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# MYO1F Regulates IgE and MRGPRX2-Dependent Mast Cell Exocytosis

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The activation and degranulation of mast cells is critical in the pathogenesis of allergic inflammation and modulation of inflammation. Recently, we demonstrated that the unconventional long-tailed myosin, MYO1F, localizes with cortical F-actin and mediates adhesion and migration of mast cells. In this study, we show that knockdown of MYO1F by short hairpin RNA reduces human mast cell degranulation induced by both IgE crosslinking and by stimulation of the Mas-related G protein-coupled receptor X2 (MRGPRX2), which has been associated with allergic and pseudoallergic drug reactions, respectively. Defective degranulation was accompanied by a reduced reassembly of the cortical actin ring after activation but reversed by inhibition of actin polymerization. Our data show that MYO1F is required for full Cdc42 GTPase activation, a critical step in exocytosis. Furthermore, MYO1F knockdown resulted in less granule localization in the cell membrane and fewer fissioned mitochondria along with deficient mitochondria translocation to exocytic sites. Consistent with that, AKT and DRP1 phosphorylation are diminished in MYO1F knockdown cells. Altogether, our data point to MYO1F as an important regulator of mast cell degranulation by contributing to the dynamics of the cortical actin ring and the distribution of both the secretory granules and mitochondria. *The Journal of Immunology*, 2021, 206: 2277–2289.

Human mast cells (huMCs) perform a variety of biological activities that are closely linked to their ability to release and secrete several mediators like histamine, leukotrienes, PGs (1), and different cytokines. These mediators are important in host defense, innate and acquired immunity, and homeostasis. Mast cell activation can induce the immediate release of preformed mediators present in secretory granules but also the synthesis and secretion of de novo mediators. Depending upon the stimuli involved, these events can be discriminated in a chronological manner and can involve different mediators and secretory pathways. The best studied mechanism is IgE-mediated degranulation which is mediated via the high affinity receptor for IgE (FcεRI). Crosslinking of receptor-bound IgE by a specific Ag results in the activation of different signaling pathways that promote the degranulation event (2). Various inflammatory products such as cytokines, chemokines, and sphingosine-1-phosphate (3) can also induce mast cell activation and they are important in the pathophysiology of various inflammatory conditions. Mast cells can recognize invasive pathogens through complement and pattern recognition receptor-dependent pathways (4). They can also be triggered by neuropeptides such as corticotrophin-releasing hormone, neurotrophins, and substance P (SP). The

Mas-related G protein-coupled receptor X2 (MRGPRX2), is activated by SP (5), and several drugs such as neuromuscular relaxants, opioids, and vancomycin (6, 7).

Mast cell degranulation is a highly regulated process that relies on close interaction with receptors and signaling proteins, as well as fine regulation of the microtubule and actin cytoskeleton (8). On the one hand, the movement of secretory granules depends on intact microtubules (9), which are stably anchored in the centrosome and grow from the center of the cell to the periphery. In contrast, microfilaments, also called actin filaments, mostly concentrate just beneath the plasma membrane, forming the cortical actin cytoskeleton. Actin filaments are involved in cell movement, changes in cell shape and organization of cytoplasmic components (10).

Recently, our group described the role of a long-tailed unconventional type I myosin called myosin 1f (MYO1F) as a modulator of mast cell adhesion through the adaptor protein Src homology 3 (SH3)-binding protein 2 (3BP2) (11, 12). The interaction between these two proteins was modulated by stem cell factor (SCF), the ligand of the KIT receptor, and MYO1F silencing led to downregulation of β1 and β7 integrins on the mast cell membrane. In the same article, we also described the role of 3BP2 in mast cell migration, as

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Abbreviations used in this article: 3BP2, SH3-binding protein 2; DRP1, dynamin-related protein 1; huMC, human mast cell; MRGPRX2, Mas-related G protein-coupled receptor X2; MYO1F, myosin 1f; SCF, stem cell factor; SH3, Src homology 3; shRNA, short hairpin RNA; SP, substance P.

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this molecule has already been reported to have a role in mast cell survival via KIT (13) and mast cell degranulation via FcεRI (14). This unconventional myosin contains a pleckstrin domain that allows the localization to the plasma membrane through phosphorylated phosphatidyl-inositols, the usual motor domain with an ATP binding site, and a calmodulin binding site. Furthermore, the presence of an SH3 protein-binding domain in the C-terminal region of MYO1F as well as MYO1E differentiates them from short-tailed myosins and mediates the interaction between MYO1F and the adaptor protein 3BP2 (11). The role of MYO1F in mast cell degranulation, however, remains unknown.

MYO1F was initially described as a modulator of cell adhesion and motility in neutrophils through the augmentation of exocytosis of β2 integrin-containing granules (15). A recent study argued that in fact, the migration and extravasation defect in MYO1F knockout neutrophils was due to its role in regulating squeezing and dynamic deformation of the nucleus during migration through physical barriers (16). In macrophages, MYO1F regulates M1-polarization by stimulating intercellular adhesion through integrin-αVβ3 (17). Along with MYO1E, MYO1F regulates adhesion turnover during phagocytosis, creating membrane-cytoskeleton crosstalk through Fc-receptors that is indispensable for correct phagocytosis (18).

In this study, we describe the role of MYO1F in regulating IgE-dependent and -independent degranulation through MRGPRX2 and the involvement of the actin cytoskeleton in these processes. We also describe MYO1F as a key regulator of mitochondrial movement and fission after mast cell activation. Altogether, these results shed some light on the involvement of long-tailed, unconventional myosins in the dynamic events that are crucial for mast cell function and pathophysiology.

## Materials and Methods

### Cell lines and reagents

The LAD2 huMC line kindly provided by Drs. A. Kirshenbaum and D.D. Metcalfe (National Institutes of Health, Bethesda, MD) was grown in Stem-Pro-34 media (Life Technologies, Carlsbad, CA), supplemented with Stem-Pro-34 nutrient and L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 100 ng/ml SCF (Amgen, Thousand Oaks, CA) (19). Primary huMCs derived from CD34-positive peripheral blood cells were obtained from healthy donors following informed consent using a protocol (98-I-0027) approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board, and differentiated *in vitro* for 8 wk in the presence of 100 ng/ml IL-6 and 100 ng/ml SCF as previously described (20). After 8 wk, culture purity was assessed by surface FcεRI and KIT expression. The HEK 293LTV cell line (Cell Biolabs, San Diego, CA) was used for lentivirus production.

### Abs and other reagents

Mouse Abs anti-MYO1F C5, anti-KIT (clone Ab81), anti-DRP1 C5, anti-pAKT (Ser<sup>473</sup>) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pDRP1 (Ser<sup>616</sup>) and anti-pERK (Thr<sup>202</sup>/Tyr<sup>204</sup>) were from Cell Signaling Technology (Danvers, MA). Mouse anti-Cdc42 from Cytoskeleton (Denver, CO). Goat anti-mouse Alexa 647 and goat anti-rabbit Alexa 488 were from Life Technologies (Carlsbad, CA), and mouse anti-human-Fc R1PE were from eBioscience (San Diego, CA). PE anti-human MRGPRX2 clone K125H4 was from BioLegend (San Diego, CA). TOM20 rabbit polyclonal Ab was from ProteinTech (Rosemont, IL), and mouse monoclonal CD63-allophycocyanin (clone MEM-259) was from Immunotools (Friesoythe, Germany).

Biotinylated human IgE (IgE<sup>B</sup>) was obtained from Abbiotec (San Diego, CA). Anti-mouse-HRP Ab was obtained from DAKO (Carpinteria, CA). Streptavidin, puromycin, doxycycline hyclate, mouse anti-tubulin (DM1A) and anti-β-actin (clone AC-40) were purchased from Sigma-Aldrich (St. Louis, MO). SP was from AnaSpec (Fremont, CA).

### Lentiviral transduction

Lentiviral particles were generated according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO) as described elsewhere (11). We used two constructs for MYO1F knockdown: the lentiviral plasmid used in

huMCs, with the short hairpin RNA (shRNA) sequence for MYO1F silencing, was from Origene Technologies (sequence D).

For LAD2, we used the TRCN0000158118 shRNA sequence (which partly overlaps with sequence D), CGTCTTCAAGACCGAGTTTGT, cloned into a doxycycline-inducible lentiviral Tet-pLKO-puro plasmid (Addgene plasmid 21915) (21). Knockdown of MYO1F in cells was induced by adding 0.5 μg/ml doxycycline.

### Degranulation assays

For IgE-dependent activation we preincubated cells with or without IgEB (0.1 μg/ml) overnight in culture media without SCF. Cells were stimulated at 37°C with streptavidin (10–1000 ng/ml) to induce IgE crosslinking, with SP (SP, 0.05–5 μM) or with ionomycin (200 μg/ml) + PMA (10 ng/ml). Degranulation was analyzed based on the levels of β-hexosaminidase activity at the supernatant or on CD63 expression on the cell membrane, assessed by flow cytometry, as described in previous studies (7). To inhibit actin polymerization cells were preincubated with latrunculin B (1 μM) or cytochalasin D (2 μM) for 20 min before stimulation.

### Imaging flow cytometry assay

Cells were activated or inhibited as described above. Afterward, cells were fixed in paraformaldehyde 4% and PBS at 4°C. Cells were permeabilized with saponin buffer (PBS, 0.05% saponin) for 15 min and blocked with blocking buffer (0.2% skimmed milk, 22% FCS, 1% BSA, 0.01% Triton X-100, 0.01% NaN<sub>3</sub>, dissolved in PBS) for 1 h at 4°C. We used primary Abs (TOM20 or CD63) (0.1–0.2 μg/100,000 cells) for 2 h of incubation at 4°C. At last, we used goat anti-rabbit or goat anti-mouse secondary Abs labeled with Alexa 488 or Alexa 647, respectively, for 45 min (dilution 1:300 to 1:500) at 4°C. For actin staining, we used phalloidin-TRITC (dilution 1:500 to 1:1000).

Preparations were visualized and analyzed with an Amnis ImageStream (Luminex). All images were processed with AMNIS IDEAS software. Cytoplasmic area versus membrane area were determined with a mask and the cells were divided into two groups: cells with fluorescence associated to plasma membrane or cells with fluorescence on the cytoplasm. Then, we quantified the percentage of cells showing localization mostly near the cell membrane in control (nontarget shRNA) or MYO1F shRNA mast cells before or after stimulation.

### Immunoblotting

Cells were sensitized overnight with biotinylated IgE (100 ng/ml) in culture media. The following day, the cells were stimulated at 37°C with 400 ng/ml SA to induce IgE crosslinking; alternatively, cells were treated with 2 μM SP in Tyrode buffer for the indicated times. Whole-cell lysate were solubilized in RIPA lysis buffer (1% Triton X-100, 5mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 7.4], 150 mM NaCl, 100 mM NaF, 1 mM Na<sub>3</sub>VO<sub>3</sub>). Total cell lysates were separated by SDS-PAGE and electrotransferred to PVDF membranes (MilliporeSigma, Bedford, MA). Blots were probed with the indicated Abs. In all blots, proteins were visualized by ECL (Santa Cruz Biotechnology).

### FACS staining

FcεRI and MRGPRX2 expression was detected by direct staining with the indicated Abs for 30 min at 4°C. KIT expression was detected using the primary Ab plus a secondary goat anti-mouse Ab with Alexa 647. Cells were analyzed using a FACScalibur Flow Cytometer (FACScan; BD Biosciences). In all cases, dead cells were excluded based on their forward and side-scattering profile.

### Calcium release and mitochondrial membrane potential determination using tetramethylrhodamine methyl ester

Cells were preincubated for activation as described above and then washed and incubated with Fluo-4 AM (Invitrogen) or tetramethylrhodamine methyl ester (Thermo Fisher Scientific) for 20 min prior to cell activation with IgE<sup>B</sup> plus streptavidin, SP, or just ionomycin. Fluorescence was determined using a TECAN SPARK microplate reader (Tecan Group, Männedorf, Switzerland).

### PG D2 release and TNF-α secretion assay

PG D2 (PGD<sub>2</sub>) and TNF-α release were determined from the supernatants of cells activated for 4 h or 24 h, respectively, using a specific enzyme immunoassay for PGD<sub>2</sub> (Cayman Chemical) and a specific ELISA for TNF-α (R&D Systems) according to the manufacturer's instructions.

### Active GTPase assays

GTPase activation assay was performed using Cdc42 G-LISA kits following the manufacturer's instructions (Cytoskeleton) as well as described elsewhere (22). Briefly, cell lysates ( $1.5 \times 10^5$  cells in triplicate) were prepared using G-LISA lysis buffer, snap-frozen in liquid nitrogen, and processed within 2 wk after preparation. Lysate aliquots corresponding to Cdc42 assay were applied, respectively, to wells coated with Cdc42-GTP-binding protein. Active, GTP-bound GTPases bound to the wells were detected with the corresponding specific Ab. Constitutively active proteins were used as a standard.

### Transmission electronic microscopy

LAD2 mast cells, previously incubated with IgE<sup>B</sup> were activated with streptavidin (200 ng/ml) for 30 min, washed with PBS, and pelleted. The pellet was fixed in 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer. After that, samples were processed for transmission electronic microscopy and images were taken with a JEOL JEM 1010 100kv transmission electronic microscopy microscope.

### Data analysis

All results are expressed as mean  $\pm$  SEM. Unpaired Student *t* test or one-way ANOVA was used to determine significant differences (*p* value) between two or several experimental groups, respectively, after determination of normal distribution of the sample and variance analysis.

## Results

### MYO1F is critical for IgE-dependent mast cell degranulation

The doxycycline-inducible MYO1F shRNA already described elsewhere (11) was used to determine the effect of MYO1F downregulation on mast cell degranulation, with nontarget shRNA as a control. MYO1F protein knockdown in LAD2 mast cells was confirmed by Western blot (Fig. 1A). We measured degranulation as  $\beta$ -hexosaminidase release after IgE<sup>B</sup> crosslinking at different doses of streptavidin after 30 min in LAD2 mast cells (Fig. 1B). MYO1F shRNA cells showed reduced degranulation compared with nontarget shRNA cells in the doxycycline-induced group. To determine whether this effect was delayed, we analyzed mast cell degranulation at several time points using CD63 as a marker. CD63 is found in granule membranes and it is exposed to the plasma membrane during degranulation. As demonstrated by FACS analysis (Fig. 1C), MYO1F shRNA cells were unable to achieve similar degranulation levels as nontarget shRNA cells, even after 3 h.

We extended the study to CD34<sup>+</sup>-derived huMCs. For this purpose, CD34<sup>+</sup>-derived huMCs were induced to differentiate with IL-3, IL-6, and SCF as described in the *Materials and Methods* section. Cells were transduced with an MYO1F shRNA plasmid or scrambled shRNA (nontarget shRNA) using lentivirus at week 7 of mast cell differentiation and transduced cells were selected with puromycin. Knockdown was confirmed by Western blot and the levels of KIT and Fc $\epsilon$ RI were checked by flow cytometry (Fig. 1D). Similar to LAD2 mast cells, we observed a decrease in the extent of degranulation in MYO1F shRNA cells compared with nontarget shRNA cells after 30 min (Fig. 1E).

### MYO1F is also necessary for MRGPRX2 mast cell degranulation

MRGPRX2 (Mas-related G protein-coupled receptor X2) has emerged in recent years as an important receptor in pseudoallergic drug reactions and has been implicated in innate and adaptive responses (23). Degranulation induced by MRGPRX2 and other G protein-coupled receptors is a rapid piecemeal process that, unlike degranulation induced by IgE and IgG receptors, does not involve granule fusion before exocytosis. We used SP to activate MRGPRX2. Similar to the results shown in Fig. 1, we observed that mast cell degranulation was impaired in MYO1F shRNA cells at several doses (Fig. 2A). In addition, CD63 release from MYO1F-silenced cells was lower at all assessed times (Fig. 2B). In CD34<sup>+</sup>-derived huMCs, MRGPRX2 expression was similar in

nontarget shRNA cells and MYO1F shRNA cells (Fig. 2C); however, SP-induced degranulation was significantly lower in MYO1F knockdown cells (Fig. 2D).

### TNF secretion but not PGD<sub>2</sub> release is impaired in MYO1F-knockdown cells

Apart from the release of preformed proinflammatory mediators, activated mast cells can induce the early synthesis of PGs and leukotrienes and, subsequently, the synthesis of cytokines like IL-4 and TNF- $\alpha$  (24, 25). To examine whether MYO1F shRNA also affects the release of the de novo synthesized proinflammatory components we measured PGD<sub>2</sub> release at 4 h after IgE crosslinking or SP stimulation. PGD<sub>2</sub> release, which is an example of transporter-mediated secretion, was similar in both groups (Supplemental Fig. 1A). However, TNF- $\alpha$  secretion, reported to occur by constitutive exocytosis (26), was diminished 24 h after SP activation in MYO1F shRNA cells compared with nontarget shRNA cells (Supplemental Fig. 1B). Release of TNF- $\alpha$  induced pharmacologically by ionomycin plus PMA was not different in MYO1F shRNA cells, indicating that there was a defect in release rather than a different TNF content within the cells. These results show a differential role for MYO1F in regulating the exocytosis of preformed proinflammatory mediators and cytokines versus lipidic de novo proinflammatory mediators.

### MYO1F is required for Cdc42 GTPases activation through Fc $\epsilon$ RI and MRGPRX2 in mast cells

Rho family GTPases play an important role in rearrangement of actin cytoskeleton (8, 27) and exocytosis (28). Specifically, it has been shown that GTPase Cdc42 regulates and coordinate vesicle transport with actin cytoskeletal rearrangements, events that trigger exocytosis (29, 30). Indeed, GTPase Cdc42 activation was maximal 2–5 min after MRGPRX2 activation in LAD2 cells (Fig. 3A), whereas the activation pattern via Fc $\epsilon$ RI was slower, with maximal activation after 5–10 min (11). Thus, we examined the activation of small GTPases Cdc42 by IgE<sup>B</sup>-streptavidin (400 ng/ml) or SP (2  $\mu$ M) at the peak of activation in MYO1F-knockdown LAD2 mast cells. Our results showed that knockdown of MYO1F significantly reduced Cdc42 activity 5–10 min after IgE<sup>B</sup>-stv activation (Fig. 3B), or 2–5 min after MRGPRX2 activation (Fig. 3C) compared with nontarget control cells. Although the basal levels of Cdc42 activity are also significantly reduced in MYO1F knockdown cells, the total level of Cdc42 GTPase was not affected by MYO1F knockdown (Fig. 3D). Taken together, our results suggest the involvement of MYO1F in the axis GTPases-actin rearrangement and exocytosis.

### Inhibition of actin polymerization can restore the loss of degranulation of MYO1F knockdown mast cells

The phenotype observed during mast cell degranulation by IgE suggested that MYO1F cooperates with the actin cytoskeleton, specifically in the cortical actomyosin ring that is necessary for mast cell degranulation (31). It has been reported that mast cell degranulation is enhanced after inhibition of actin polymerization (32). Taking this into consideration, we measured degranulation after preincubating nontarget shRNA and MYO1F-shRNA cells with the actin polymerization inhibitors latrunculin B and cytochalasin D. Mast cells were then stimulated by IgE crosslinking (Fig. 4A) or SP (Fig. 4B). We observed that mast cell degranulation was enhanced using these inhibitors in control cells and that the degranulation capacity of MYO1F shRNA cells could be significantly recovered, although not up to the same levels as in control cells.

### MYO1F knockdown reduces cortical actin rearrangement

Like all unconventional myosins, MYO1F colocalizes with the cortical actin ring in neutrophils and mast cells (11, 15). Mast cell

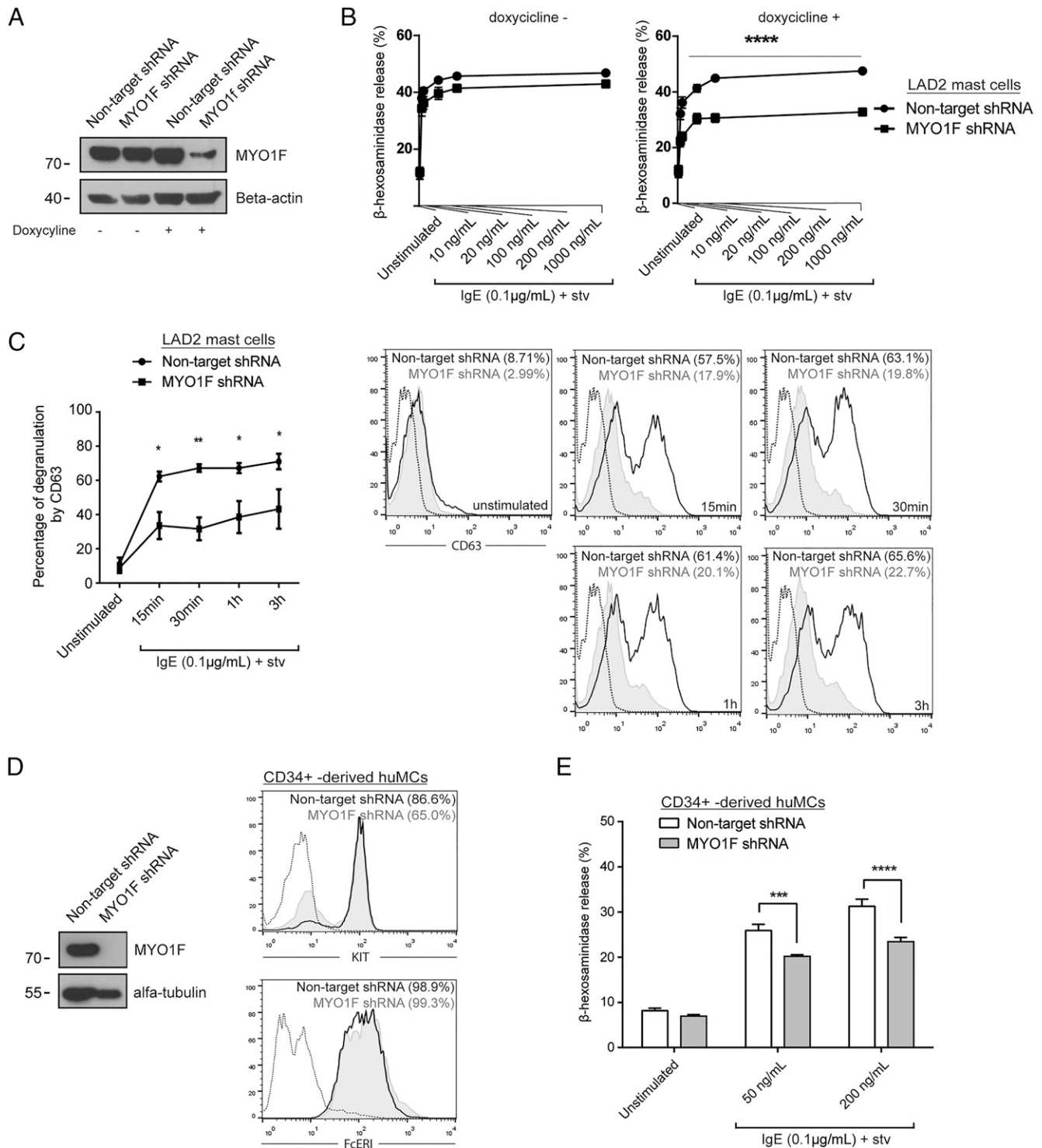


**FIGURE 1.** MYO1F-knockdown reduces IgE-mediated degranulation. **(A)** Doxycycline was used in the culture media to induce MYO1F silencing, and doxycycline-untreated cells were used as a control. Knockdown was confirmed by Western blot. **(B)** Control (nontarget shRNA) and MYO1F-deficient (MYO1F-shRNA) prebound with IgE<sup>B</sup> LAD2 mast cells were stimulated with different doses of streptavidin for 30 min to induce IgE crosslinking. β-Hexosaminidase activity in the supernatant was measured as a percentage of total activity. **(C)** Nontarget and MYO1F-shRNA prebound with IgE<sup>B</sup> LAD2 mast cells were stimulated at different times with streptavidin (200 ng/mL) to induce IgE crosslinking. The percentage of positive cells was determined in relation to the isotype control. Nontarget shRNA is represented by an empty black curve and MYO1F-shRNA is represented by a gray-filled curve. **(D)** CD34<sup>+</sup>-derived huMCs were transduced with MYO1F shRNA or nontarget shRNA and the silencing was confirmed by Western blot. Expression of KIT and FcεRI was determined by flow cytometry. Nontarget shRNA is represented by an empty black curve and MYO1F-shRNA is represented by a gray-filled curve. **(E)** Control (nontarget shRNA) and MYO1F-deficient (MYO1F-shRNA) huMCs were stimulated with different doses of streptavidin for 30 min to induce IgE crosslinking. β-Hexosaminidase activity in the supernatant was measured as a percentage of total activity. One-way ANOVA test was used for the statistical analysis. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

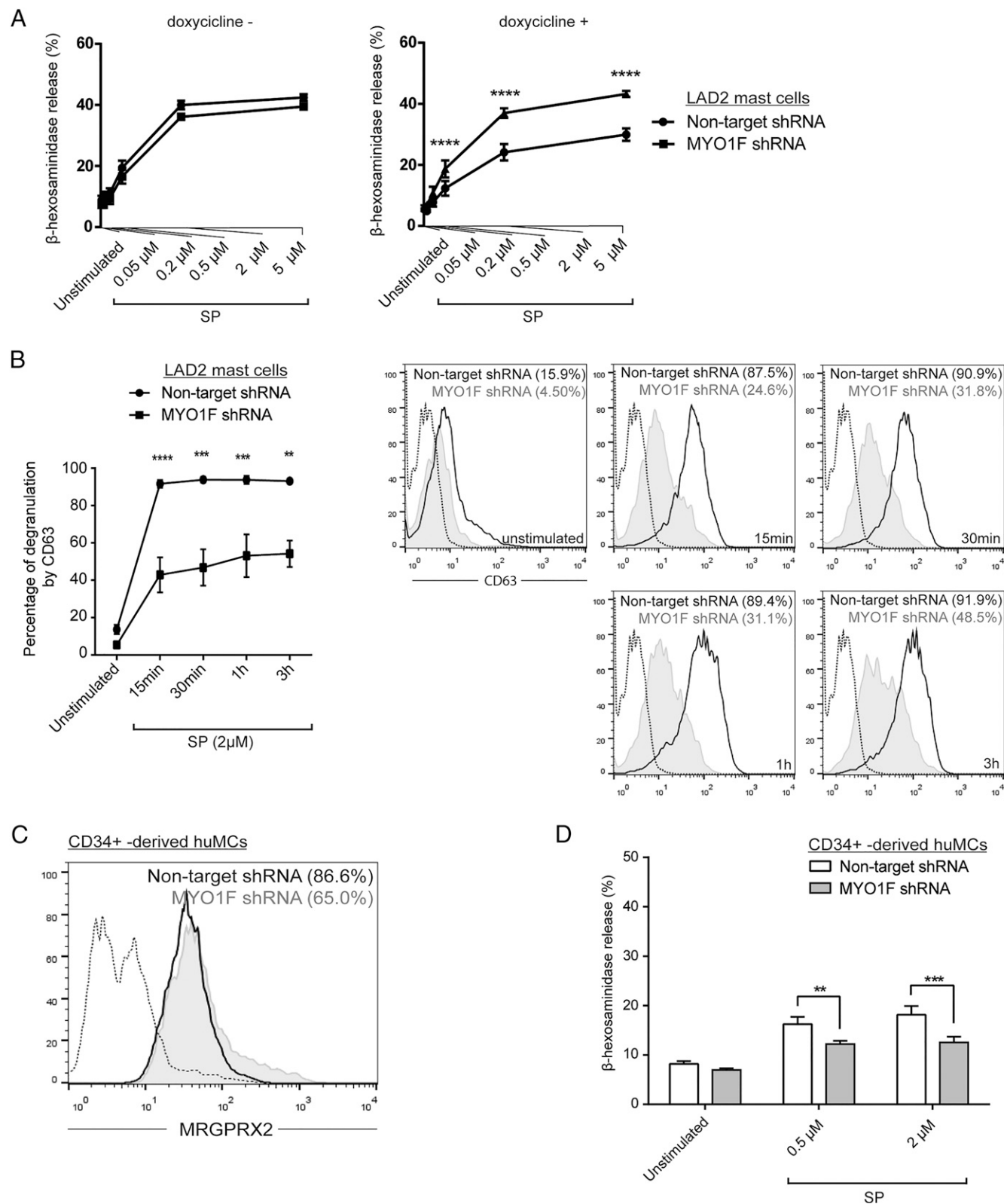


**FIGURE 2.** MYO1F knockdown reduces MRGPRX2-mediated degranulation. **(A)** Doxycycline was used in the culture media to induce MYO1F silencing, and doxycycline-untreated cells were used as a control. Control (nontarget shRNA) and MYO1F-deficient (MYO1F-shRNA) LAD2 mast cells were stimulated with different doses of SP for 30 min.  $\beta$ -Hexosaminidase activity in the supernatant was measured as a percentage of total activity. **(B)** Nontarget and MYO1F-shRNA LAD2 mast cells were stimulated at different times with SP (2  $\mu$ M). The percentage of positive cells was determined in relation to the isotype control. Nontarget shRNA is represented by an empty black curve and MYO1F-shRNA is represented by a gray-filled curve. **(C)** Expression of MRGPRX2 receptor in CD34<sup>+</sup>-derived huMCs transduced with MYO1F shRNA or nontarget shRNA was determined by flow cytometry. Nontarget shRNA is represented by an empty black curve and MYO1F-shRNA is represented by a gray-filled curve. **(D)** Control (nontarget shRNA) and MYO1F-deficient (MYO1F-shRNA) huMCs were stimulated with different doses of SP for 30 min.  $\beta$ -Hexosaminidase activity in the supernatant was measured as a percentage of total activity. One-way ANOVA test was used for the statistical analysis. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001.

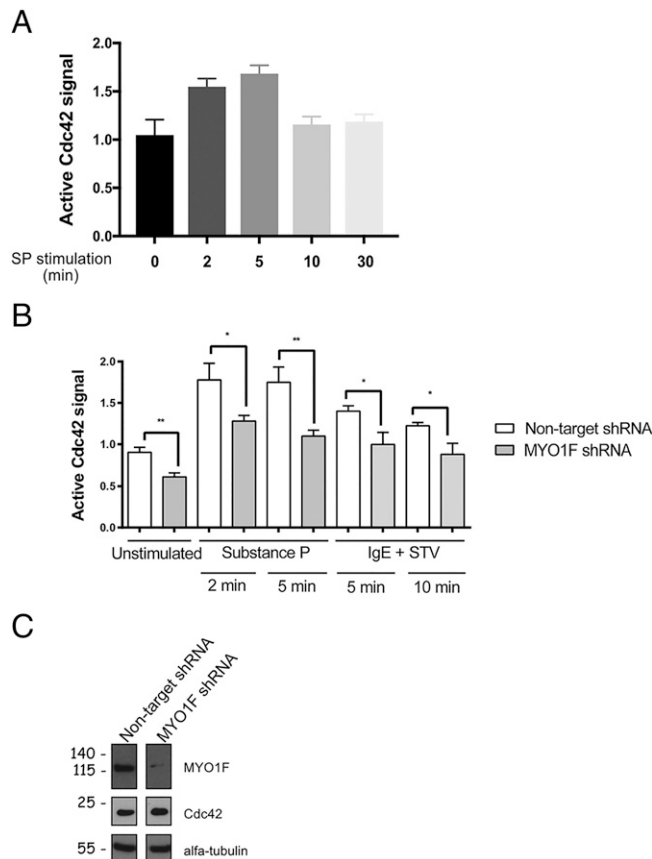


**FIGURE 3.** MYO1F is required for Cdc42 activation in mast cells. G-Lisa quantification of GTP-bound (active) small GTPases Cdc42 was performed after SP activation for several time points in LAD2 cells (**A**). IgE<sup>B</sup> + streptavidin (400 ng/ml) or SP (2  $\mu$ M) cell activation for the indicated times in control (nontarget shRNA) and MYO1F-knockdown (MYO1F-shRNA) LAD2 mast cells (**B**). Western blot showing Cdc42 GTPase levels in NT and MYO1F-silenced cells (**C**). The Student *t* test was used for the statistical analysis. Data are the mean of three independent experiments. \**p* < 0.05, \*\**p* < 0.01.

activation by Fc $\epsilon$ RI causes degranulation in a process that involves the reorganization of the actin cytoskeleton. Pfeiffer et al. (33) first reported that the actin content of mast cells during degranulation decreases during the first 10–30 s of Ag binding but then increases to almost double the control levels after 1 min. These rearrangements are mediated by a plethora of proteins but seem to be critical for exocytosis (34). To determine the effect of MYO1F knockdown on the pattern of actin reorganization, we looked at the actin cytoskeleton by imaging flow cytometry after IgE-dependent activation of LAD2 mast cells for 15 min, using phalloidin-TRITC. As seen in Fig. 5A, the cortical actin ring was clearly defined in control cells after IgE-dependent activation whereas in MYO1F shRNA cells the cortical actin ring was indistinct. Indeed, in those cells the actin ring was rearranged in clusters. The same phenotype was seen in CD34<sup>+</sup>-derived huMC in which MYO1F was silenced (Fig. 5B). Granule distribution in those cells was also determined using CD63 as a marker. After IgE-dependent stimulation granule translocation to the membrane could be observed by a shift in the CD63 fluorescence from the center of the cell (internal) to the periphery (external). As expected, MYO1F shRNA cells showed less external translocation than nontarget shRNA cells (Fig. 5C).

#### MYO1F silencing does not impair calcium signaling

Calcium mobilization in response to IgE crosslinking and MRGPRX2 is crucial for the degranulation process, and there is a relationship between Ca<sup>2+</sup> flux and exocytosis after mast cell stimulation (35). We therefore wanted to determine whether calcium signaling on MYO1F shRNA cells is affected. For this reason, we activated mast cells via IgE or with SP stimuli and determined the calcium signal using the fluorescent marker Fluo-4. Our data show that the calcium signal in nontarget shRNA cells and MYO1F shRNA cells was similar (Supplemental Fig. 2A). Elevations in cytosolic calcium after activation of mast cells occurs by release of calcium from intracellular stores and by store-operated Ca<sup>2+</sup> entry, which is activated in response to endoplasmic reticulum Ca<sup>2+</sup> store depletion. To measure the contribution of calcium release from intracellular stores, we stimulated cells in the absence of calcium in the cell media. As shown in Supplemental Fig. 2B, in the absence of extracellular calcium the calcium peaks were of lower magnitude than those in Supplemental Fig. 2A. Under these conditions, we observed slight but significant differences in the calcium responses to IgE and SP in MYO1F-knockdown cells compared with nontarget shRNA cells (right panels). These results indicate that intracellular calcium release is partly impaired by MYO1F knockdown, although the impairment is not sufficient to inhibit store-operated Ca<sup>2+</sup> entry.

#### Mitochondrial fission and translocation to the cell membrane are altered in MYO1F knockdown mast cells after IgE-dependent activation

Mitochondria not only generate ATP but also regulate cellular calcium signals by accumulating and releasing Ca<sup>2+</sup> (36). Mitochondria undergo fission into independent structures and translocation to the cell membrane after cell activation (37). Translocation may be necessary to provide energy locally or to maintain the calcium signal (37), whereas fission generates new organelles in stress conditions (38) and enhances mitochondrial mobility (39). Because mitochondria fission and translocation are important for degranulation and TNF production (37), we analyzed the shape of mitochondria using electron microscopy. As shown in Fig. 6A, MYO1F-deficient cells showed more elongated mitochondria after stimulation, indicating fewer fissioned mitochondria than in nontarget cells. We also determined mitochondrial translocation after IgE-dependent degranulation using imaging flow cytometry, with TOM20 as a marker. In this case, the percentage of mitochondrial translocation in MYO1F-silenced cells was lower than that in control cells (Fig. 6B).

Despite the differences in mitochondrial shape and translocation after activation in MYO1F knockdown cells, we did not observe any significant differences in mitochondrial membrane potential after IgE or SP activation (Supplemental Fig. 3), as determined using tetramethylrhodamine methyl ester. Altogether, our data suggest that the mitochondrial morphology and localization during activation is, at least partly, regulated by MYO1F.

#### MYO1F knockdown regulates DRP1 phosphorylation leading to a block in mitochondrial fission

It is known that mitochondria can undergo fusion or fission depending on the dynamics of the cell (40, 41). During mast cell degranulation and cytokine secretion, the mitochondria will provide energy to perform these processes. The mitochondria will fission and translocate to the membrane to supply energy locally (42). As shown in Fig. 7, MYO1F-deficient cells exhibited less mitochondrial fission compared with nontarget cells. A key player in the fission process is a dynamin-related protein 1 (DRP1) a conserved dynamin member of a GTPase superfamily protein that facilitates membrane remodeling in a variety of cellular membranes (43, 44) and their

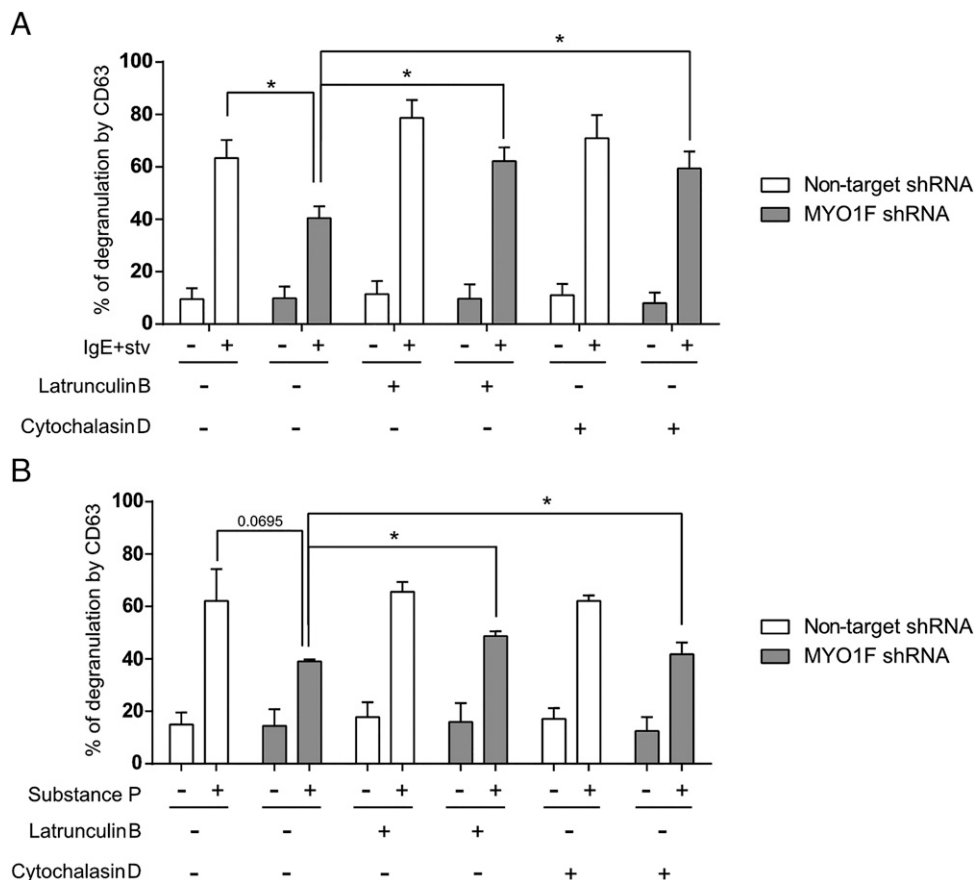


**FIGURE 4.** Inhibition of actin polymerization recovers the degranulation ability of MYO1F-silenced cells. Control (nontarget shRNA) and MYO1F-knock-down (MYO1F shRNA) LAD2 mast cells in the presence of doxycycline (0.5  $\mu$ g/ml) were treated for 20 min with latrunculin B or cytochalasin D before stimulating them with (A) IgE<sup>B</sup> + streptavidin (200 ng/ml) or (B) SP (2  $\mu$ M) for 30 min. The percentage of degranulation was determined by flow cytometry using CD63 staining. One-way ANOVA test was used for the statistical analysis. \* $p < 0.05$ .

phosphorylation on Ser<sup>616</sup> is needed for mitochondrial division to occur (40, 45, 46). Thus, we explored the effect of DRP1 phosphorylation in MYO1F-silenced cells. Western blot analysis revealed that deficiency in MYO1F resulted in marked reductions in the phosphorylation of DRP1 after activation with either IgE<sup>B</sup>-streptavidin (400 ng/ml) or SP (2  $\mu$ M). Effector pathways downstream of AKT and MAPK have also been reported to directly activate DRP1 and promote mitochondria fission (47). Thus, the levels of AKT and ERK phosphorylated were assessed. AKT phosphorylation, unlike ERK phosphorylation, was also markedly reduced in MYO1F-silenced cells (Fig. 7). Taken together, our results suggest that MYO1F may play a role in mitochondria fission by regulating DRP1 phosphorylation.

## Discussion

The hallmark of the allergic reaction is the immediate release of inflammatory mediators located in the secretory granules of mast cells by regulated exocytosis. This event may occur by diverse mechanisms: full degranulation, kiss-and-run exocytosis, piecemeal degranulation, and compound exocytosis. All of these models occur in mast cells, although in vivo the predominant form is the compound exocytosis, mediated by IgE, in which several secretory granules merge together to form larger and heterogeneous secretory granules that then fuse with the plasma membrane (48). The mode of exocytosis also depends on the type of stimulus. Certain signals, including SP, the complement anaphylatoxins C3a and C5a, and endothelin 1,

induce huMCs to rapidly secrete small and relatively spherical granule structures, a pattern consistent with the secretion of individual granules (49). Thus, IgE-dependent mast cell activation produces patterns of degranulation that vary in spatiotemporal features and in the structural characteristics of the released granules from G protein-coupled receptor-dependent degranulation. In this study, we show that in MYO1F-deficient cells, both types of exocytosis are impaired, indicating a relevant role of this myosin in the release of preformed granules.

Apart from the immediate release of preformed mediators, mast cells also release lipid mediators that are de novo synthesized and released immediately from activated cells rather than stored. One example of that is PGD<sub>2</sub>, a mast cell-specific eicosanoid derived from arachidonic acid. PGD<sub>2</sub> has bronchoconstricting and vasoactive properties and has an important role in host defense (50). Little is known about their secretion mechanism, although it has been proposed that the mode of secretion is not only stimulus dependent but also cell type dependent (48). It is known that eicosanoids are negatively charged at physiological pH and thus their secretion involves facilitated transport across the membrane through organic or anion transporters of the ATP-binding cassette type C family (51) or anion transporter solute carrier superfamily (52). Our results show that PGD<sub>2</sub> is synthesized after IgE and MRGPRX2 activation and the reduction of MYO1F expression do not impair its secretion, suggesting a selective action of MYO1F in the exocytic pathways of proinflammatory mediators. Among the large number of cytokines and chemokines released after cell activation, TNF is known to be

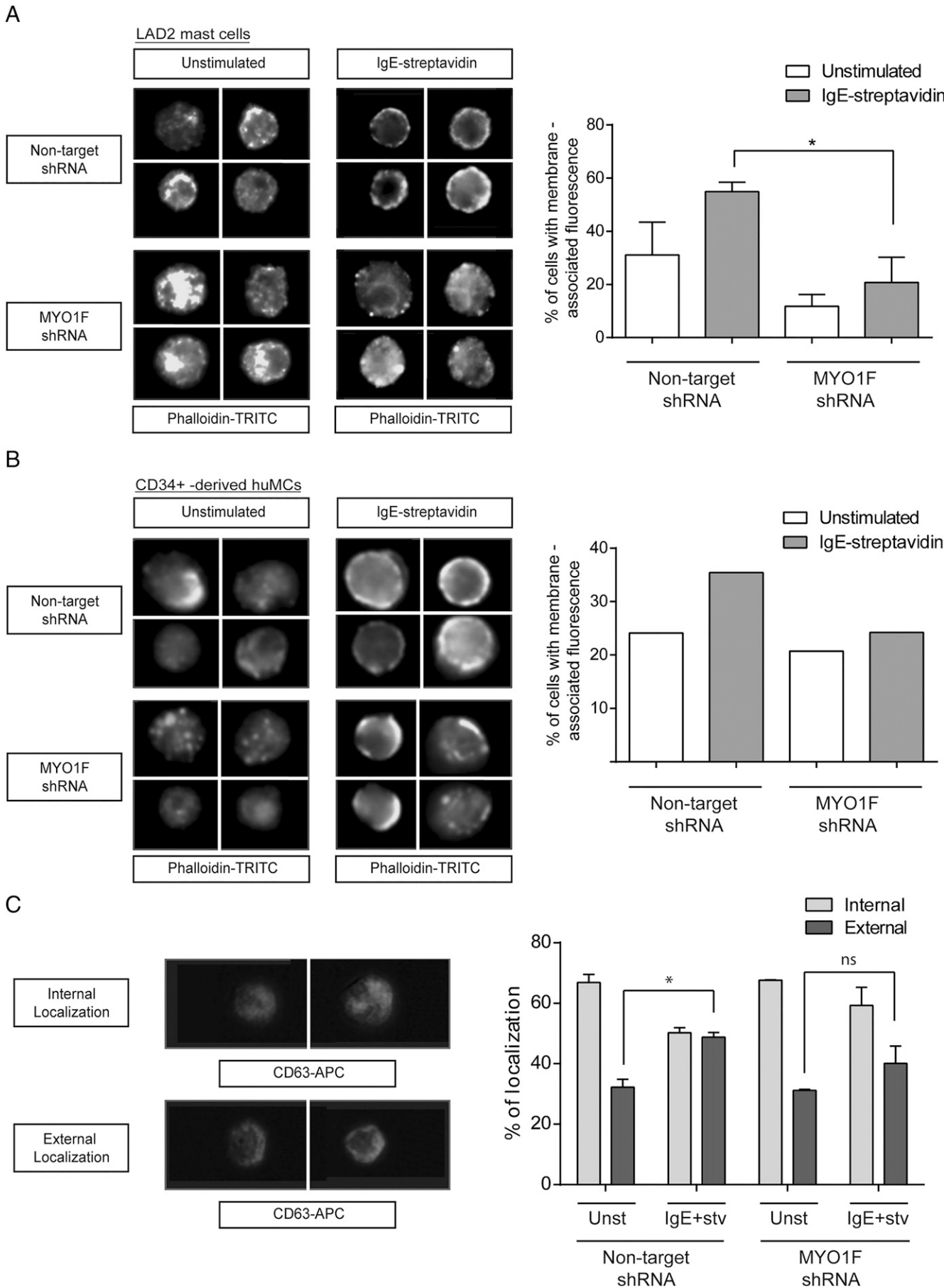


**FIGURE 5.** The downregulation of MYO1F alters the cortical actin ring after FcεRI activation and reduces granule localization close to the cell membrane after IgE-dependent activation. **(A)** Control (nontarget shRNA) and MYO1F-knockdown (MYO1F shRNA) LAD2 mast cells in (Figure legend continues)

prestored in mast cell granules (53), and can be released by regulated exocytosis, as well as synthesized following mast cell activation and released through constitutive exocytosis (26). Interestingly, in this case, we found a dependence of MYO1F expression in TNF secretion. Altogether, we found that the role of MYO1F varies according to the specific type of secretion.

The Rho family GTPases play key roles in exocytotic processes and in actin polymerization (28, 54). It is known that Cdc42-WASP-actin polymerization axis is important for degranulation and TNF secretion mediated by FcεRI activation (55). Our results adds to this pathway by involving MYO1F as a player in the regulation of active Cdc42 levels not only in cells stimulated via IgE/Ag but also via MRGPRX2 receptor activation, thus promoting degranulation and secretion of certain cytokines.

MYO1F, like all unconventional myosins, binds to cortical F-actin (as demonstrated by colocalization studies in neutrophils and mast cells) (11, 15). The actin cytoskeleton is part of the secretory machinery. Actin has been proposed to serve as a barrier to exocytosis, preventing fusion of the secretory granules with the plasma membrane, but may also play a role in the trafficking of these granules and assisting in the expulsion of their contents outside of the cell (24). In unicellular models like *Dyctiostelium*, it has been shown that MYO1F is responsible for the inhibition of actin waves with dependence of its membrane binding site but not the motor site (56). These actin waves are seen in mast cells during IgE Ag stimulation of FcεRI, driving a coordinated transformation of F-actin structures necessary for granule secretion (34). This process is a dynamic assembly and disassembly of cortical actin accompanied by changes in cell surface polarization (57). We demonstrated that cortical actin reorganization was impaired after IgE activation in MYO1F-knockdown cells, leading to a defective granule extrusion. Furthermore, inhibition of actin polymerization significantly reversed the impaired degranulation in MYO1F-silenced cells, suggesting that MYO1F is important for cortical actin reorganization after IgE activation.

In macrophages, MYO1F and the closely related MYO1E are required for phagocytosis of Ab-opsonized targets by macrophages. In the absence of these myosins, the organization of individual adhesions is compromised, leading to excessive actin polymerization, slower adhesion turnover, and deficient cup closure during phagocytosis (18). Also, the M1-phenotype polarization of macrophages, which is mediated by integrin-αVβ3, relies on the presence of MYO1F (17), which collaborates with MYO1E for efficient LPS-induced spreading (58). In a zebrafish model, MYO1F appears to be crucial for maintaining the blastodisc morphology and the actin cortex thickness (59). Altogether, the role of MYO1F in these different models point to an involvement of this myosin in actin reorganization in agreement with our data in mast cells.

Although total calcium responses were not altered in MYO1F-silenced cells, calcium mobilization in the absence of extracellular calcium, which represent release from intracellular stores, was slightly but significantly reduced in the first few minutes of mobilization. Mitochondria contribute to the regulation of Ca<sup>2+</sup> mobilization by coupling with the endoplasmic reticulum and maintaining Ca<sup>2+</sup> oscillations (60). Moreover, mitochondrial structure is

necessary for degranulation, as shown by the use of the antimicrobial agent triclosan, which disrupts mitochondrial nanostructure, leading to a defective function that decreases mast cell degranulation capacity (61). Furthermore, it has been reported that mitochondria undergo fission and translocation to the cell membrane after mast cell activation and that this translocation is necessary for degranulation and TNF secretion (37). Interestingly, we observed a diminished mitochondria fission capacity after stimulation and defective translocation to the plasma membrane in MYO1F-knockdown cells, which could have contributed to the defective degranulation and TNF secretion. Additionally, blockade of F-actin polymerization/depolymerization has been reported to alter the mitochondrial network (62). Our results show that inhibitors of polymerization significantly reverse the defective degranulation in MYO1F-silenced cells, so we hypothesize that MYO1F downregulation may alter actin filament dynamics and subsequently alter mitochondrial organization. Reinforcing a role of MYO1F in mitochondrial network, a mutation in MYO1F (Gly134Ser) in thyroid cancer cells has been reported to confer an altered mitochondrial network and induce tumor proliferation. MYO1F G134S increased mitochondrial mass but did not impair mitochondrial membrane potential or DRP1 phosphorylation, a protein involved in mitochondrial fission (63, 64). These data are consistent with our results in MYO1F-deficient cells, which showed defective degranulation but a normal mitochondrial membrane potential.

DRP1 is essential for mitochondria fission process (43, 64). According to Ji et al. (65), DRP1-dependent fission process can be explained in three steps: recruitment, maturation, and conversion. During the recruitment, DRP1 binds F-actin in the cytosol and translocates to the mitochondria. Actin-dependent localization of DRP1 to (17) mitochondria requires a myosin (66–68). Then, DRP1 is phosphorylated of Ser<sup>616</sup> and this modification contributes to mitochondrial localization of DRP1 and promotes mitochondrial fission (41). Our results show that after IgE- and MRGPRX2-dependent mast cell activation, the phosphorylation levels of DRP1 are significantly reduced in MYO1F-silenced cells. However, ERK1/2 activity (involved in DRP1 phosphorylation) (69) remained comparable in control and silenced cells. In contrast, MYO1F silencing reduced AKT phosphorylation. Consistent with our data, MYO1F has been reported to regulate AKT phosphorylation in macrophages (17). The way to do that could be related to actin dynamics where the role of a myosin stabilizing actin cytoskeleton could play a key role. In this context, it has been reported that AKT associates with the actin cytoskeleton, through the N-terminal pleckstrin domain of AKT (70). The same authors show that concomitant with association there is an increase in AKT phosphorylation and suggest that Cdc42 seems to facilitate AKT actin binding, possibly increasing AKT phosphorylation. Although AKT activity has been directly linked to DRP1 phosphorylation and mitochondrial fission in an Alzheimer disease model (71) more experiments are needed to fully understand the AKT-DRP1 pathway. Altogether, our data point out that MYO1F is crucial for AKT activation and DRP1 phosphorylation controlling mitochondrial fission. Further experiments will be needed to dissect this signaling.

the presence of doxycycline (0.5 μg/ml) with or without stimulation with IgE<sup>B</sup> + streptavidin (200 ng/ml) for 15 min were fixed and analyzed in the imaging flow cytometer. Each group was divided into two subgroups, one with low translocation of actin to the cell membrane and another with high translocation and a clearly visible cortical actin ring (actin is shown in yellow). The percentage of cells with a visible actin ring is represented and compared with the other groups. (B) Control (nontarget shRNA) and MYO1F-knockdown (MYO1F shRNA) CD34<sup>+</sup>-derived huMCs stimulated with or not with IgE<sup>B</sup> + streptavidin (200 ng/ml) were fixed and analyzed in the imaging flow cytometer as described in (A). (C) Control (nontarget shRNA) and MYO1F-knockdown (MYO1F shRNA) LAD2 mast cells in the presence of doxycycline (0.5 μg/ml) stimulated with or not with IgE<sup>B</sup> + streptavidin (200 ng/ml) were fixed and analyzed in the imaging flow cytometer. They were divided into two subgroups, one in which CD63 expression was close to the center of the cell (internal) and the other in which CD63 expression was at the periphery (external). The percentages are represented and compared between groups. One-way ANOVA test was used for the statistical analysis. \**p* < 0.05. ns, not significant.

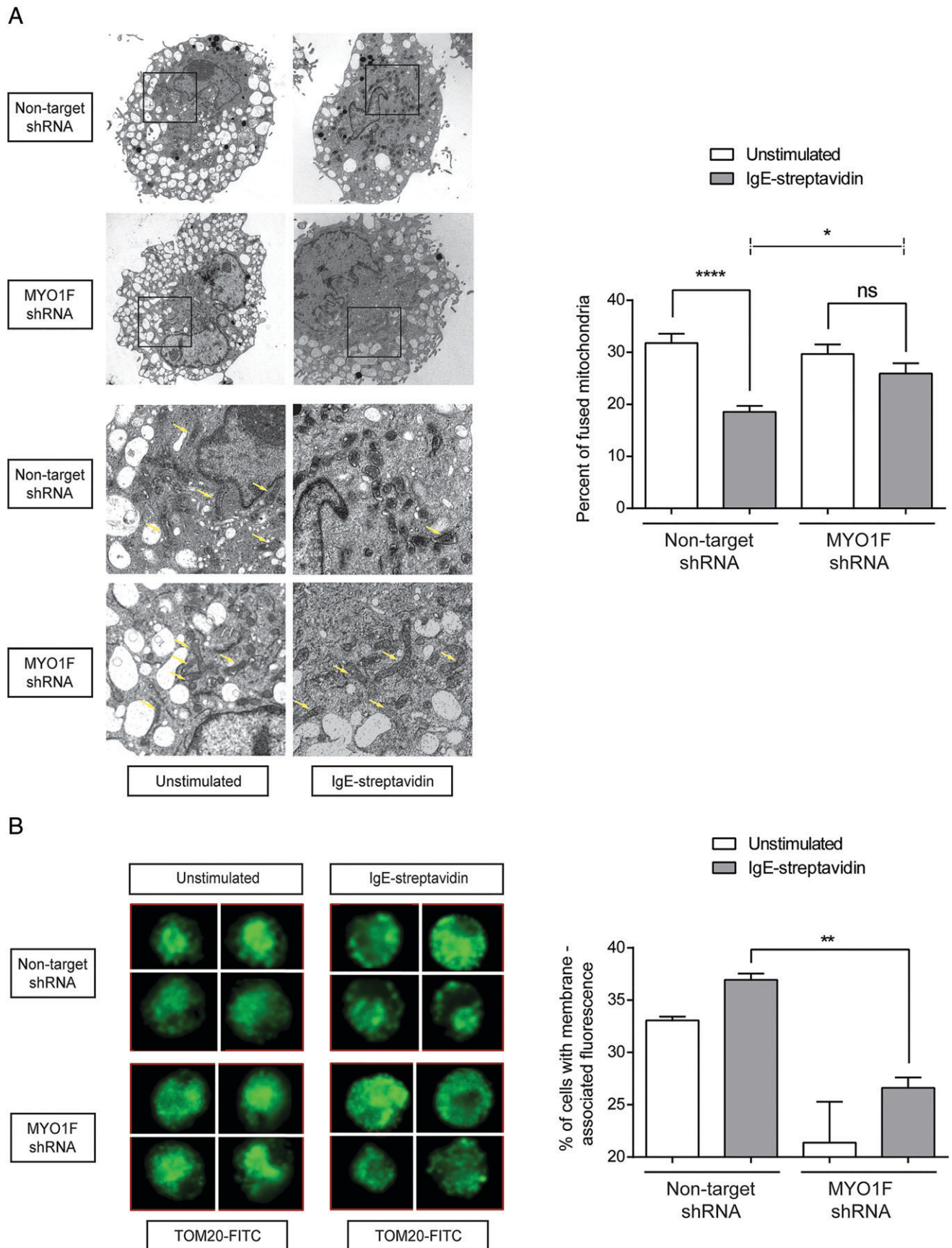


**FIGURE 6.** Mitochondria do not translocate to the cell membrane and present fewer fission events in MYO1F-silenced cells after FcεRI activation. **(A)** Nontarget shRNA and MYO1F shRNA LAD2 mast cells in the presence of doxycycline (0.5 μg/ml) were stimulated with IgE<sup>B</sup> + streptavidin (200 ng/ml) for 30 min, fixed, and prepared for electronic microscopy. Total mitochondria were counted using ImageJ software and the percentage of elongated mitochondria (elongated mitochondria are indicated by yellow arrows in the magnified image) was compared between groups. **(B)** (Figure legend continues)

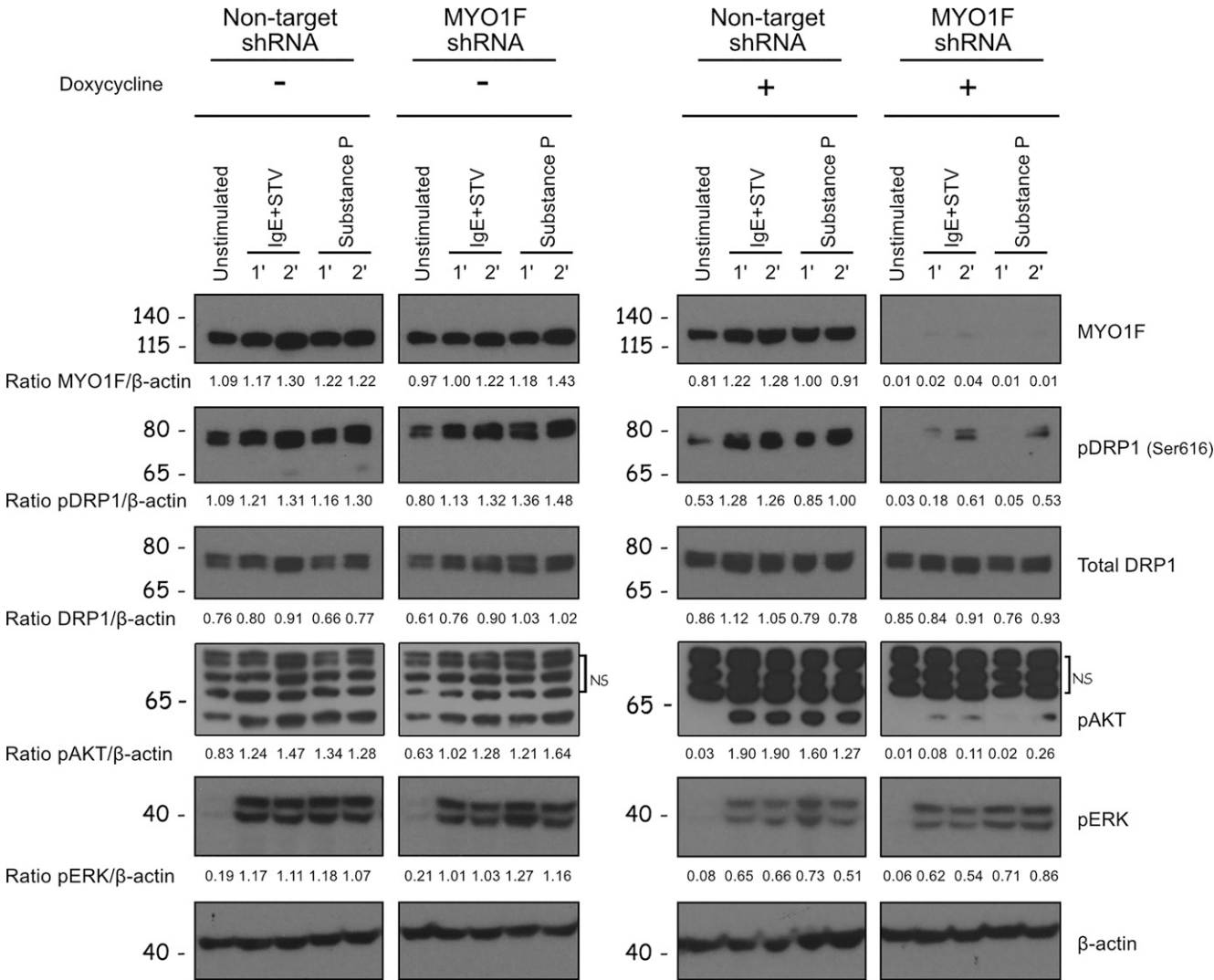


**FIGURE 7.** MYO1F knockdown does affect DRP1 phosphorylation on Ser<sup>616</sup> after Fc $\epsilon$ RI and MRGPRX2 activation. Control (nontarget shRNA) and MYO1F-knockdown (MYO1F-shRNA) LAD2 mast cells in the presence or not of doxycycline (0.5  $\mu$ g/ml) were sensitized with IgE<sup>B</sup> or left untreated. The next day, cells were challenged with streptavidin (400 ng/ml). In contrast, cells were treated with SP (2  $\mu$ M) for the indicated times. Western blots probed with the following Abs: anti-MYO1F, anti-pDRP1 (Ser<sup>616</sup>), anti-DRP1, anti-pAKT (Ser<sup>473</sup>), anti-pERK, and anti- $\beta$ -actin (used as loading control). Band intensity for each blot was quantified by densitometry. NS, nonspecific bands.

MYO1F apart from the N-terminal motor domain (actin binding), L chain-binding IQ motifs (calmodulin binding) and a basic tail homology 1 (TH1) thought to interact with membranes has an additional proline-rich TH2 domain and a TH3 domain containing a single Src homology (SH3) domain (72). Recently, our group reported that the SH3 domain of MYO1F binds the adaptor protein 3BP2, which regulates both KIT migration and survival as well as Fc $\epsilon$ RI-dependent degranulation (11, 13, 14). Our study links MYO1F to IgE and non-IgE receptor-induced signaling pathways and their function and describes a role for this protein on actin mesh dynamics. Because MRGPRX2 has emerged as an important receptor in mast cells contributing to innate and adaptive immunity, chronic allergic diseases and potentially neurogenic inflammation, pain, and itch (73), our data may have wider implications in mast

cell biology beyond allergic inflammation. In macrophages, MYO1F is specifically localized to the Fc-receptor adhesions and is required for efficient phagocytosis of Ab-opsonized targets (18). Interestingly, the adaptor protein 3BP2 is also required for optimal FcR $\gamma$ -mediated phagocytosis and chemokine expression in macrophages (74) which points that both partners may play a role in cytoskeleton dynamics in macrophages and mast cells.

A better understanding of mediator-specific trafficking and its regulatory pathways by cytoskeleton-interacting motor proteins may bring us new information and hopefully therapeutic targets for the treatment of increasingly widespread mast cell-related diseases.

Nontarget shRNA and MYO1F shRNA LAD2 mast cells unstimulated or activated with IgE<sup>B</sup> + streptavidin (200 ng/ml) for 15 min were fixed and analyzed in the imaging flow cytometer. Each group was divided into two subgroups, one with low translocation of mitochondria to the cell membrane and another with high translocation (mitochondria are shown in green). The percentage of cells with high translocation is represented and compared with the other groups. One-way ANOVA was used for the statistical analysis. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ . ns, = not significant.

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## Disclosures

The authors have no financial conflicts of interest.

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## Corrections

Navinés-Ferrer, A., E. Ainsua-Enrich, E. Serrano-Candelas, E. Proaño-Pérez, R. Muñoz-Cano, G. Gastaminza, A. Olivera, and M. Martín. 2021. MYO1F regulates IgE and MRGPRX2-dependent mast cell exocytosis. *J. Immunol.* 206: 2277–2289.

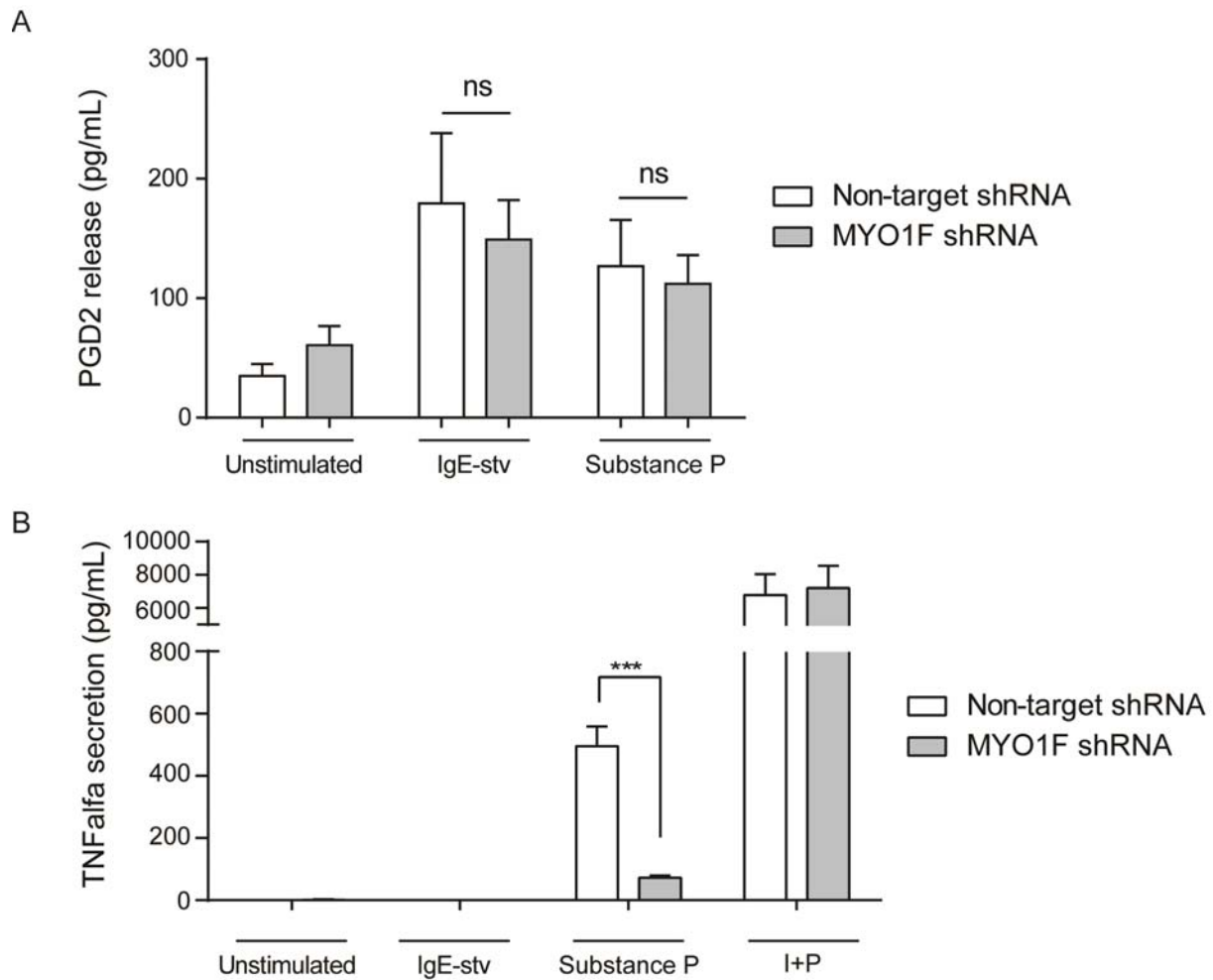
An affiliation was omitted from the fourth author's affiliations. The corrected author list and affiliations are shown below. This has been corrected in the online version of the article, which now differs from the print version as originally published.

Arnau Navinés-Ferrer,<sup>\*,†</sup> Erola Ainsua-Enrich,<sup>\*,†</sup> Eva Serrano-Candelas,<sup>\*,†</sup> Elizabeth Proaño-Pérez,<sup>\*,†,‡</sup> Rosa Muñoz-Cano,<sup>†,§,¶</sup> Gabriel Gastaminza,<sup>¶,||</sup> Ana Olivera,<sup>#</sup> and Margarita Martín<sup>\*,†,¶</sup>

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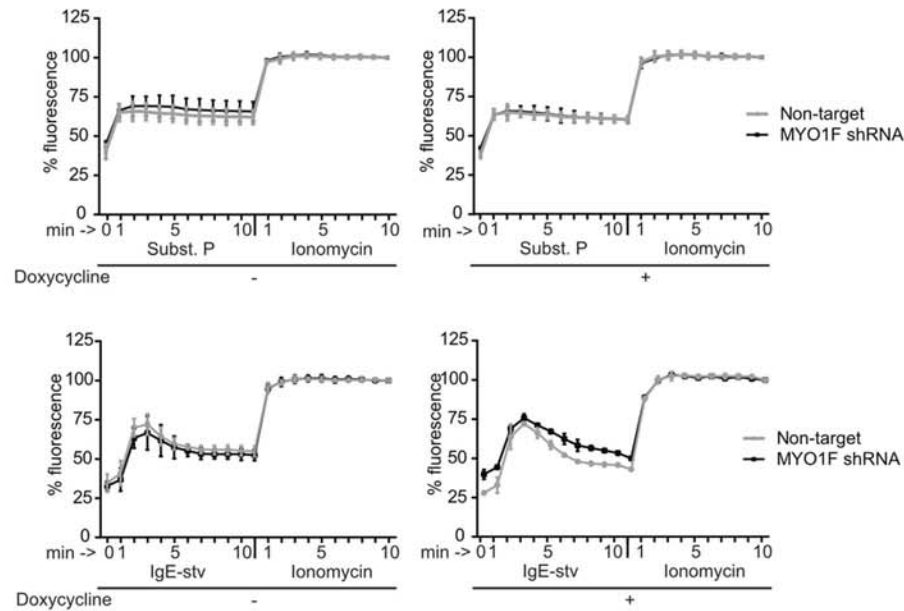
Supplementary Figure 1



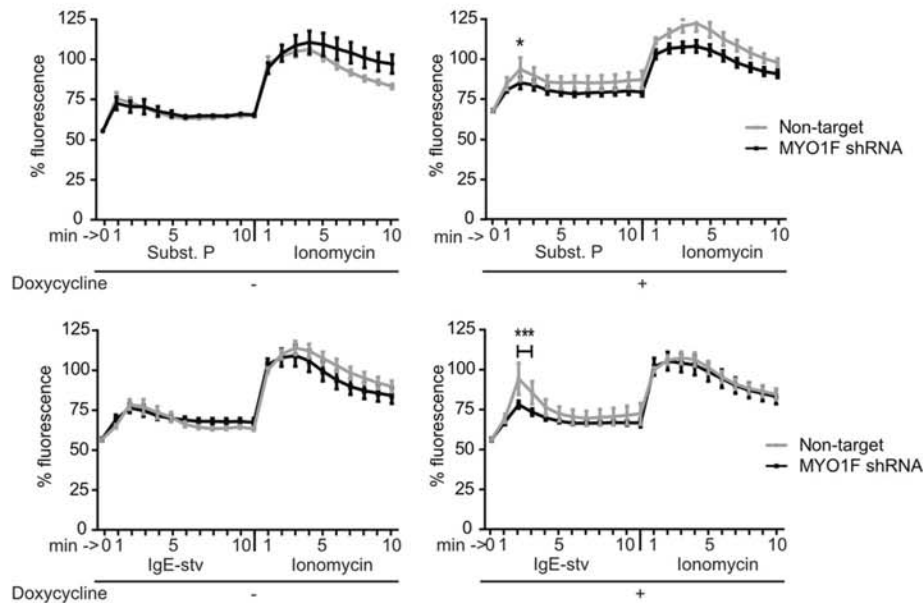
**Supplementary Figure 1. PGD2 release is not affected but TNF $\alpha$  secretion is diminished in MYO1F-silenced cells.** (A) PGD2 release was determined in the supernatant of control (Non-target shRNA) and MYO1F-deficient (MYO1F-shRNA) LAD2 mast cells in the presence of doxycycline (0.5  $\mu$ g/mL) with or without stimulation with IgE<sup>B</sup> + streptavidin (200 ng/mL) or substance P (2  $\mu$ M) for 4 h. (B) TNF $\alpha$  secretion was determined in the supernatant of control (non-target shRNA) and MYO1F-deficient (MYO1F-shRNA) LAD2 mast cells in the presence of doxycycline (0.5  $\mu$ g/mL) after stimulation with IgE<sup>B</sup> + streptavidin (200ng/mL), substance P (2  $\mu$ M) or Ionomycin (200  $\mu$ g/mL) plus PMA (10 ng/mL) (I+P) for 24 h. One-way ANOVA test was used for the statistical analysis (ns= not significant, \*\*\*p<0.001).

## Supplementary Figure 2

A



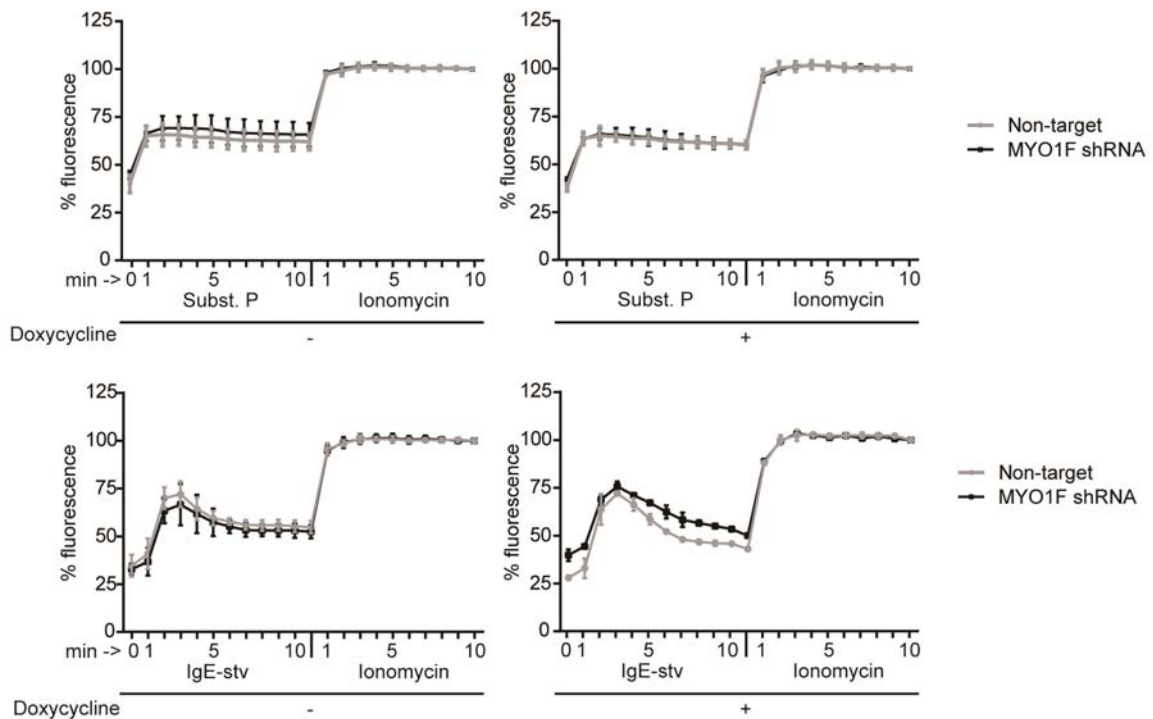
B



### Supplementary Figure 2. Intracellular calcium signaling is affected in MYO1F-knockdown cells (A)

Control (non-target shRNA) and MYO1F-knockdown (MYO1F shRNA) LAD2 mast cells were preincubated with Fluo4 and activated with Substance P (2 μM) or IgE<sup>B</sup> + streptavidin (200 ng/mL) for 10 minutes and then with Ionomycin (200 μg/mL) for another 10 minutes in Tyrodes buffer. The percentage of fluorescence is represented as 100% for the first minute after Ionomycin activation. (B) Control (non-target shRNA) and MYO1F-knockdown (MYO1F shRNA) LAD2 mast cells were treated as described above in a calcium-free Tyrodes buffer. The percentage of fluorescence is represented as 100% for the first minute after Ionomycin activation. The student-t test was used for the statistical analysis (\*p<0.05, \*\*\*p<0.001).

Supplementary Figure 3



**Supplementary Figure 3. MYO1F-knockdown does not affect membrane potential of mitochondria after activation.**

Control (Non-target shRNA) and MYO1F-knockdown (MYO1F shRNA) LAD2 mast cells were preincubated with TMRM and activated with Substance P (2  $\mu$ M) or IgE + streptavidin (200 ng/mL) for 10 minutes and then with Ionomycin (200  $\mu$ g/mL) for another 10 minutes in Tyrodes buffer. Percentage of fluorescence is represented as a 100% for the first minute after Ionomycin activation.



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### Correction: MYO1F Regulates IgE and MRGPRX2-Dependent Mast Cell Exocytosis

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## Corrections

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An affiliation was omitted from the fourth author's affiliations. The corrected author list and affiliations are shown below. This has been corrected in the online version of the article, which now differs from the print version as originally published.

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