# Zinc is a novel intracellular second messenger

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Zinc is an essential trace element required for enzymatic activity and for maintaining the conformation of many transcription factors; thus, zinc homeostasis is tightly regulated. Although zinc affects several signaling molecules and may act as a neurotransmitter, it remains unknown whether zinc acts as an intracellular second messenger capable of transducing extracellular stimuli into intracellular signaling events. In this study, we report that the cross-linking of the high affinity immunoglobin E receptor (Fcε receptor I [FcεRI]) induced a release of free zinc from the perinuclear area, including the endoplasmic reticulum in mast cells, a phenomenon we call the zinc wave. The zinc wave was dependent on calcium influx and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase activation. The results suggest that the zinc wave is involved in intracellular signaling events, at least in part by modulating the duration and strength of FcεRI-mediated signaling. Collectively, our findings indicate that zinc is a novel intracellular second messenger.

## Introduction

Zinc is a structural constituent of a great number of proteins, including enzymes belonging to cellular signaling pathways and transcription factors, and it is essential for their biological activity (Vallee and Auld, 1993; Prasad, 1995). Zinc has a variety of effects on the immune and nervous systems in vivo and vitro, and these effects mainly depend on the zinc concentration (Rink and Gabriel, 2000; Frederickson et al., 2005). Many researchers have reported that immune function decreases after zinc depletion. Zinc-deficient mice exhibit reduced natural killer cell–mediated cytotoxic activity, antibody-mediated responses, and host defense against pathogens and tumors (Fernandes et al., 1979; Fraker et al., 1982; Keen and Gershwin, 1990). The requirement for zinc is most likely because of its essential constitutive role in maintaining the conformation or enzymatic activity of many important components of these processes, including enzymes, transcription factors, and signaling molecules. On the other hand, zinc itself is cytotoxic: zinc induces apoptosis in T and B cells (Telford and Fraker, 1995; Ibs and Rink, 2003) and neuronal death (Koh et al., 1996; Sensi and Jeng, 2004). Therefore, the intracellular zinc concentration is tightly controlled by zinc importers (ZIPs/SLA39s; Eide, 2004), exporters (zinc transporters/SLC30s; Palmiter and Huang, 2004), and binding proteins such as metallothioneins (Vallee, 1995). In addition, zinc-sensing molecules such as metal response element–binding transcription factor-1 respond to free zinc levels by regulating gene expression to maintain zinc homeostasis (Andrews, 2001).

Zinc has been shown to act as a neurotransmitter (Colvin et al., 2003; Frederickson, 2003). In neurons, exocytotic stimuli induce zinc release into the surrounding milieu and its uptake into the cytoplasm through gated zinc channels on neighboring cells. Synaptically released zinc probably travels to adjacent cells such as postsynaptic neurons and glial cells and functions as a modulator and mediator of cell-to-cell signaling (Xie and Smart, 1994; Hershfinkel et al., 2001; Li et al., 2001). In this role, zinc acts as an autocrine or paracrine, transcellular, transmembrane signaling factor, like a neurotransmitter.

Zinc mimics the actions of hormones, growth factors, and cytokines, which suggests that zinc may act on intracellular signaling molecules (Beyersmann and Haase, 2001). In fact, zinc is a well-known inhibitor of protein tyrosine phosphatases (Brautigan et al., 1981). The inhibition constant is reported to be in the nanomolar range (Maret et al., 1999). In addition, zinc affects the regulation of transcription factors. Zinc can induce the expression of some genes, including those coding for molecules involved in zinc homeostasis, like zinc transporters and metallothioneins (Palmiter, 2004). The gene expression of metallothioneins by zinc is regulated by metal response element–binding

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Abbreviations used in this paper: BMMC, bone marrow–derived mast cell; DTPA, diethylenetriaminepentaacetic acid; ERK, extracellular signal-regulated kinase; FcεRI, Fcε receptor I; IL, interleukin; $IP_3R$, inositol 1,4,5-triphosphate receptor; MEK, MAPK/ERK kinase; TIRF, total internal reflection fluorescence.

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transcription factor-1 (Lichtlen and Schaffner, 2001). We previously reported that the nuclear localization of the transcription factor Snail is dependent on the zinc transporter Zip6, suggesting that zinc plays a role in the nuclear localization of Snail and may act as an intracellular signaling molecule (Yamashita et al., 2004). This notion was further supported by the finding that toll-like receptor 4–mediated dendritic cell maturation is, at least in part, dependent on a toll-like receptor 4–induced decrease in intracellular free zinc (Kitamura et al., 2006). Collectively, this evidence suggests that zinc may act as an intracellular signaling molecule. However, the toll-like receptor 4–mediated decrease in intracellular free zinc is dependent on the change in the expression profile of zinc transporters. Therefore, it remains unknown whether zinc acts as an intracellular second messenger like calcium and cAMP. A second messenger is defined as a molecule whose intracellular status is directly altered by extracellular stimuli and that can transduce the extracellular stimuli into intracellular signaling events.

In this study, we report that an extracellular stimulus such as high affinity IgE receptor (Fcε receptor I [FcεRI]) cross-linking directly induces a release of free zinc from the area of the ER in mast cells, a phenomenon we call the zinc wave. The zinc wave occurred in a manner dependent on calcium influx and MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) activation. Based on our results, we suggest that one of the roles of the zinc wave is to inhibit phosphatase activity, resulting in the modulation of MAPK activation and the expression of the genes for interleukin-6 (IL-6) and TNFα in mast cells. Our results show that zinc is a novel intracellular second messenger.

## Results

### Increase in intracellular free zinc after FcεRI activation in mast cells

To investigate whether the level of intracellular free zinc changes after FcεRI stimulation, we observed its level over time using the zinc indicator Newport green DCF. The fluorescent signal was observed mainly in the cytoplasm, and a gradual enhancement of fluorescence intensity was observed several minutes after stimulation in the center of the cell rather than in its peripheral region, suggesting that the zinc was released from intracellular stores (Fig. 1, A and B; and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200702081/DC1). No obvious change was seen in the absence of stimulation (Fig. 1 B and Video 2). The cell-impermeable zinc chelator diethylenetriaminepentaacetic acid (DTPA) did not inhibit the enhancement of the Newport green fluorescence, further supporting the idea that the zinc was released from intracellular stores (Fig. 1 C). Treatment of the cells by 10 μM of cell-permeable metal chelator *N,N,N,N*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) decreased the enhancement of Newport green fluorescence, but 10 μM of chelators for copper (ammonium tetrathiomolybdate), iron (2,2'-dipyridyl), or manganese (*p*-aminosalicylic acid) did not, indicating that the increased fluorescence was specific for changes in zinc levels (Fig. 1 D). These results were supported by the relative increase of fluorescence intensity of Newport green for each metal ion (for $Zn^{2+} = 1.0$, $Cu^{2+} = -0.35$, $Fe^{2+} = 0.30$, and $Mn^{2+} = 0.22$, respectively). This increase in intracellular free zinc was observed several minutes after the stimulation, in contrast to the rapid increase in intracellular calcium, which occurred seconds afterward (Fig. 1 E). These results indicated that the FcεRI stimulation induced an increase in intracellular free zinc. We called this phenomenon the zinc wave.

FcεRI-mediated signal transduction occurs by two pathways: the Lyn–Syk–SLP-76–PLCγ2 pathway and the Fyn–Gab2 (Grb2-associated binder 2) pathway (Rivera, 2002; Nishida et al., 2005). The Lyn–Syk–SLP-76–PLCγ2 pathway is required for inositol 1,4,5-triphosphate receptor ($IP_3R$)–dependent calcium release (Turner and Kinet, 1999), whereas the Fyn–Gab2 pathway has little or no effect on intracellular calcium (Parravicini et al., 2002). To investigate which pathway is required for the FcεRI-induced zinc wave, we used mast cells defective in various signaling molecules. As shown in Fig. 1 (F–H), the zinc wave was diminished in Syk- and PLCγ2-deficient mast cells but not in Gab2-deficient mast cells. Thus, the zinc wave was induced by FcεRI via the Syk–PLCγ2-dependent pathway.

### Both calcium and MAPK activation are required for the zinc wave

Because Syk–PLCγ2 is required for calcium signaling and the zinc wave was observed after calcium influx, we next investigated whether the zinc wave requires calcium signaling. As shown in Fig. 2 A, under calcium-free conditions, the zinc wave was suppressed. Furthermore, Xestospongin C (an inhibitor of $IP_3R$) inhibited the zinc wave (Fig. 2 B), and this suppression was reversed by simultaneous treatment with a calcium ionophore, ionomycin (Fig. 2 C). These results suggested that the entry of external calcium induced by FcεRI stimulation is required to trigger the zinc wave. However, ionomycin, which induces calcium influx, could not induce the zinc wave by itself (Fig. 2 D). Collectively, these results indicated that calcium is essential but not sufficient for the FcεRI-induced zinc wave.

To reveal what signals, in addition to calcium, are required for the zinc wave, we tested whether the Ras–MAPK pathway is involved by examining the effect of the MEK inhibitors PD98059 and U0126. As shown in Fig. 2 E, both PD98059 and U0126 inhibited the zinc wave without any effect on the calcium influx. These results indicated that both calcium and MEK signaling are involved in the zinc wave. Consistent with this notion, as shown in Fig. 2 F, simultaneous treatment with ionomycin and EGF induced the zinc wave, although neither treatment alone did so. Furthermore, the ionomycin- and EGF-induced zinc wave was blocked by a MEK inhibitor. We confirmed that EGF alone could not induce calcium influx (unpublished data). These results clearly indicated that the zinc wave is regulated by calcium- and MEK-dependent pathways.

### Identification of regions where the zinc wave originates

Fluorescence laser scanning is known to induce reactive oxygen species (Jou et al., 2004), which may induce the release of free zinc from zinc-binding proteins such as metallothionein,

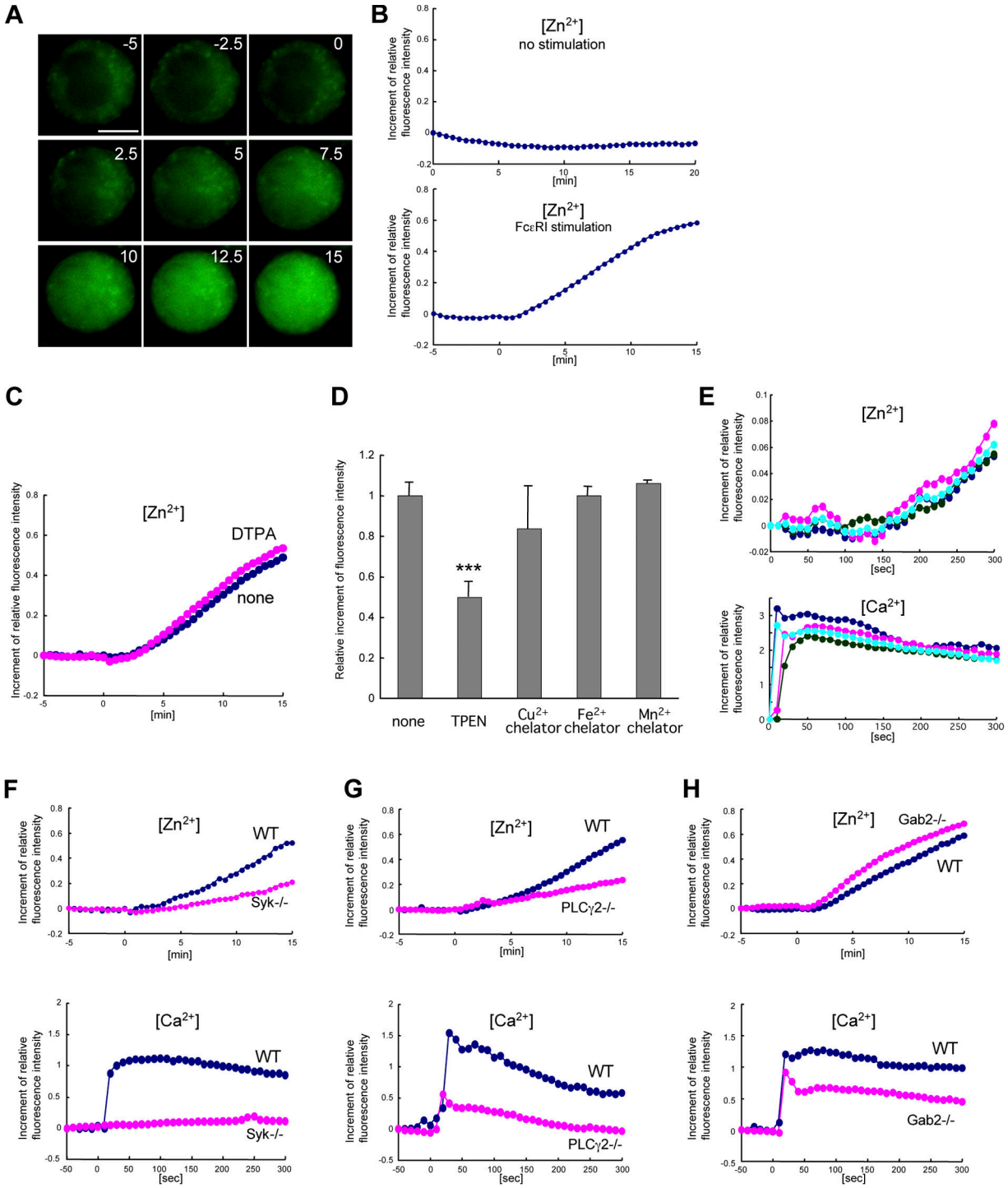


Figure 1. **Elevation of intracellular zinc level upon FcεRI stimulation.** (A) BMMCs containing Newport green were stimulated with FcεRI, and intracellular fluorescence was assessed every 30 s at 25°C. The numbers represent time after stimulation (in minutes). Bar, 5 μm. (B) Newport green fluorescence was quantified relative to the signal intensity of the first image observed. To rule out spontaneous Newport green elevation caused only by fluorescence emissions, a 5-min preobservation period was included. Quantification was performed for at least 10 cells in the observed field, and one representative of three independent experiments is shown. (C) The effect of a cell-impermeable zinc chelator, diethylenetriaminepentaacetic acid (DTPA), on the FcεRI-mediated elevation of the intracellular Newport green signal. Cells were stimulated with FcεRI in the presence or absence of 1 mM DTPA. (D) The effect of chelators for $Zn^{2+}$, $Fe^{2+}$, $Cu^{2+}$, and $Mn^{2+}$ on the FcεRI-mediated change in the Newport green signal detected by flow cytometry. Cells were pretreated with 10 μM each of chelator and were stimulated with FcεRI for 15 min. The affinity of TPEN for the metal ions is $Ka = 10^{15.58}\ M^{-1}$, $10^{14.61}\ M^{-1}$, $10^{20}\ M^{-1}$, and $10^{10.27}\ M^{-1}$ for $Zn^{2+}$, $Fe^{2+}$, $Cu^{2+}$, and $Mn^{2+}$, respectively. We also used 2,2'-dipyridyl ($Ka = 10^{17.2}\ M^{-1}$ for $Fe^{2+}$), ammonium tetrathiomolybdate, and *p*-aminosalicylic acid to chelate $Fe^{2+}$, $Cu^{2+}$ and $Mn^{2+}$, respectively. Data are means ± SD (error bars) of three independent experiments. ***, $P < 0.001$ compared with no chelator (*t* test). (E) Newport green (for $Zn^{2+}$) or Fluo-4 (for $Ca^{2+}$) was incorporated into cells, and the fluorescence after stimulation was observed every 10 s. (F–H) BMMCs from Syk-, PLCγ2-, and Gab2-deficient mice containing Newport green or Fluo-4 were stimulated. One representative of three independent experiments is shown for each panel.

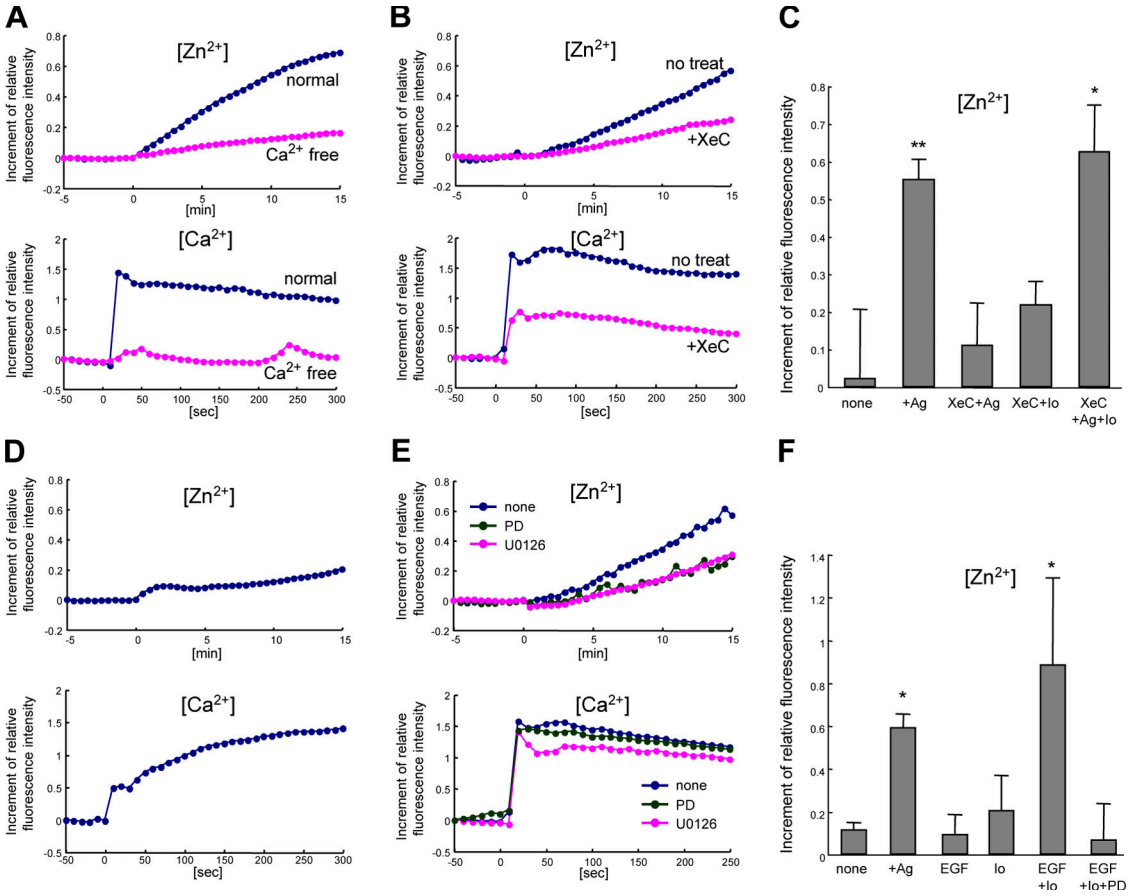


Figure 2. **Zinc wave requires both calcium and MAPK activation.** (A) BMMCs containing Newport green or Fluo-4 were stimulated with FcεRI in calcium-free Tyrode's buffer. (B) 10 μM $IP_3R$ inhibitor (XeC) was added 30 min before FcεRI stimulation. (C) Zinc wave in the presence of 10 μM XeC and 300 nM ionomycin (Io). The relative fluorescence intensity 15 min after stimulation is shown. Ag represents DNP–human serum albumin stimulation–dependent FcεRI activation. (D) Ionomycin-induced zinc and calcium waves were observed. (E) Effect of MEK1/2 inhibitors U0126 and PD98059 on zinc and calcium waves. Cells were pretreated with 50 μM U0126 or 100 μM PD98059 and Newport green for 30 min and were stimulated with FcεRI. (F) Effect on the zinc wave of EGF stimulation and ionomycin treatment, alone and in combination, and with PD98059 pretreatment. Data are means ± SD (error bars) of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with no stimulation or additions (none; *t* test).

independent of receptor-mediated signaling events. To identify the subcellular regions where the zinc wave originates while avoiding secondary effects caused by cell stress, we used a thin-layer illumination microscope system that was based on the total internal reflection fluorescence (TIRF) microscope (Tokunaga et al., 1997). By recording images with highly sensitive CCD video cameras, we could detect fluorescence signals with a lower laser power than is usual for observation under a confocal laser microscope. Using this modified TIRF microscope, we observed the FcεRI stimulation–dependent zinc wave (Fig. 3 A). To learn where the zinc wave was generated, we performed 3D imaging of mast cells costained with Newport green and the ER-specific marker ER-tracker red. As shown in Fig. 3 A, the fluorescence signal of the Newport green was enhanced mainly in the perinuclear and nuclear areas after FcεRI stimulation. Importantly, the fluorescence intensity of the Newport green initially increased specifically in the area stained by the ER marker (Fig. 3 B, 0.5 min). This observation suggested that the zinc wave most likely originated in regions that include the ER, although we do not neglect the possible involvement of other sources, such as the nucleus.

### Role of the zinc wave on the modulation of phosphatase activity

To determine whether the zinc wave can affect signaling events, we investigated the effect of zinc depletion by TPEN and of enforced zinc influx using the zinc ionophore pyrithione on FcεRI-induced signaling events. TPEN treatment inhibited the zinc wave (Fig. 1 D), whereas pyrithione/zinc (Py/Zn) treatment rapidly increased the free zinc in cells (Fig. 4 A). TPEN treatment decreased the FcεRI-induced IL-6 and TNFα mRNA expression (Fig. 4 B) and the activation of ERK and JNK (Fig. 4 C). In particular, 5–60 min after stimulation, ERK activation was still impaired (Fig. 4 C). On the other hand, Py/Zn prolonged the expression of IL-6 and TNFα mRNA as well as the activation of the MAPKs induced by FcεRI stimulation (Fig. 4, B and C). We confirmed that the Py/Zn-induced sustained MAPK activation was canceled by TPEN (Fig. 4 C). These results suggested that one of roles of the zinc wave is to regulate the duration of MAPK activation and modulation of the late phase of these signaling events.

Zinc is reported to inhibit phosphatase activity (Brautigan et al., 1981); therefore, one likely target of the zinc wave is

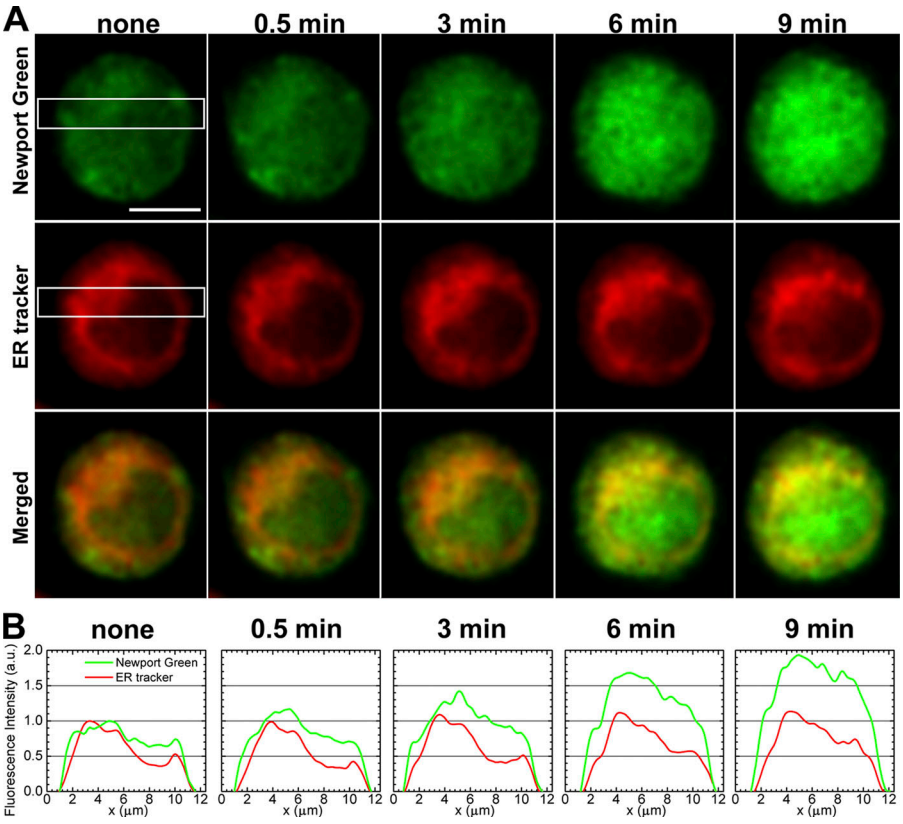


Figure 3. **Initiation of the zinc wave in the perinuclear region, especially near the ER.** Observation of the zinc wave by a thin-layer illumination microscope system based on a TIRF microscope. (A) Simultaneous images of Newport green signals (top), ER-tracker red (middle), and merged images (bottom) in a cross section 4 μm above the bottom of the costained BMMCs. Time after FcεRI stimulation is shown above the images. Bar, 5 μm. (B) Fluorescence intensity profile of the rectangular areas indicated in A is displayed for each time point after FcεRI stimulation. The fluorescence intensity was vertically integrated and normalized with the maximum intensity and plotted against the horizontal distance through the selected area.

phosphatase. In fact, Py/Zn treatment enhanced the total FcεRI-induced tyrosine phosphorylation in mast cells (Fig. 4 D). Furthermore, the phosphatase activity of the FcεRI-stimulated mast cells was inhibited by the addition of Py/Zn, and this inhibitory effect was rescued by TPEN (Fig. 4 E). To verify this result, we tested whether zinc directly inhibits tyrosine phosphatase activity using mast cell lysate. The results showed that zinc addition inhibited tyrosine phosphatase activity in a dose-dependent manner (Fig. 4 F). Collectively, the results support the hypothesis that the zinc wave plays a role, at least in part, in the modulation of signaling efficiency, with phosphatase as one of its targets.

## Discussion

### The zinc wave originated in the perinuclear region that includes the ER in a manner dependent on calcium influx and MEK activation

In this study, we showed that the FcεRI stimulation of mast cells induced the zinc wave, which originated in the perinuclear region that includes the ER, several minutes after the stimulation. This zinc wave was dependent on both calcium and MEK signaling. We further showed that the zinc wave was dependent on calcium influx, although calcium influx alone was not sufficient to induce it. In addition to the calcium influx, we showed that MEK activation was essential to induce the zinc wave. Thus, we have provided the molecular framework of the zinc wave regulation, although we still do not know the precise mechanisms by which calcium and MEK signaling are integrated to induce the zinc wave. An interesting hypothesis is that an unknown molecule whose activity is regulated by both calcium and MEK plays a role in inducing the zinc wave. This issue needs to be clarified to fully understand the zinc wave.

Several studies have shown that many secretory cells, such as nerve, pancreatic, and mast cells, contain granules with high concentrations of zinc (Gustafson, 1967; Frederickson and Moncrieff, 1994; Kristiansen et al., 2001). In nerve cells, zinc released by exocytotic stimuli can be taken up into the cytoplasm, resulting in an increase of free zinc (Sensi et al., 1999; Aizenman et al., 2000; Yin et al., 2002; Gyulkhandanyan et al., 2006). In these studies, the source of the increased zinc ion was extracellular. In contrast to these observations, we showed that the source of the zinc wave induced by FcεR1 stimulation was intracellular, not extracellular. The possibility of extracellular zinc influx was unlikely because (1) the cell-impermeable zinc chelator DTPA did not inhibit the zinc wave (Fig. 1 C), and (2) as shown in Fig. 1 H, the zinc wave was still observed in mast cells derived from Gab2-deficient mice, which are defective in FcεRI-mediated degranulation (Nishida et al., 2005), negating the possibility that zinc released from mast cells upon degranulation was the source of the zinc wave. Moreover, we showed that upon FcεRI stimulation, the zinc wave originated in the perinuclear region that includes the ER (Fig. 1 A). This observation was confirmed using a thin-layer illumination microscope and ER marker. The zinc wave was first seen in the region that was stained with an ER marker, such as ER-tracker red, and then the increased free zinc was observed in the nucleus (Fig. 3, A and B). At present, however, it remains possible that other intracellular compartments, including the nucleus and

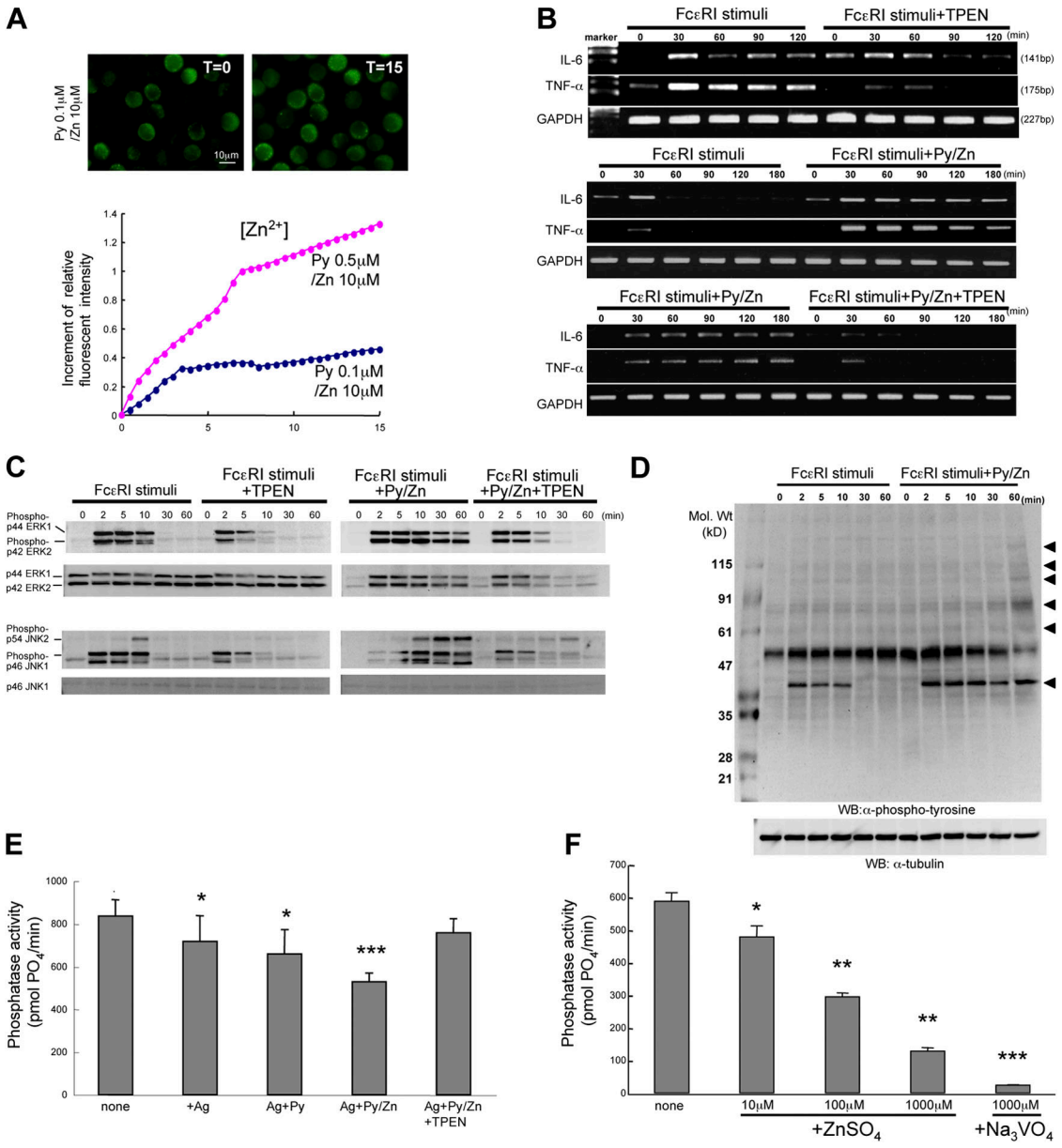


Figure 4. **The zinc wave has a role in cytokine production through the modulation of phosphatase activity.** (A) BMMCs were treated with the zinc ionophore pyrithione (Py) and $ZnSO_4$ (Zn). Images show Newport green–containing mast cells before and after (15 min) the addition of 0.1 μM Py and 10 μM Zn. (bottom) Relative fluorescence intensity in cells treated with 10 μM Zn and 0.1 or 0.5 μM Py. (B) The effect of simultaneous zinc chelation or zinc influx with FcεRI activation on IL-6 and TNF-α mRNA induction was determined by RT-PCR. Cells were treated with 10 μM TPEN or 10 μM Zn and 0.1 μM Py simultaneously with FcεRI stimulation. (C) Effect of TPEN or zinc influx on FcεRI-mediated ERK and JNK activation. Cells were treated as in B. (D) Cells were stimulated with FcεRI, 10 μM Zn, and 0.1 μM Py for the indicated times, and protein phosphorylation was detected. Arrowheads indicate proteins showing enhanced phosphorylation. (E) Intracellular phosphatase activity was measured using phosphopeptide as a substrate. Whole-cell lysate from unstimulated (none) or FcεRI-stimulated cells treated with 1 μM Py alone, 10 μM Zn alone, Py and Zn combined, or the combined Py and Zn with TPEN were incubated with phosphopeptide at 37°C for 30 min. (F) Phosphatase activity was measured using whole-cell lysate without any treatment, with 1 mM vanadate, or with 50–1,000 μM Zn in vitro. Data are means ± SD (error bars). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with no stimulation or additions (none; *t* test).

mitochondria, contribute to the zinc wave. Although the precise intracellular source of the zinc wave is still uncertain, it would be interesting if the zinc wave originates in the ER, considering that calcium signaling is elicited through $IP_3R$ stimulation on the ER membrane. Although the mechanism is still an open question, an attractive hypothesis is that the integration of calcium- and MEK-dependent events causes the activation of unknown molecules capable of stimulating $IP_3R$-like molecules or ZIP family members present on the ER membrane to elicit the zinc wave.

## Possible role of the zinc wave

The interpretation of many of the effects of zinc on phosphorylation-dependent signal transduction events, including those in insulin signaling, requires an evaluation of whether or not they occur at physiological zinc concentrations (Beyersmann and Haase, 2001). However, it is still unclear how zinc regulates signaling events downstream of receptor-mediated activation that lead to changes in biological activity, such as cytokine production. Regarding cytokine gene expression, we previously

showed that the FcεRI-induced gene expression of IL-6 and TNFα requires zinc-dependent mechanisms because the pretreatment of mast cells with TPEN inhibited this gene induction, at least in part, by inhibiting PKC activation (Kabu et al., 2006). Our previous results clearly showed the presence of zinc-dependent mechanisms in the PKC–nuclear factor κB signaling pathway (Kabu et al., 2006). Because Py/Zn treatment, which mimicked the zinc wave, induced neither PKC nor nuclear factor κB activation (unpublished data), that requirement for zinc is most likely in its previously recognized role as a constituent of signaling molecules essential for maintaining their proper conformation or enzymatic activity. In this study, we show that free zinc at the levels elicited by the zinc wave after FcεRI stimulation enhances the transcription of the genes for the cytokines IL-6 and TNFα. This enhancing effect of free zinc was cancelled by simultaneous treatment with TPEN, negating the possibility that this was an artificial effect of adding zinc to the culture medium. Furthermore, we showed that free zinc could inhibit tyrosine phosphatase activity, suggesting that tyrosine phosphatases are possible targets of the zinc wave.

Calcium signaling is rapidly induced seconds after stimulation, whereas the zinc wave was elicited in minutes. We showed that the zinc wave might modulate signaling events by affecting several molecules, including tyrosine phosphatase activity, which is consistent with reports that zinc inhibits phosphatase activity (Brautigan et al., 1981). It is generally thought that the final output of signaling is dependent not only on the quality of the signal but also on its quantity (for instance, its duration and the signal strength). For example, NGF-induced dendrite outgrowth is totally dependent on the duration of MAPK activation (Marshall, 1995; Sasagawa et al., 2005). We hypothesize that one of the roles of the zinc wave is to modulate signaling quantity, thereby playing a critical role in determining the final output of the signaling pathway.

### Zinc is a novel intracellular second messenger

Zinc is a structural constituent of a great number of proteins, including enzymes of cellular signaling pathways and transcription factors, and it is essential for their biological activity. In these cases, zinc binds tightly to proteins containing the zinc finger motif and maintains their structure. However, zinc has not been thought to play a role as an intracellular second messenger capable of transducing extracellular stimuli into intracellular signaling pathways, like calcium and cAMP. In neurons, exocytotic stimuli induce zinc release into the surrounding milieu, and it is then taken up into the cytoplasm of neighboring cells through gated zinc channels. In this case, the action of zinc is very similar to that of neurotransmitters, which are stored in membrane-enclosed synaptic vesicles and released by exocytosis, activating postsynaptic cells through transmitter-gated ion channels (Xie and Smart, 1994; Hershfinkel et al., 2001; Li et al., 2001; Colvin et al., 2003; Frederickson, 2003). However, the action of zinc as a neurotransmitter is different from the conventional concept of a second messenger. In this study, we showed that an extracellular stimulus such as FcεRI stimulation induced an increase in intracellular free zinc, which we called the zinc wave, originating in the region of the ER. Furthermore, the zinc wave was observed under conditions in which either the extracellular zinc influx or the exocytosis of granules, which are rich in zinc, was inhibited. Collectively, our observations indicate that the zinc wave is a completely different phenomenon from that already reported in neurons; rather, zinc is a novel intracellular second messenger. This conclusion is drawn from the following results. (1) An extracellular stimulus, such as FcεRI cross-linking, directly induced an increase in intracellular free zinc, the zinc wave. (2) The source of zinc was an intracellular compartment, possibly the ER. (3) Free zinc at a level similar to that elicited by the zinc wave affects intracellular signaling molecules, such as tyrosine phosphatase, and, therefore, it could modulate the final output triggered by extracellular stimuli.

We previously showed that the Stat3-Liv1 (Zip6) cascade is critically involved in the epithelial-mesenchymal transition and is required for the nuclear localization of Snail1, a zinc finger–containing repressor (Yamashita et al., 2004). In addition, toll-like receptor–mediated signaling decreases the intracellular free zinc in dendritic cells, and this decrease is required for dendritic cell activation (Kitamura et al., 2006), suggesting that zinc acts as a signaling molecule. An important difference between our current observations and our previous findings is that the zinc wave was observed several minutes after the stimulation, whereas the change in free zinc induced by toll-like receptor ligand was observed several hours after stimulation. In addition, the latter was totally dependent on a change in zinc transporter expression. We propose that intracellular zinc signaling can be classified into at least two categories: one is late zinc signaling that is dependent on a transcriptional change in zinc transporter expression, and the other is the zinc wave, an early zinc signaling pathway that is directly induced by an extracellular stimulus, such as FcεRI. Under the latter condition, zinc acts as an intracellular second messenger capable of directly transducing the extracellular stimulus into intracellular signaling events.

cAMP was the first intracellular second messenger to be discovered, by Berthet et al. (1957); calcium was the second. At present, a limited number of intracellular signaling effectors or modes are known, including cAMP, calcium, NO, lipid mediators, G proteins, and related molecular mediators, protein phosphorylation, and dephosphorylation (Gomperts et al., 2002). We do not know whether the zinc wave occurs in cell types other than mast cells, and this important issue remains to be resolved in the future. Nevertheless, our results support the idea that zinc is a novel second messenger/signaling ion that has the potential to influence many aspects of cellular signaling through its effect on zinc-binding proteins because there are many transcription factors and enzymes containing zinc-binding sites. This novel finding yields new insight into the areas of cell signaling and biological response.

## Materials and methods

#### Cell culture and mice

Bone marrow–derived mast cells (BMMCs) were prepared as described previously (Nishida et al., 2005). Syk-, Gab2-, and PLCγ2-deficient mice, which are crosses of C57BL6 and 129Sv, were generated as described previously (Turner et al., 1995; Hashimoto et al., 2000; Nishida et al., 2002).

Syk-deficient mice were provided by V.L.J. Tybulewicz (Division of Immune Cell Biology, National Institute for Medical Research, London, UK).

### Reagents and antibodies

TPEN ($Ka = 10^{15.58}\ M^{-1}$, $10^{14.61}\ M^{-1}$, $10^{20}\ M^{-1}$, and $10^{10.27}\ M^{-1}$ for $Zn^{2+}$, $Fe^{2+}$, $Cu^{2+}$, and $Mn^{2+}$, respectively; Arslan et al., 1985; McCabe et al., 1993), 2,2′-dipyridyl ($Ka = 10^{17.2}\ M^{-1}$ for $Fe^{2+}$; Dehne et al., 2001), *p*-aminosalicylic acid ($Mn^{2+}$ chelator; Ky et al., 1992), DTPA (membrane-impermeable zinc chelator; Tandon and Singh, 1975; Yui et al., 2002), Xestospongin C, and ionomycin were purchased from Sigma-Aldrich. Ammonium tetrathiomolybdate ($Cu^{2+}$ chelator; Armstrong et al., 2001) was purchased from Sigma-Aldrich. Newport green DCF diacetate, Fluo-4 AM, ER-tracker red, and pyrithione were purchased from Invitrogen. The relative increase of fluorescence intensity of Newport green DCF is calculated from the fluorescence intensity of 1 μM ion-containing solution and that of reference solution containing 10 μM EGTA and 10 μM TPEN. PD98059 was obtained from Calbiochem. U0126 was obtained from Cell Signaling, and EGF was purchased from PeproTech. The rabbit antiactive MAPK, anti-ERK1/2, and antiactive JNK polyclonal antibody were purchased from Promega. The rabbit anti-JNK1 polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc..

### Microscopy

$1 \times 10^6$/ml BMMCs were sensitized with 1 μg/ml IgE (anti-DNP IgE clone SPE-7; Sigma-Aldrich) for 6 h at 37°C. IgE-sensitized mast cells were washed three times, resuspended in Tyrode's buffer (10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.6 mM glucose), allowed to adhere to a poly-L-lysine–coated glass-bottom dish, and incubated with 10 μM Newport green or 5 μM Fluo-4 for 30 min at 37°C. These dyes are cell permeant. Surplus fluorescence indicator and floating cells were removed by at least three washes with Tyrode's buffer. Cells were stimulated with 100 ng/ml dinitrophenylated human serum albumin (Sigma-Aldrich) at 37°C. The images of fluorescent signals were captured every 10 or 30 s by an inverted microscope (Axiovert 200 MO; Carl Zeiss MicroImaging, Inc.) with an oil plan Neofluar 100× NA 1.3 objective (Carl Zeiss MicroImaging, Inc.), CCD camera (CoolSnap HQ; Roper Scientific), and the system control application SlideBook (Intelligent Imaging Innovation) at 25°C. Obtained images were processed with Photoshop software (Adobe) to adjust for size and contrast.

Newport green and ER tracker were detected using a thin-layer illumination microscope system based on the TIRF microscope (Tokunaga et al., 1997) at 25°C. For illumination, two laser lines of 488 nm (Sapphire 488–20-OPS; Coherent) and 558 nm (YA11-558; Megaopto) were directed through a 100× NA 1.45 oil immersion objective (PLAPAO100X-OTIRFM; Olympus) to an inverted microscope (IX-81; Olympus). The fluorescence images were collected at every 0.5-μm slice from the bottom to the top of the cells during scanning along the z direction using two electron-bombarded CCD cameras (C-7190-23; Hamamatsu Photonics), each equipped with an image intensifier (C8600-05; Hamamatsu Photonics). The images were captured using AQUACOSMOS software (Hamamatsu Photonics) and processed to obtain 3D images using an averaging method of AQUACOSMOS software, a deconvolution method of Volicity software (Improvision), and γ adjustments (Adobe Photoshop).

### Zinc quantification by flow cytometry

Anti-DNP IgE-sensitized mast cells were washed, resuspended in Tyrode's buffer, and incubated with 10 μM Newport green for 30 min in suspension at 37°C. The cells were washed twice with Tyrode's buffer and incubated with the indicated metal chelators at 10 μM for 10 min at 37°C. The cells were stimulated with DNP–human serum albumin for 15 min at 37°C and fixed with 4% PFA in PBS on ice. The intensity of the Newport green fluorescence was analyzed by flow cytometry.

### Cell lysates and immunoblotting

Anti-DNP IgE-sensitized BMMCs were stimulated with DNP–human serum albumin at 37°C. After the indicated times, the cells were harvested and lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, proteinase inhibitors, 5 μg/ml pepstatin, and 10 μg/ml leupeptin) for 30 min at 4°C and spun at 12,000 *g* at 4°C for 30 min. The eluted and reduced samples were resolved by SDS-PAGE using a 4–20% gradient polyacrylamide gel (Dai-ichi Kagaku) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). For immunoblotting, the membranes were incubated with antiphosphotyrosine, antiphospho-ERK, antiphospho-JNK, anti-ERK, or anti-JNK. After the reaction with the first antibody, the membranes were incubated with HRP-conjugated anti–mouse or rabbit IgG (Zymed Laboratories) for 1 h at room temperature. After extensive washing of the membranes, immunoreactive proteins were visualized using the Renaissance chemiluminescence system (Dupont NEN) according to the manufacturer's recommendations. The chemiluminescence images of the polyvinylidene difluoride membranes were captured with a chemiluminescence and fluorescence imaging system (LAS-1000; Fuji) and analyzed with Image Gauge software (Fuji).

### RT-PCR analysis

Cells were homogenized, and total RNA was isolated with the RNeasy Protect kit (QIAGEN) according to the manufacturer's instructions. For standard RT-PCR, cDNA was synthesized from 500 ng of the total RNA by incubation with reverse transcriptase (ReverTra Ace; Toyobo) and 500 ng of oligonucleotide (dT) primer for 30 min at 42°C (Invitrogen). A portion of the cDNA (typically a 1/20 volume) was used for the standard PCR to detect IL-6, TNFα, and glyceraldehyde-3-phosphate dehydrogenase. 25 cycles of PCR were performed with 0.5 U rTaq DNA polymerase and 10 pmol of gene-specific sense and antisense primers. Amplified segments of RT-PCR for IL-6, TNFα, and glyceraldehyde-3-phosphate dehydrogenase were 141 bp, 175 bp, and 227 bp, respectively. Primers used in these experiments were purchased from Invitrogen, and sequences were as follows: IL-6, forward primer (5′-GAGGATACCACTCCCAACAGACC-3′) and reverse primer (5′-AAGTGCATCATCGTTGTTCATACA-3′); TNFα, forward primer (5′-CATCTTCTCAAAATTCGAGTGACAA-3′) and reverse primer (5′-TGGGAGTAGACAAGGTACAACCC-3′); and glyceraldehyde-3-phosphate dehydrogenase, forward primer (5′-TTCACCACCATGGAGAAGGCCG-3′) and reverse primer (5′-GGCATGGACTGTGGTCATGA-3′).

### Measurement of phosphatase activity

Anti-DNP IgE-sensitized BMMCs were stimulated with DNP–human serum albumin at 37°C for 30 min and lysed with lysis buffer as described in Cell lysates and immunoblotting. The cell lysate was analyzed using the Tyrosine Phosphatase Assay System (Promega) according to the manufacturer's recommendations.

### Online supplemental material

Videos 1 and 2 show time-lapse images of Newport green in mast cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200702081/DC1.

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## References

Aizenman, E., A.K. Stout, K.A. Hartnett, K.E. Dineley, B. McLaughlin, and I.J. Reynolds. 2000. Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release. *J. Neurochem.* 75:1878–1888.

Andrews, G.K. 2001. Cellular zinc sensors: MTF-1 regulation of gene expression. *Biometals*. 14:223–237.

Armstrong, C., W. Leong, and G.J. Lees. 2001. Comparative effects of metal chelating agents on the neuronal cytotoxicity induced by copper (Cu+2), iron (Fe+3) and zinc in the hippocampus. *Brain Res.* 892:51–62.

Arslan, P., F. Di Virgilio, M. Beltrame, R.Y. Tsien, and T. Pozzan. 1985. Cytosolic Ca2+ homeostasis in Ehrlich and Yoshida carcinomas. A new, membrane-permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free Ca2+. *J. Biol. Chem.* 260:2719–2727.

Berthet, J., T.W. Rall, and E.W. Sutherland. 1957. The relationship of epinephrine and glucagon to liver phosphorylase. IV. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. *J. Biol. Chem.* 224:463–475.

Beyersmann, D., and H. Haase. 2001. Functions of zinc in signaling, proliferation and differentiation of mammalian cells. *Biometals*. 14:331–341.

Brautigan, D.L., P. Bornstein, and B. Gallis. 1981. Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn. *J. Biol. Chem.* 256:6519–6522.

Colvin, R.A., C.P. Fontaine, M. Laskowski, and D. Thomas. 2003. Zn2+ transporters and Zn2+ homeostasis in neurons. *Eur. J. Pharmacol.* 479:171–185.

Dehne, N., J. Lautermann, F. Petrat, U. Rauen, and H. de Groot. 2001. Cisplatin ototoxicity: involvement of iron and enhanced formation of superoxide anion radicals. *Toxicol. Appl. Pharmacol.* 174:27–34.

Eide, D.J. 2004. The SLC39 family of metal ion transporters. *Pflugers Arch.* 447:796–800.

Fernandes, G., M. Nair, K. Onoe, T. Tanaka, R. Floyd, and R.A. Good. 1979. Impairment of cell-mediated immunity functions by dietary zinc deficiency in mice. *Proc. Natl. Acad. Sci. USA*. 76:457–461.

Fraker, P.J., R. Caruso, and F. Kierszenbaum. 1982. Alteration of the immune and nutritional status of mice by synergy between zinc deficiency and infection with *Trypanosoma cruzi*. *J. Nutr.* 112:1224–1229.

Frederickson, C. 2003. Imaging zinc: old and new tools. *Sci. STKE*. 2003:pe18.

Frederickson, C.J., and D.W. Moncrieff. 1994. Zinc-containing neurons. *Biol. Signals*. 3:127–139.

Frederickson, C.J., J.Y. Koh, and A.I. Bush. 2005. The neurobiology of zinc in health and disease. *Nat. Rev. Neurosci.* 6:449–462.

Gomperts, B.D., P.E.R. Tatham, and I.M. Kramer. 2002. Signal Transduction. Academic Press, San Diego. 424 pp.

Gustafson, G.T. 1967. Heavy metals in rat mast cell granules. *Lab. Invest.* 17:588–598.

Gyulkhandanyan, A.V., S.C. Lee, G. Bikopoulos, F. Dai, and M.B. Wheeler. 2006. The Zn2+-transporting pathways in pancreatic beta-cells: a role for the L-type voltage-gated Ca2+ channel. *J. Biol. Chem.* 281:9361–9372.

Hashimoto, A., K. Takeda, M. Inaba, M. Sekimata, T. Kaisho, S. Ikehara, Y. Homma, S. Akira, and T. Kurosaki. 2000. Cutting edge: essential role of phospholipase C-gamma 2 in B cell development and function. *J. Immunol.* 165:1738–1742.

Hershfinkel, M., A. Moran, N. Grossman, and I. Sekler. 2001. A zinc-sensing receptor triggers the release of intracellular Ca2+ and regulates ion transport. *Proc. Natl. Acad. Sci. USA*. 98:11749–11754.

Ibs, K.H., and L. Rink. 2003. Zinc-altered immune function. *J. Nutr.* 133:1452S–1456S.

Jou, M.J., S.B. Jou, M.J. Guo, H.Y. Wu, and T.I. Peng. 2004. Mitochondrial reactive oxygen species generation and calcium increase induced by visible light in astrocytes. *Ann. NY Acad. Sci.* 1011:45–56.

Kabu, K., S. Yamasaki, D. Kamimura, Y. Ito, A. Hasegawa, E. Sato, H. Kitamura, K. Nishida, and T. Hirano. 2006. Zinc is required for FcepsilonRI-mediated mast cell activation. *J. Immunol.* 177:1296–1305.

Keen, C.L., and M.E. Gershwin. 1990. Zinc deficiency and immune function. *Annu. Rev. Nutr.* 10:415–431.

Kitamura, H., H. Morikawa, H. Kamon, M. Iguchi, S. Hojyo, T. Fukada, S. Yamashita, T. Kaisho, S. Akira, M. Murakami, and T. Hirano. 2006. Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. *Nat. Immunol.* 7:971–977.

Koh, J.Y., S.W. Suh, B.J. Gwag, Y.Y. He, C.Y. Hsu, and D.W. Choi. 1996. The role of zinc in selective neuronal death after transient global cerebral ischemia. *Science*. 272:1013–1016.

Kristiansen, L.H., J. Rungby, L.G. Sondergaard, M. Stoltenberg, and G. Danscher. 2001. Autometallography allows ultrastructural monitoring of zinc in the endocrine pancreas. *Histochem. Cell Biol.* 115:125–129.

Ky, S.Q., H.S. Deng, P.Y. Xie, and W. Hu. 1992. A report of two cases of chronic serious manganese poisoning treated with sodium para-aminosalicylic acid. *Br. J. Ind. Med.* 49:66–69.

Li, Y., C.J. Hough, S.W. Suh, J.M. Sarvey, and C.J. Frederickson. 2001. Rapid translocation of Zn(2+) from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation. *J. Neurophysiol.* 86:2597–2604.

Lichtlen, P., and W. Schaffner. 2001. Putting its fingers on stressful situations: the heavy metal-regulatory transcription factor MTF-1. *Bioessays*. 23:1010–1017.

Maret, W., C. Jacob, B.L. Vallee, and E.H. Fischer. 1999. Inhibitory sites in enzymes: zinc removal and reactivation by thionein. *Proc. Natl. Acad. Sci. USA*. 96:1936–1940.

Marshall, C.J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*. 80:179–185.

McCabe, M.J., Jr., S.A. Jiang, and S. Orrenius. 1993. Chelation of intracellular zinc triggers apoptosis in mature thymocytes. *Lab. Invest.* 69:101–110.

Nishida, K., L. Wang, E. Morii, S.J. Park, M. Narimatsu, S. Itoh, S. Yamasaki, M. Fujishima, K. Ishihara, M. Hibi, et al. 2002. Requirement of Gab2 for mast cell development and KitL/c-Kit signaling. *Blood*. 99:1866–1869.

Nishida, K., S. Yamasaki, Y. Ito, K. Kabu, K. Hattori, T. Tezuka, H. Nishizumi, D. Kitamura, R. Goitsuka, R.S. Geha, et al. 2005. Fc{epsilon}RI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. *J. Cell Biol.* 170:115–126.

Palmiter, R.D. 2004. Protection against zinc toxicity by metallothionein and zinc transporter 1. *Proc. Natl. Acad. Sci. USA*. 101:4918–4923.

Palmiter, R.D., and L. Huang. 2004. Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. *Pflugers Arch.* 447:744–751.

Parravicini, V., M. Gadina, M. Kovarova, S. Odom, C. Gonzalez-Espinosa, Y. Furumoto, S. Saitoh, L.E. Samelson, J.J. O'Shea, and J. Rivera. 2002. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* 3:741–748.

Prasad, A.S. 1995. Zinc: an overview. *Nutrition*. 11:93–99.

Rink, L., and P. Gabriel. 2000. Zinc and the immune system. *Proc. Nutr. Soc.* 59:541–552.

Rivera, J. 2002. Molecular adapters in Fc(epsilon)RI signaling and the allergic response. *Curr. Opin. Immunol.* 14:688–693.

Sasagawa, S., Y. Ozaki, K. Fujita, and S. Kuroda. 2005. Prediction and validation of the distinct dynamics of transient and sustained ERK activation. *Nat. Cell Biol.* 7:365–373.

Sensi, S.L., and J.M. Jeng. 2004. Rethinking the excitotoxic ionic milieu: the emerging role of Zn(2+) in ischemic neuronal injury. *Curr. Mol. Med.* 4:87–111.

Sensi, S.L., H.Z. Yin, and J.H. Weiss. 1999. Glutamate triggers preferential Zn2+ flux through Ca2+ permeable AMPA channels and consequent ROS production. *Neuroreport*. 10:1723–1727.

Tandon, S.K., and J. Singh. 1975. Removal of manganese by chelating agents from brain and liver of manganese treated rats: as in vitro and an in vivo study. *Toxicology*. 5:237–241.

Telford, W.G., and P.J. Fraker. 1995. Preferential induction of apoptosis in mouse CD4+CD8+ alpha beta TCRloCD3 epsilon lo thymocytes by zinc. *J. Cell. Physiol.* 164:259–270.

Tokunaga, M., K. Kitamura, K. Saito, A.H. Iwane, and T. Yanagida. 1997. Single molecule imaging of fluorophores and enzymatic reactions achieved by objective-type total internal reflection fluorescence microscopy. *Biochem. Biophys. Res. Commun.* 235:47–53.

Turner, H., and J.P. Kinet. 1999. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature*. 402:B24–B30.

Turner, M., P.J. Mee, P.S. Costello, O. Williams, A.A. Price, L.P. Duddy, M.T. Furlong, R.L. Geahlen, and V.L. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*. 378:298–302.

Vallee, B.L. 1995. The function of metallothionein. *Neurochem. Int.* 27:23–33.

Vallee, B.L., and D.S. Auld. 1993. Cocatalytic zinc motifs in enzyme catalysis. *Proc. Natl. Acad. Sci. USA*. 90:2715–2718.

Xie, X., and T.G. Smart. 1994. Modulation of long-term potentiation in rat hippocampal pyramidal neurons by zinc. *Pflugers Arch.* 427:481–486.

Yamashita, S., C. Miyagi, T. Fukada, N. Kagara, Y.S. Che, and T. Hirano. 2004. Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. *Nature*. 429:298–302.

Yin, H.Z., S.L. Sensi, F. Ogoshi, and J.H. Weiss. 2002. Blockade of Ca2+-permeable AMPA/kainate channels decreases oxygen-glucose deprivation-induced Zn2+ accumulation and neuronal loss in hippocampal pyramidal neurons. *J. Neurosci.* 22:1273–1279.

Yui, S., Y. Nakatani, M.J. Hunter, W.J. Chazin, and M. Yamazaki. 2002. Implication of extracellular zinc exclusion by recombinant human calprotectin (MRP8 and MRP14) from target cells in its apoptosis-inducing activity. *Mediators Inflamm.* 11:165–172.