LAD2 cells. (C) Distribution of Lyn and DJ-1 in sucrose density gradient centrifugation fractions from non-stimulated (NS) or SA-stimulated (SA) LAD2 cell lysates. (D) Lyn was immunoprecipitated from 1% Triton X-100 soluble and insoluble fractions of IgE-sensitized LAD2 cells stimulated or not with 100 ng/ml SA for 7 min, and the presence of DJ-1 or oxidized DJ-1 determined by Western blotting. (E) LAD2 cells transduced with lentiviral DJ-1 shRNA or non-target shRNA were stimulated with 100 ng/ml SA for 7 min. Lyn was immunoprecipitated

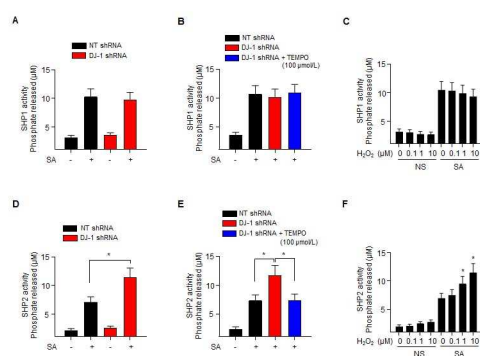
from Triton X-100 soluble and insoluble fractions and DJ-1 or Lyn localization and Lyn phosphorylation determined by Western blotting. Quantification of phospho-Lyn and Lyn for are shown. The values are means  $\pm$  SEM from 3 independent experiments.  $^{**}P < 0.01$ .

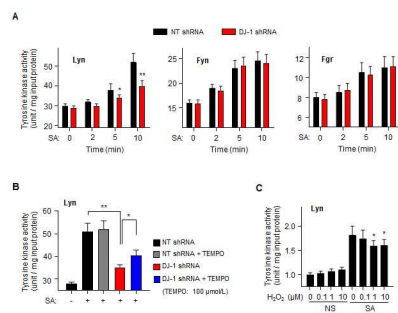
**Figure 8. Human DJ-1 directly interacts with Lyn, but not Fyn or Syk, and enhances its tyrosine kinase activity.** (A) Lyn activity in immunoprecipitates from unstimulated LAD2 cells incubated with or without various amounts of purified human recombinant DJ-1 or oxidized DJ-1 protein (OX-DJ-1) for 30 min. Values are means  $\pm$  SEM from 3 independent experiments.  $^{*}P < 0.05$ . (B,C) Human recombinant Lyn, Fyn, Syk and DJ-1 were immobilized on a nitrocellulose membrane and incubated with a solution of 100 ng/ml human recombinant DJ-1 (B) or human recombinant Lyn (C). After washing, positive interactions were revealed using antibodies against DJ-1 (B) or anti-Lyn (C). C, right panel: positive controls for recognition of dotted proteins to the corresponding antibodies. (D) Dot blots demonstrating that only non-oxidized human recombinant DJ-1 (h R DJ-1) interacts with human recombinant Lyn. Blots are representative of three experiments. (E) Model summarizing the findings. After activation of the Fc $\epsilon$ RI, non-oxidized DJ-1 is translocated to lipid rafts where it interacts with Lyn facilitating its activity and downstream signaling. In addition, at later times after Fc $\epsilon$ RI engagement, DJ-1 has a positive effect on MC signaling acting as an antioxidant protein that reduces ROS levels, thus limiting SHP-2 activation and enhancing Lyn activity, both of which favor Syk activation and MC responses.

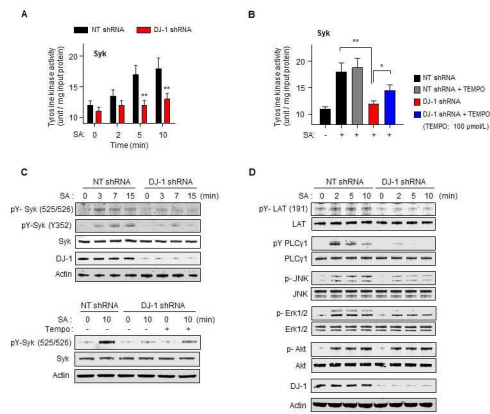


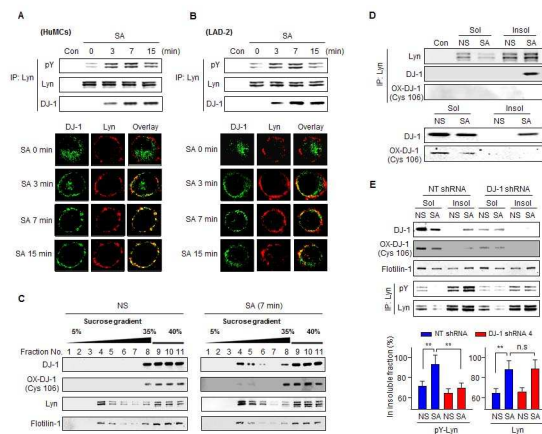


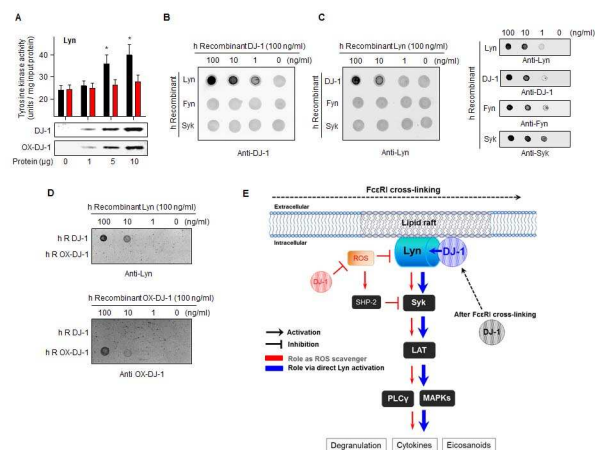


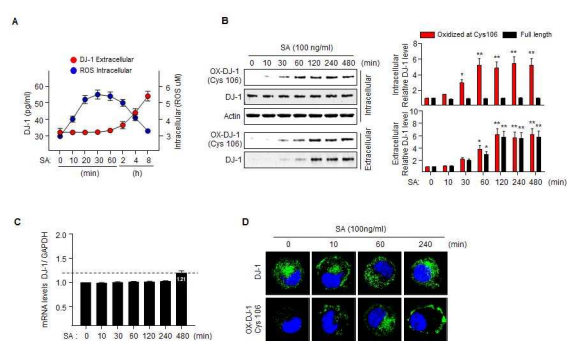


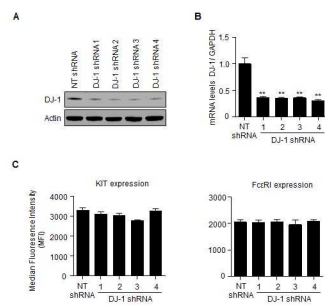


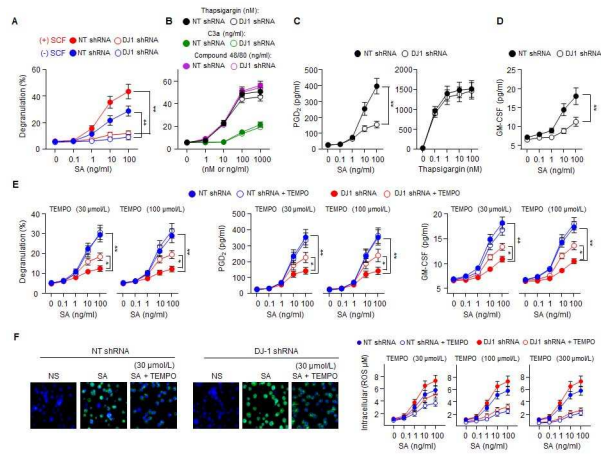


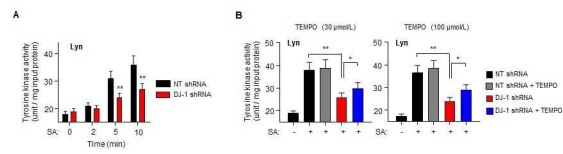


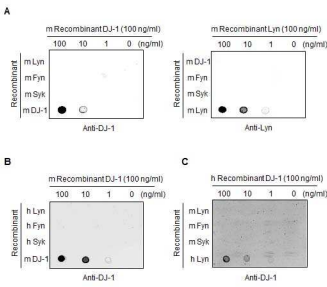


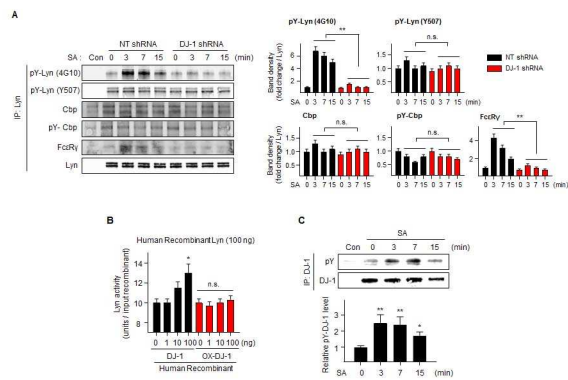












**SUPPLEMENTARY INFORMATION TO:****Interaction of DJ-1 with Lyn is essential for IgE-mediated stimulation of human mast cells****Table of Contents**

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## METHODS

**Human Mast Cell Cultures (HuMCs).** Primary HuMCs were derived from CD34<sup>+</sup> peripheral blood progenitors isolated from healthy volunteers after informed consent under a protocol (NCT00001756) approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases (NIH). CD34<sup>+</sup> cells were cultured in StemPro-34 SFM Complete Medium (Invitrogen, Grand Island, NY), L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), 100 ng/ml human recombinant SCF and 100 ng/ml human recombinant IL-6 (R&D Systems, Minneapolis, MN), except for the initial week of culture, when 30 ng/ml human recombinant IL-3 (PeproTech, Rocky Hill, NJ) was also present, as described<sup>E1</sup>. Cells were used for experiments by 6 to 8 weeks when 99% of cells were identifiable as FcεRI<sup>+</sup>/KIT<sup>+</sup> MCs. LAD2 cells were maintained in complete StemPro-34 SFM Complete Medium supplemented with 100 ng/ml human recombinant SCF, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 mg/ml) as previously described<sup>E2</sup>. For activation, HuMCs and LAD2 cells were sensitized overnight with biotinylated IgE (0.1 µg/ml), washed and the IgE-bound FcεRI crosslinked with 100 ng/ml streptavidin (SA) for the indicated times.

**Antibodies and Recombinant proteins.** Antibodies against DJ-1 (sc-27006) and Lyn (sc-15) were from Santa Cruz Biotechnology (Dallas, TX) and used at a dilution of 1:1,000 for western blotting. Antibodies against oxidized DJ-1 (Cys106) (MABN1773, diluted to 1:200) and phosphotyrosine (pY) residues (05-321, diluted to 1:1,000) were from EMD Millipore (Billerica, MA). Anti- phospho-Syk (Tyr525/526) (#2711), phospho-LAT (Tyr191) (#3584), phospho-Akt (Thr308) (#9275), phospho-SAPK/JNK (Thr183/Tyr185) (#9251), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9101), phospho-PLCγ1 (Y783) (#2821) and Flotillin-1 (#3253) antibodies were from Cell Signaling Technology (Beverly, MA) and diluted to 1:2,000 for

western blotting, except for anti-flotillin which was diluted to 1:1,000. Anti-  $\beta$ -actin (A5316), used as loading control at 1:5,000 dilution, was purchased from Sigma-Aldrich (St Louis, MO). For immunoprecipitations, antibodies anti-Lyn (sc-7274), Fyn (sc28791), Syk (sc-929), and SHP-1 (sc-287) were from Santa Cruz Biotechnology; anti-Fgr (sc-17) was from Cell Signaling Technology and anti-SHP2 (ab32083) from Abcam (Cambridge, Mass). For confocal microscopy anti-Lyn antibody (628102; diluted to 1:1,000) was purchased from BioLegend (San Diego, Calif); anti-DJ-1 (sc-32874 diluted to 1:1,000) from Santa Cruz Biotechnology and anti-oxDJ-1 (Cys106) (HCA024, diluted to 1:500) from Bio-Rad (formerly AbD Serotec; Raleigh, NC). Recombinant human Lyn, Fyn, Syk and recombinant mouse Lyn proteins for dot blots were from EMD Millipore. Human DJ-1 recombinant protein was from AnaSpec (San Jose, CA) and mouse DJ-1, Fyn, Syk recombinant proteins from MyBioSource (San Diego, CA).

**Knockdown of DJ-1 by sh-RNA.** Non-target control vector (SHC002) or DJ-1-targeted short hairpin RNAs (sh-RNAs) (TRCN0000004921) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). This DJ-1 sh-RNA construct was selected among other available constructs (TRCN0000004918, TRCN0000004919 and TRCN0000004920) for its consistency in silencing DJ-1 in HuMCs and LAD2 cells, although they all reduced DJ-1 expression (Supplementary Figure 2). The Mission Lentiviral packaging mix (Sigma-Aldrich) and the pLKO1 vectors with sh-DJ-1 or control sh-RNA (3.4  $\mu$ g) were cotransfected into 293T cells ( $4 \times 10^6$  cells) with FuGENE6 transfection reagent (Roche, Indianapolis, Ind) in Opti-MEM and the viral particles were collected by centrifugation as described<sup>E3</sup>. The resulting pellet was resuspended in 3 ml of pre-warmed complete StemPro medium (Invitrogen) and 0.5 to 1.0 ml of this volume transferred to a T25 culture flask containing 1 to  $2 \times 10^6$  HuMC or LAD2 in 10 ml

of complete StemPro medium. Two days after transduction, cells were placed in fresh complete media for two additional days. Transduced cells were selected in media containing 0.2 µg/ml puromycin (Sigma-Aldrich) for 5 days. After a recovery period of 2 days, the cells were used for experiment. Most experiments were also repeated in cells using DJ-1 sh-RNA knockdown 3-4 days after transduction without puromycin selection with very similar results (not shown).

**Measurement of ROS and DJ-1 levels.** Intracellular ROS levels were measured with the OxiSelect™ Intracellular ROS Assay kit (Cell Biolabs, Inc., San Diego, CA). HuMCs ( $2 \times 10^5$  cells/1.5 ml) and LAD2 ( $2 \times 10^5$  cells/1.5 ml) cells were sensitized overnight with biotinylated IgE (0.1 µg/ml), washed and the IgE-bound FcεRI crosslinked with 100 ng/ml streptavidin (SA) for various time periods. Cells were incubated at 37°C with 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (20 µM) for the last 15 min of stimulation with SA and ROS was quantified as described<sup>E4</sup>. For cell imaging of intracellular ROS, dichlorofluorescein-loaded cells treated as above were fixed in 4% formaldehyde for 10 min. In some experiments, cells were pretreated with the antioxidant TEMPO (30µM) for 10 min before stimulation with SA. Confocal images were obtained in a Leica SP5 X confocal laser scanning microscope with an Apochromat 60× objective (Leica Microsystems, Buffalo Grove, IL).

For DJ-1 measurements, HuMCs or LAD2 ( $5 \times 10^5$  cells/1.0 ml) cells were sensitized with IgE and stimulated with SA as described above. Aliquots of the supernatants were taken at the indicated times and DJ-1 measured by ELISA (R&D Systems). Alternatively, supernatants were concentrated to 10 µl by ultrafiltration (Amicon Ultra-4 Centrifugal Filter Units; 10 kDa) (EMD Millipore; Billerica, MA), the proteins solubilized with 2X SDS sample buffer and separated by

SDS-PAGE using 4-12% gels. For detection of intracellular DJ-1 and oxidized DJ-1, cells were lysed in 200  $\mu$ l of lysis buffer and 10  $\mu$ l per sample used for Western blot determinations.

**Measurement of DJ-1 mRNA.** Total cellular RNA was extracted from  $5 \times 10^5$  cells using an RNeasy Plus Mini kit (Qiagen, Valencia, CA). A 1  $\mu$ g aliquot of RNA, was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Quantitative PCR was executed in 96-well plates using ABI Prism 7500 Real Time PCR system (Foster City, CA) and TaqMan gene expression assays for DJ-1 (AI15HL) and GAPDH as the endogenous control gene (Assay ID: Hs99999905\_m1) (Life Technologies, Grand Island, NY) was used for normalization.

**Immunoprecipitation and Western blotting analysis.** IgE-primed HuMCs and LAD2 cells were stimulated with SA in HEPES-BSA buffer (10 mmol/L HEPES [pH 7.4], 137 mmol/L NaCl, 2.7 mmol/L KCl, 0.4 mmol/L  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 5.6 mmol/L glucose, 1.8 mmol/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1.3 mmol/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.04% BSA) for 0, 3, 7, and 15 min, or as indicated in the figure legends, washed twice with  $1 \times$  PBS, and lysed on ice with lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM  $\text{Na}_3\text{VO}_4$ , 0.5 mM PMSF, 5 mg/ml aprotinin, 5 mg/ml leupeptin, complete protease inhibitor cocktail (Roche, Indianapolis, IN) and 1% NP-40 for 15 min. Samples were then clarified at 13,000 g for 5 min at 4°C. Protein content in the lysates was determined using a Bradford assay (Bio-Rad, Hercules, CA) and equal amounts of protein (1 mg in ml lysis buffer) were used for the immunoprecipitation of the indicated tyrosine kinases and Src kinases. Cell lysates were incubated at 4°C with the indicated specific antibodies with gentle rocking for 4h and the immunocomplexes were captured with

protein A/G-agarose overnight at 4°C. The agarose beads were washed five times with washing buffer (lysis buffer diluted 1:10 in PBS [pH 7.4]), resuspended in 2X Laemmli buffer, and then boiled for 10 min.

For western blotting analysis, equal amounts of proteins (20 µg) were separated by SDS-PAGE using 4-12% gels and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA in Tris-buffered saline for 1 h, and then incubated with specific primary antibodies overnight at 4°C. Immunoreactive bands were detected using infrared dye-conjugated secondary antibodies conjugated (LI-COR Biosciences; Lincoln, NE, USA) (1:20,000) and imaged and quantified using an Odyssey imager (LI-COR Biosciences).

**Measurement of phosphatase activity of SHP-1 and SHP-2.** SHP-1 and SHP-2 were immunoprecipitated from SA-stimulated LAD2 cells in HEPES-BSA buffer for 7 min. SHP-1 and SHP-2 phosphatase activities in the immunoprecipitates were measured using a Duoset IC Phosphatase Assay (R&D Systems, Minneapolis, Minn) by determining the amount of free phosphate after incubation with a synthetic phosphopeptide substrate for 30 min following manufacturer's protocol.

**In vitro kinase assay.** Immunoprecipitates of Lyn, Fyn, Fgr or Syk from 1 mg whole-cell lysates were assayed for kinase activity by using the ELISA-based Universal Tyrosine Kinase Assay Kit (Gen Way, San Diego, Calif) according to the manufacturer's instructions. One unit of tyrosine kinase represents incorporation of 1 pmol of phosphate into the substrate (a p34cdc2 peptide fragment of about 6-20 aminoacids) per minute.

115  
116 **Dot blots.** Recombinant Lyn, Fyn, and Syk were dotted on nitrocellulose membranes (0.45  $\mu$ m  
117 pore size; Invitrogen). The membranes were blocked with TBST (150 mM NaCl, 25 mM Tris  
118 [pH 7.5], 0.05% Tween 20) containing 5% BSA for 1 h at room temperature. Recombinant DJ-1  
119 was diluted in TBST plus 0.05% BSA and incubated with the membrane for 1 h to allow for  
120 recognition of the spotted proteins. After washing (three times in TBST plus 0.1% milk), the  
121 membrane was incubated with anti-DJ-1 antibody overnight at 4°C. The membranes were  
122 detected by LI-COR Odyssey using IR-conjugated secondary antibodies. Oxidized recombinant  
123 DJ-1 was prepared by incubating recombinant DJ-1 with H<sub>2</sub>O<sub>2</sub> for 30 min and then removing  
124 H<sub>2</sub>O<sub>2</sub> by ultrafiltration (Amicon Ultra-4 Centrifugal Filter Units; 10 kDa) (EMD Millipore;  
125 Billerica, MA).

126  
127 **Confocal microscopy.** Non-stimulated and SA-stimulated HuMC and LAD2 cells were seeded  
128 into 8-well Lab-Tek chamber slides at a cell density of  $3 \times 10^4$  cells/well, and centrifuged at 600  
129 rpm for 5 min. After washing with PBS, HuMCs or LAD2 cells were fixed with 4%  
130 formaldehyde in PBS for 10 min, washed with PBS, and permeabilized with 0.1% Triton X-100  
131 for 10 min. Fixed cells were incubated with 1% BSA in PBS for 60 min at room temperature and  
132 then overnight at 4°C with antibodies against Lyn, DJ-1, or oxidized-DJ-1 with gentle rocking.  
133 Cells were washed five times and then incubated for 50 min with Alexa Fluor® 594-conjugated  
134 goat anti-mouse IgG to detect Lyn or oxidized-DJ-1, or with Alexa Fluor® 488-conjugated  
135 Donkey anti-Rabbit IgG to detect DJ-1. The slides were then mounted with ProLonged Antifade  
136 Kit mounting solution (Molecular Probes) and confocal images were obtained with a Leica SP5  
137 X confocal laser scanning microscope (Apochromat 60× objective lens) (Leica Microsystems)

within the Biological Imaging Section of the Research Technologies Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health,

**Isolation of detergent-resistant membranes.** LAD2 cells treated as indicated in the legends to figures were washed twice with ice-cold PBS and lysed in ice-cold PBS containing 1% Triton X-100 and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 1 mM EDTA) for 20 min. The lysates were centrifuged at 16,600 g for 10 min at 4°C and the corresponding supernatants were used as soluble fractions. The pellets were washed with ice-cold PBS buffer, solubilized with 1× sample buffer and used as insoluble fraction. For sucrose density gradient centrifugation fractionation analysis LAD2 cells ( $2.0 \times 10^7$ ) were washed twice with ice-cold PBS and lysed in 2 ml of TNE buffer (50 mM tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 mg/ml), and aprotinin (1 mg/ml)) containing 0.5% Brij 58 for 30 min on ice. Lysates were mixed with an equal volume of 80% (w/v) sucrose in TNE buffer. A sucrose gradient was prepared by the sequential addition of 1.5 ml of 80% sucrose, 2 ml of a 1:1 mixture of 80% sucrose and cell lysate, 4 ml of 35% sucrose, and finally 4 ml of 5% sucrose, which was processed by ultracentrifugation at 270,000g for 18 h at 4°C. One-milliliter fractions (for a total of 12 fractions) were collected from the top to the bottom of the gradient for Western blot analysis.

**Statistical analysis.** Data were expressed as mean  $\pm$  SEM of values from 3 or more independent experiments. 1-way ANOVA was used to determine statistically significant differences between groups using Prism 6 software (Graph Pad Software, San Diego, CA, USA). Statistical significance was indicated as follows: \*P <0.05 and \*\*P <0.01.

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**SUPPLEMENTARY FIGURE LEGENDS****Supplementary Figure 1. Crosslinking of FcεRI in LAD2 cells induces early ROS production and DJ-1 oxidation followed by DJ-1 release into the extracellular space. (A)**

Changes in intracellular ROS and release of DJ-1 into the extracellular media following stimulation with 100 ng/ml streptavidin (SA) of IgE-sensitized LAD2 cells. (B) Immunoblots showing changes in total DJ-1 and oxidized DJ-1 (at Cys106) inside cells or secreted to the media, as indicated. Quantification of blots is shown as bar graphs representing the average of 3 independent experiments. As in Fig. 1, the loaded amounts of lysates (intracellular DJ-1) and of supernatants (extracellular) are not equivalent in “per cell” basis, being the latter 5 times more concentrated than those for the intracellular (see methods). (C) Changes in the expression of DJ-1 mRNA after treatment of LAD2 cells with 100 ng/ml SA for the indicated times. (D) Confocal microscopic analysis of the intracellular localization of DJ-1 and oxidized DJ-1 in non-stimulated or SA-stimulated LAD2 cells for 0, 10, 60, and 240 min. Data are from a representative experiment of three independent experiments.

**Supplementary Figure 2. Validation of DJ-1 Silencing in LAD-2 cells. (A,B) DJ-1 protein (A) and mRNA expression (B) levels in LAD2 cells transduced with four different lentiviral DJ-1 shRNA or non-target shRNA were analyzed by Western blot and qRT-PCR, respectively. (C) c-KIT and FcεRI expression in DJ-1 shRNA and control-transduced LAD2 cells. FcεRI and KIT expression were determined by FACS. Data are representative of three experiments.**

**Supplementary Figure 3. DJ-1 is required for normal LAD2 responses to FcεRI crosslinking by a mechanism mostly independent of ROS increases.** (A,B) Effect of DJ-1 knockdown (empty circles) in LAD2 degranulation induced by SA (A) or the indicated concentrations of thapsigargin, complement component 3a (C3a) and Compound 48/80 (B). (A) IgE-sensitized LAD2 cells were stimulated with various concentrations of SA in media with (red lines) or without (blue line) 100 ng/ml SCF. Degranulation was measured as  $\beta$ -hexosaminidase activity released into the extracellular medium after 30 min of stimulation and expressed as percentage of the total cellular  $\beta$ -hexosaminidase activity. (C,D) Effect of DJ-1 knockdown (empty circles) in the production of  $\text{PGD}_2$  induced by SA (C, left panel) or by thapsigargin (C, right panel) and in the production of the cytokine GM-CSF (D) induced by SA in LAD2 cells. (E) IgE-sensitized LAD2 cells were pretreated with 30 or 100  $\mu\text{mol/L}$  of the antioxidant TEMPO for 10 min and then incubated with or without the indicated concentrations of SA. The extent of degranulation after 30 min (left panels), and release of  $\text{PGD}_2$  (middle panels) and GM-CSF (right panels) after 6 hours were determined as in A-D. (F) Representative confocal microscopy images showing the intracellular ROS levels in control and DJ-1 knockdown LAD2 cells pretreated or not with 30  $\mu\text{mol/L}$  TEMPO for 10 min prior to SA stimulation (100 ng/ml) for 30 min. Right panels show determinations of ROS levels in LAD2 cells after pretreatment with 30, 100 and 300  $\mu\text{mol/L}$  TEMPO 10 min before stimulation with the indicated concentrations of SA. All values are means  $\pm$  SEM from 3 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

**Supplementary Figure 4. DJ-1 knockdown reduced Lyn activity in primary human mast cells, and this effect is only partially restored by increased ROS.** (A) HuMCs cells transduced with lentiviral DJ-1 shRNA or non-target (NT) shRNA were sensitized with IgE and stimulated

with 100 ng/ml SA for the indicated times. (B) Control or DJ-1 shRNA-silenced HuMCs cells were stimulated with SA for 10 min in the absence and presence of 30 (left panel) or 100  $\mu$ mol/L TEMPO (right panel) for 10 min before SA stimulation. Lyn was immunoprecipitated from the indicated cell lysates and Lyn activity was measured with an ELISA-based Tyrosine Kinase Assay Kit. All values are means  $\pm$  SEM from 3 independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01.

**Supplementary Figure 5. Mouse DJ-1, unlike the human homologs, does not interact with Lyn.** (A) Recombinant mouse Lyn and recombinant mouse DJ-1 do not show direct interaction. Various amounts of mouse recombinant Lyn, Fyn and Syk and DJ-1 were immobilized on a nitrocellulose membrane as indicated. Membranes were incubated with a solution of mouse recombinant DJ-1 (left) or mouse recombinant Lyn (right) at a concentration of 100 mg/ml. Interaction between the dotted protein and the protein in solution were revealed using antibodies against DJ-1 or anti-Lyn as indicated. (B,C) Dot blots demonstrating lack of interaction between mouse DJ-1 and human Lyn (B) or between human DJ-1 and mouse Lyn (C).

**Supplementary Figure 6. (A) DJ-1 knockdown does not affect Cbp phosphorylation or association with Lyn but markedly reduced total tyrosine phosphorylation of Lyn and associated Fc $\epsilon$ RI.** LAD2 cells transduced with lentiviral DJ-1 shRNA or non-target (NT) shRNA were stimulated with 100 ng/ml SA for the indicated times. Lyn was immunoprecipitated and Lyn phosphorylation as well as associated Cbp and Fc $\gamma$  subunits were analyzed by Western blotting with antibodies specific for the indicated proteins. Quantification of phospho-Lyn and Lyn for the indicated fractions and treatments is shown as bar graphs. Data represents means  $\pm$

SEM from 2 independent experiments.  $**P < 0.01$ . (B) **Purified DJ-1 enhances activity of recombinant purified Lyn.** Human recombinant Lyn (100 ng) was incubated with various amounts of purified human recombinant DJ-1 or human recombinant oxidized DJ-1 protein (OX-DJ-1) for 30 min, and Lyn activity measured with an ELISA-based Tyrosine Kinase Assay Kit. (C) **DJ-1 is phosphorylated in tyrosine after activation of FcεRI.** DJ-1 was immunoprecipitated from IgE-sensitized LAD2 cells and stimulated 100 ng/ml SA for the indicated times. Tyrosine phosphorylation of DJ-1 in the immunoprecipitates was assessed by Western blotting using a phospho-tyrosine specific antibody. The values are means  $\pm$  SEM from 3 independent experiments.  $*P < 0.05$ .

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