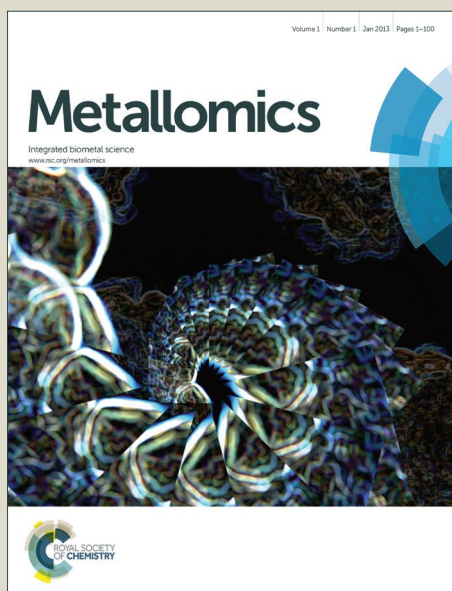


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Journal Name

ARTICLE

## Monitoring Cytosolic and ER Zn<sup>2+</sup> in Stimulated Breast Cancer Cells using Genetically Encoded FRET Sensors

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**Abstract.** The Zn<sup>2+</sup>-specific ion channel ZIP7 has been implicated to play an important role in releasing Zn<sup>2+</sup> from the ER. External stimulation of breast cancer cells has been proposed to induce phosphorylation of ZIP7 by CK2α, resulting in ZIP7-mediated Zn<sup>2+</sup> release from the ER into the cytosol. Here, we examined whether changes in cytosolic and ER Zn<sup>2+</sup> concentrations can be detected upon such external stimuli. Two previously developed FRET sensors for Zn<sup>2+</sup>, eZinCh-2 (*K<sub>d</sub>* = 1 nM at pH 7.1) and eCALWY-4 (*K<sub>d</sub>* = 0.63 nM at pH 7.1), were expressed in both the cytosol and the ER of wild-type MCF-7 and TamR cells. Treatment of MCF-7 and TamR cells with external Zn<sup>2+</sup> and pyrithione, one of the previously used triggers, resulted in an immediate increase in free Zn<sup>2+</sup> in both cytosol and ER, suggesting that Zn<sup>2+</sup> was directly transferred across the cellular membranes by pyrithione. Cells treated with a second trigger, EGF/ionomycin, showed no changes in intracellular Zn<sup>2+</sup> levels, neither in multicolor imaging experiments that allowed simultaneous imaging of cytosolic and ER Zn<sup>2+</sup>, nor in experiments in which cytosolic and ER Zn<sup>2+</sup> were monitored separately. In contrast to previous work using small-molecule fluorescent dyes, these results indicate that EGF-ionomycin treatment does not result in significant changes in cytosolic Zn<sup>2+</sup> levels as a result from Zn<sup>2+</sup> release from the ER. These results underline the importance of using genetically encoded fluorescent sensors to complement and verify intracellular imaging experiments with synthetic fluorescent Zn<sup>2+</sup> dyes.

### Introduction

Zn<sup>2+</sup> is essential for proper cell function and cell growth<sup>[1]</sup>. However, excess Zn<sup>2+</sup> can be toxic to cells, affecting growth and causing serious metabolic disorders<sup>[2]</sup>. How Zn<sup>2+</sup> participates in controlling cellular processes is an emerging area of interest. In addition to its well established roles as a Lewis acid cofactor in enzyme catalysis and its structural role in protein folding, several studies have recently proposed a role for Zn<sup>2+</sup> as a secondary messenger in intracellular signal transduction pathways<sup>[3-5]</sup>. Fluorescent sensors that allow intracellular imaging of Zn<sup>2+</sup> concentrations in living cells have proven crucial in our understanding of the molecular mechanisms of intracellular Zn<sup>2+</sup> homeostasis and its putative signaling.

Whereas small molecule sensors continue to contribute valuable insights into the role of Zn<sup>2+</sup> in numerous biological processes, their intracellular concentration and localization cannot be easily controlled. Therefore, we and others have developed fluorescent Zn<sup>2+</sup> sensor proteins based on the

modulation of Fluorescence Energy Transfer (FRET) between a donor and acceptor fluorescent domain<sup>[6-8]</sup>. These genetically-encoded sensors do not require cell-invasive procedures, their concentration and intracellular location can be tightly controlled, and the use of FRET allows for ratiometric detection. The eCALWY-sensors that were developed by our group consist of two small metal binding domains, ATOX1 and WD4, connected by a long flexible linker, with self-associating variants of cerulean and citrine fluorescent domains fused to the N- and C-terminus, respectively. A toolbox of 6 eCALWY variants has been developed that range in Zn<sup>2+</sup> affinity between 2 pM and 5 nM. Of these, eCALWY-4 with a *K<sub>d</sub>* of 630 pM was found to be optimal for measuring cytosolic Zn<sup>2+</sup> concentrations, which typically reside in the 0.2-1 nM range, in a wide variety of cells<sup>[6,9]</sup>. Replacement of Cerulean and Citrine by self-associating variants of mOrange and mCherry yielded the red-shifted redCALWY variants, whose spectral properties allowed them to be used in combination with CFP-YFP-based reporters<sup>[10]</sup>. Recently, we also reported another versatile FRET sensor containing a de novo Cys<sub>2</sub>His<sub>2</sub> Zn<sup>2+</sup> binding pocket created directly on the surface of Cerulean and Citrine domains<sup>[11]</sup>. This eZinCh-2 sensor displays a large increase in emission ratio upon binding Zn<sup>2+</sup> at the interface of the two fluorescent domains, with an affinity that is similar to that of eCALWY-4. By default eCALWY-4 and eZinCh-2 reside in the cytosol, but both have been successfully targeted to the ER, mitochondria and secretory vesicles by the introduction of organelle specific targeting sequences<sup>[11]</sup>.

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Because  $\text{Zn}^{2+}$  is unable to passively diffuse across cell membranes, active transport between the cytosol, the extracellular space and intracellular compartments is required to allow control over intracellular  $\text{Zn}^{2+}$  homeostasis. Two families of mammalian  $\text{Zn}^{2+}$  transporters are known that transport  $\text{Zn}^{2+}$  across cellular membranes. The ZnT (SLC30A) family exports  $\text{Zn}^{2+}$  from the cytosol into organelles or to the extracellular space<sup>[12]</sup>. The ZIP family (SLC39A) acts as an importer protein and is responsible for  $\text{Zn}^{2+}$  import from either the extracellular space or from different cellular compartments<sup>[13]</sup>. One of the ZIP family members, ZIP7, was found to localize on the membrane of the endoplasmic reticulum<sup>[14, 15]</sup> and is believed to play a role in the transportation of  $\text{Zn}^{2+}$  from the ER into the cytosol.  $\text{Zn}^{2+}$  transporters play a crucial role in maintaining the delicate balance between cell growth and cell death and aberrant functioning of  $\text{Zn}^{2+}$  transporters has been linked to several disease states including cancer. For example,  $\text{Zn}^{2+}$  was found to be increased in human breast tumors<sup>[16, 17]</sup>. A widely used model to investigate the development of breast cancer<sup>[18]</sup> showed an up to 19-fold increase in total  $\text{Zn}^{2+}$  levels in mammary tumors in mice, rats and humans<sup>[19]</sup>. One mechanism by which increased levels of cytosolic  $\text{Zn}^{2+}$  could enhance cell proliferation is by the inhibition of protein tyrosine phosphatases, which could lead to a net increase in phosphorylation of well-known cancer associated downstream effectors such as AKT and MAPK (mitogen-activated protein kinase)<sup>[20, 21]</sup>.

Taylor and coworkers have proposed that phosphorylation of ZIP7 might induce release of  $\text{Zn}^{2+}$  from the ER to the cytosol in Tamoxifen-resistant MCF-7 breast cancer cells (TamR)<sup>[22]</sup>. Using the membrane-permeable  $\text{Zn}^{2+}$ -specific indicator Newport Green DCF a 2-fold higher fluorescence was observed in TamR cells compared to wild-type MCF-7 cells, suggesting a higher intracellular free  $\text{Zn}^{2+}$  concentration<sup>[22]</sup>. Comparison of the expression levels of several  $\text{Zn}^{2+}$  importers (ZIPs) revealed a significant increase in expression level of ZIP7 in TamR cells compared to wild-type MCF-7 cells. Since ZIP7 is almost exclusively localized on the ER membrane<sup>[14]</sup>, ZIP7 has been implicated to control the release of  $\text{Zn}^{2+}$  from the ER to the cytosol. Two types of triggers were used to study the involvement of ZIP7 in  $\text{Zn}^{2+}$  signaling in both wild-type MCF-7 and TamR cells: the addition of extracellular  $\text{Zn}^{2+}$  with pyridithione, and addition of EGF with ionomycin. Addition of external  $\text{Zn}^{2+}$  and pyridithione to MCF-7 and TamR cells loaded with the  $\text{Zn}^{2+}$ -specific fluorescent indicator FluoZin-3 showed an increase in green fluorescence within a few minutes after stimulation, which was interpreted to be a result of ZIP7-dependent  $\text{Zn}^{2+}$  release from the ER<sup>[21, 22]</sup>. Immunoprecipitation experiments with a ZIP7 antibody in  $\text{Zn}^{2+}$  treated cells showed co-immunoprecipitation of CK2 $\alpha$ , suggesting that this kinase may be responsible for phosphorylation of ZIP7. Consistent with this role, a peak in the physical association between protein kinase CK2 and ZIP7 was observed within two minutes after treatment with  $\text{Zn}^{2+}$ /pyridithione. In addition, a proximity ligation assay, in

which fluorescent dots appear when two molecules are in close proximity, showed significant association of ZIP7 and CK2 $\alpha$  in the same timeframe.

Based on the above results a model was proposed whereby the phosphorylation of ZIP7 by CK2 results in ZIP7-mediated  $\text{Zn}^{2+}$  release from ER stores into the cytosol, resulting in subsequent activation of several downstream signaling pathways<sup>[22]</sup>. However, the evidence for  $\text{Zn}^{2+}$  release from the ER upon external stimuli has remained indirect, because these studies did not directly measure ER  $\text{Zn}^{2+}$  levels. In addition, FluoZin-3 and the other synthetic fluorescent sensors that were employed to monitor intracellular  $\text{Zn}^{2+}$ , are known to not only localize in the cytosol, but also to different cellular compartments, such as the Golgi, synaptic vesicles and the nucleus<sup>[23]</sup>. Here, we report the use of two previously developed FRET sensors for  $\text{Zn}^{2+}$  (eZinCh-2 ( $K_d = 1$  nM) and eCALWY-4 ( $K_d = 0.63$  nM)) to test the hypothesis that  $\text{Zn}^{2+}$  is released from the ER into the cytosol upon external stimuli (Table 1). Unlike synthetic fluorescent sensors, these genetically encoded sensors can be targeted exclusively to either the cytosol or the ER. Moreover, the use of two different color variants allowed us to simultaneously monitor the free  $\text{Zn}^{2+}$  concentration in the cytosol and ER within the same cell. Using these sensors an immediate increase in both cytosolic and ER  $\text{Zn}^{2+}$  levels was observed upon treatment of MCF7 and TAMR cells with  $\text{Zn}^{2+}$ /pyridithione, which is consistent with direct, pyridithione-mediated exchange of  $\text{Zn}^{2+}$  between the cell exterior and intracellular compartments. In contrast, addition of EGF/ionomycin did not induce significant changes in cytosolic or ER  $\text{Zn}^{2+}$  concentrations. The implication of these findings for the putative role of ZIP7 in controlled release of  $\text{Zn}^{2+}$  from the ER will be discussed.

**Table 1: Overview of FRET sensors used in this study**

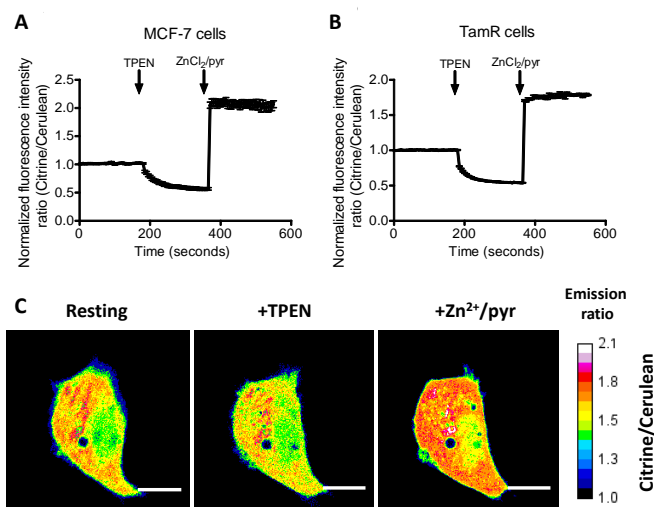
Sensor	Fluorescent domains (donor/acceptor)	$K_d$ for $\text{Zn}^{2+}$ (pH 7.0)	Cellular localization
eZinCh-2 <sup>[12]</sup>	Cerulean/Citrine <sup>a</sup>	1.0 nM	cytosol
ER-eZinCh-2 <sup>[12]</sup>	Cerulean/Citrine	1.0 nM	ER <sup>c</sup>
eCALWY-4 <sup>[6]</sup>	Cerulean/Citrine	0.63 nM	cytosol
ER-eCALWY4 <sup>[25]</sup>	Cerulean/Citrine	0.63 nM	ER <sup>c</sup>
redCALWY-4 <sup>[10]</sup>	mOrange2/mCherry <sup>b</sup>	0.24 nM	cytosol

- cerulean: excitation maximum at 435 nm, emission maximum at 475 nm; citrine: excitation maximum at 510 nm, emission maximum at 535 nm.
- mOrange2: excitation maximum at 549 nm, emission maximum at 565 nm; mCherry: excitation maximum at 587 nm, emission maximum at 610 nm.
- ER-targeted sensor variants contained an N-terminal insulin signal peptide, and a C-terminal KDEL retention sequence.

## Results

### Determination of cytosolic and ER $\text{Zn}^{2+}$ concentrations in MCF-7 and TamR cells using FRET-based $\text{Zn}^{2+}$ sensors

Previous work on MCF-7 and TamR cells using small-molecule fluorescent sensors reported increased levels of intracellular  $\text{Zn}^{2+}$  in TamR cells compared to wild-type MCF-7 cells<sup>[22]</sup>, but the concentration of free cytosolic  $\text{Zn}^{2+}$  could not be determined. We therefore expressed the eZinCh-2 sensor in the cytosol of both MCF-7 and TamR cells and monitored the *in situ* response in single living cells to the addition of the strong membrane-permeable  $\text{Zn}^{2+}$  chelator TPEN and excess  $\text{Zn}^{2+}$  together with pyrithione as a  $\text{Zn}^{2+}$ -specific ionophore. In both MCF-7 and TamR cells a robust, 3-fold change in emission ratio was observed between the  $\text{Zn}^{2+}$ -depleted and  $\text{Zn}^{2+}$ -saturated states of the sensor (Figure 1). The determination of the emission ratios under conditions of  $\text{Zn}^{2+}$  depletion ( $R_{0\%}$ ) and  $\text{Zn}^{2+}$  excess ( $R_{100\%}$ ) allowed calculation of the sensor occupancy under resting conditions, which could be translated into a free  $\text{Zn}^{2+}$  concentration using the  $K_d$  of 1 nM. The free cytosolic  $\text{Zn}^{2+}$  concentrations in wild type MCF-7 cells and TamR cells were determined to be  $0.44 \pm 0.06$  nM and  $0.65 \pm 0.06$  nM, respectively. Although the free  $\text{Zn}^{2+}$  concentration in TamR cells appears to be slightly higher, both numbers agree well with values determined previously in other mammalian cell types, and neither of them is particularly high<sup>[6]</sup>.

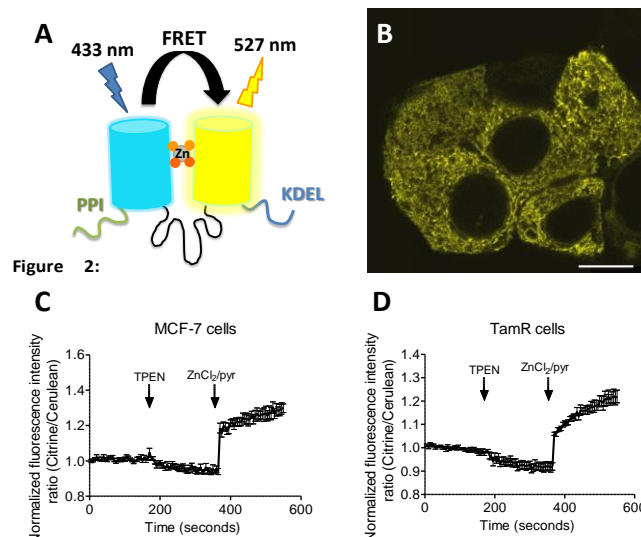


**Figure 1:** (A, B) Determination of the free cytosolic  $\text{Zn}^{2+}$  concentration in wild-type MCF-7 (A) and TamR cells (B) using eZinCh-2. Responses to the addition of 50  $\mu\text{M}$  TPEN, followed by the addition of excess 100  $\mu\text{M}$   $\text{Zn}^{2+}/5 \mu\text{M}$  pyrithione. All traces in A and B represent the average of at least four cells after normalization of the emission ratio at  $t=0$  s. Error bars represent the standard error of the mean (SEM). (C) False-colored ratiometric images of an MCF-7 cell expressing eZinCh-2 in resting state (Resting), after perfusion with 50  $\mu\text{M}$  TPEN (+TPEN), and 100  $\mu\text{M}$   $\text{ZnCl}_2/5 \mu\text{M}$  pyrithione (+ $\text{Zn}^{2+}/\text{pyr}$ ). Scale bar, 10  $\mu\text{M}$ .

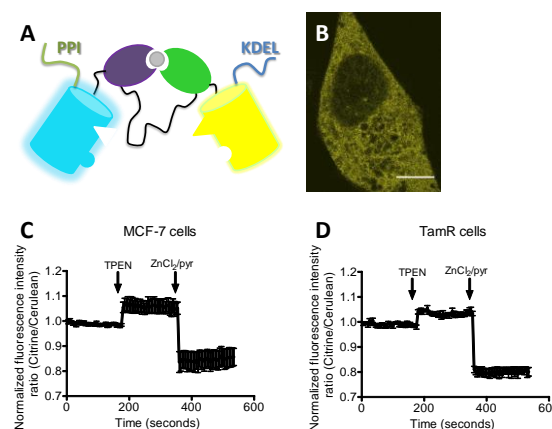
To explore a possible correlation between the increased ZIP7 expression and free ER  $\text{Zn}^{2+}$  levels in TamR cells,<sup>[22]</sup> the ER-targeted FRET-based  $\text{Zn}^{2+}$  sensors ER-eZinCh-2 and ER-eCALWY-4 were expressed in both wild-type MCF-7 and TamR cells<sup>[24]</sup> (Table 1; Figure 2 and 3). Colocalization studies showed exclusive localization of these sensors to the ER lumen. Using ER-eZinCh-1, addition of TPEN and excess  $\text{Zn}^{2+}$  yielded similar response curves as were found previously for HeLa and HEK293T cells<sup>[11]</sup>, yielding free ER  $\text{Zn}^{2+}$  concentrations of  $0.54 \pm$

$0.27$  nM for MCF-7 (Figure 2C) and  $0.75 \pm 0.49$  nM for TamR cells (Figure 2D). Measurements using ER-targeted eCALWY-4 yielded similar ER  $\text{Zn}^{2+}$  concentrations of  $0.39 \pm 0.17$  nM and  $0.21 \pm 0.05$  nM for MCF-7 (Figure 3C) and TamR cells (Figure 3D), respectively.

The average free ER  $\text{Zn}^{2+}$  concentrations that were found are similar to those found in the cytosol, although the cell-to-cell variation in free  $\text{Zn}^{2+}$  concentration was found to be larger in the ER when compared to the cytosol. The latter may reflect less efficient buffering of the free  $\text{Zn}^{2+}$  concentration due to a lack of metallothionein in the ER.



**Figure 2:**  $\text{Zn}^{2+}$  imaging using ER-targeted eZinCh-2 in both MCF-7 and TamR cells. (A) Schematic representation of eZinCh-2 containing an N-terminal signal sequence (PPI) and a C-terminal retention sequence (KDEL). (B) False-colored image showing MCF-7 cells expressing ER-eZinCh-2. (C, D) Responses of MCF-7 (C) and TamR (D) cells expressing ER-eZinCh-2 to the addition of 50  $\mu\text{M}$  TPEN, and the subsequent addition of excess  $\text{Zn}^{2+}$  together with pyrithione (100  $\mu\text{M}/5 \mu\text{M}$ ). All traces in represent the average of four cells after normalization of the emission ratio at  $t=0$  s. Error bars represent SEM.



**Figure 3:**  $\text{Zn}^{2+}$  imaging using ER-targeted eCALWY-4 in both MCF-7 and TamR cells. (A) Schematic representation of eCALWY-4 containing an N-terminal signal sequence (PPI) and a KDEL sequence. (B) False-colored image showing a TamR cells expressing ER-eCALWY-4. (C, D) Responses of MCF-7 (C) and TamR (D) cells expressing ER-eCALWY-4 to the addition of 50  $\mu\text{M}$  TPEN, and the subsequent addition of  $\text{Zn}^{2+}/\text{pyrithione}$ . All traces in represent the average of four cells after normalization of the emission ratio at  $t=0$  s. Error bars represent SEM.

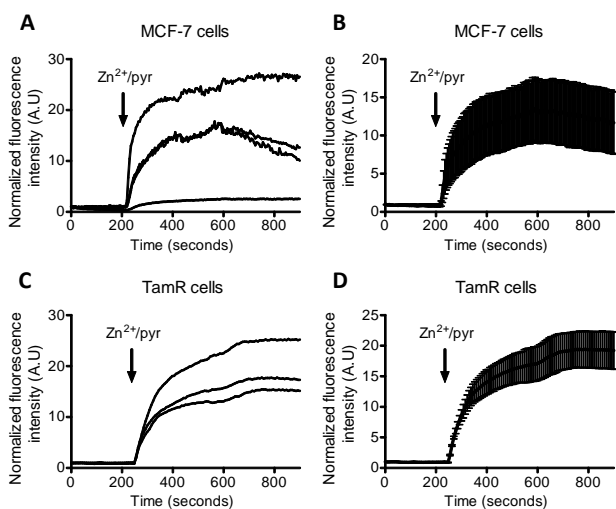


## ARTICLE

## Journal Name

Monitoring changes in intracellular  $\text{Zn}^{2+}$  levels upon external  $\text{Zn}^{2+}$  stimulation

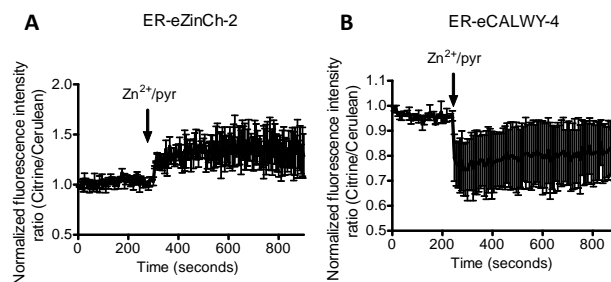
Previous studies using FluoZin-3 showed a delayed increase in intracellular  $\text{Zn}^{2+}$  upon extracellular addition of 20  $\mu\text{M}$   $\text{Zn}^{2+}$  and 10  $\mu\text{M}$  pyrithione, which was interpreted to result from ZIP7-dependent  $\text{Zn}^{2+}$  release from ER stores into the cytosol<sup>[21]</sup>. Because pyrithione is normally used to transfer  $\text{Zn}^{2+}$  ions across biological membranes, one would expect to observe a large and immediate increase in intracellular  $\text{Zn}^{2+}$  levels upon  $\text{Zn}^{2+}$ /pyrithione treatment but the cells in the previous study were fixed prior to imaging. To check whether the same results could be obtained in live cells, we repeated these imaging experiments on live MCF-7 and TamR cells loaded with FluoZin-3 AM. A perfusion setup was used to allow continuous flow over the cells during the imaging experiments. In this experimental setup, addition of external  $\text{Zn}^{2+}$  resulted in a large increase in emission ratio immediately after addition of 20  $\mu\text{M}$   $\text{Zn}^{2+}$ /10  $\mu\text{M}$  pyrithione for both wild-type MCF-7 (Figure 4A and B) and TamR cells (Figure 4C and D). A large variation in absolute fluorescent signal intensity was observed after  $\text{Zn}^{2+}$  addition between individual cells, which may reflect the difficulty to control the intracellular concentration of FluoZin-3. Nonetheless, in contrast to previous work using fixed cells, these live cell imaging experiments reveal an almost immediate increase in fluorescence intensity in all cells with a half-time of about 60 seconds after exposure to  $\text{Zn}^{2+}$ /pyrithione.



**Figure 4:** (A,B) Responses of MCF-7 cells loaded with FluoZin-3 AM to the addition of 20  $\mu\text{M}$   $\text{Zn}^{2+}$  together with the  $\text{Zn}^{2+}$ -specific ionophore pyrithione, monitored in real time. (C, D) Responses of TamR cells loaded with FluoZin-3 AM to the addition of 20  $\mu\text{M}$   $\text{Zn}^{2+}$  together with the  $\text{Zn}^{2+}$ -specific ionophore pyrithione, monitored in real time. Traces in B and D represent the average of four and three cells, respectively, after normalization at  $t=0$  s. Error bars represent SEM.

This fast increase in fluorescence is consistent with direct pyrithione-mediated transfer of  $\text{Zn}^{2+}$  from the cell exterior into the cytoplasm, without the need to invoke intracellular release of  $\text{Zn}^{2+}$  from the ER. To directly assess the effect of stimulation with  $\text{Zn}^{2+}$ /pyrithione on ER  $\text{Zn}^{2+}$  levels, we repeated these experiments in MCF-7 and TamR cells expressing either ER-ZinCh-2 or ER-eCALWY-4. Addition of 20  $\mu\text{M}$   $\text{Zn}^{2+}$ /10  $\mu\text{M}$  pyrithione to MCF-7 cells expressing ER-

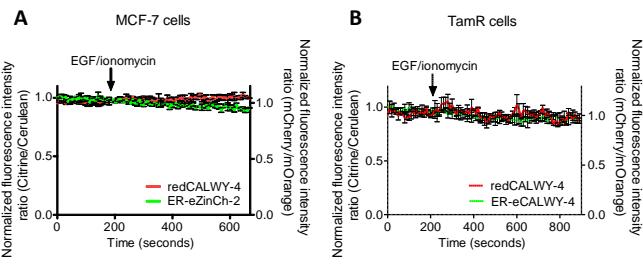
eZinCh-2 resulted in an immediate increase in emission ratio (Figure 5A), consistent with an increase in the free ER  $\text{Zn}^{2+}$  concentration. Comparable responses were observed for TamR cells expressing ER-eZinCh-2 (Figure S1). The same experiments were performed on both MCF-7 (Figure S2) and TamR (Figure 5B) cells expressing ER-eCALWY-4, resulting in a decrease in emission ratio upon  $\text{Zn}^{2+}$  addition, which again corresponds to an increase in free ER  $\text{Zn}^{2+}$  levels. The increase in ER  $\text{Zn}^{2+}$  is inconsistent with a specific release of  $\text{Zn}^{2+}$  from the ER to the cytoplasm, but is not unexpected given the fact that pyrithione can also mediate transfer of  $\text{Zn}^{2+}$  across the intracellular ER membrane.



**Figure 5:** (A) Responses of MCF-7 cells expressing ER-eZinCh-2 to the addition of 20  $\mu\text{M}$   $\text{Zn}^{2+}$ /10  $\mu\text{M}$  pyrithione. (B) Responses of TamR cells expressing ER-eCALWY-4 to the addition of 20  $\mu\text{M}$   $\text{Zn}^{2+}$ /10  $\mu\text{M}$  pyrithione. Traces in A and B represent the average of three cells after normalization at  $t=0$  s. Error bars represent SEM.

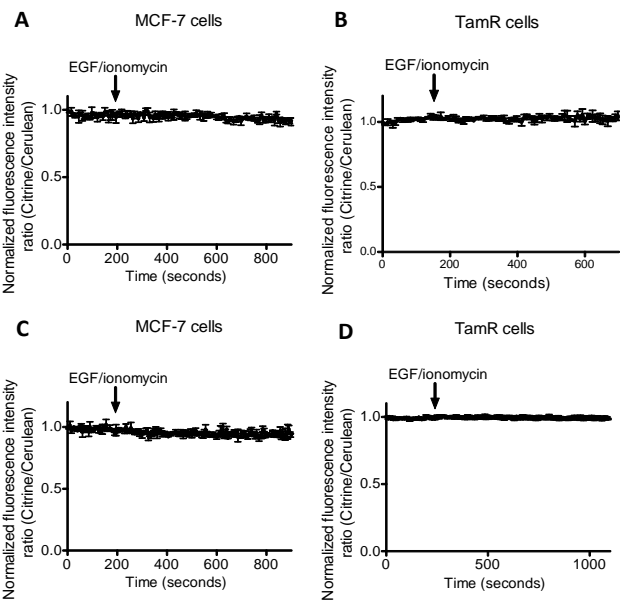
Monitoring changes in intracellular  $\text{Zn}^{2+}$  upon stimulation with EGF and ionomycin

A combination of epidermal growth factor (EGF) and the calcium ionophore ionomycin has been reported previously to trigger release of  $\text{Zn}^{2+}$  from the ER resulting in so-called  $\text{Zn}^{2+}$  waves in the cytosol of mast cells.<sup>[3]</sup> Using the synthetic  $\text{Zn}^{2+}$  probe Zinquin ( $K_d = 7 \mu\text{M}$ ), a comparable increase in cytosolic  $\text{Zn}^{2+}$  concentration has been reported in TamR cells upon addition of EGF and ionomycin<sup>[21]</sup>. To obtain independent evidence for the putative release of  $\text{Zn}^{2+}$  from the ER into the cytosol upon EGF/ionomycin stimulation, we simultaneously used redCALWY-4 to monitor cytosolic  $\text{Zn}^{2+}$  levels and either ER-eZinCh-2 or ER-eCALWY-4 to monitor changes in ER  $\text{Zn}^{2+}$  concentrations<sup>[10]</sup>. Both cell lines showed good co-expression levels of these proteins. Imaging experiments were again performed using a perfusion setup, starting with perfusion of imaging buffer followed by addition of buffer supplemented with 10 ng/mL EGF and 500 nM ionomycin (Figure 6). When  $\text{Zn}^{2+}$  would be released from the ER to the cytosol upon external stimulation, a decrease in the emission ratio of redCALWY-4 and ER-eZinCh-2 should be observed, while an increase in emission ratio would be expected for ER-eCALWY-4. However, no changes in emission ratio were observed upon perfusion with EGF and ionomycin in either the cytosol or the ER (Figure 6). Different combinations of sensors were tested for both wild-type MCF-7 and TamR cells, but none of these experiments revealed a detectable change in either cytosolic or ER  $\text{Zn}^{2+}$  levels.



**Figure 6:** (A) Responses of MCF-7 cells co-expressing redCALWY-4 and ER-eZinCh-2 to the addition of 10 ng/mL EGF together with 500 nM ionomycin. (B) Responses of TamR cells co-expressing redCALWY-4 and ER-eCALWY-4 to the addition of 10 ng/mL EGF together with 500 nM ionomycin. Traces in A and B represent the average of three cells after normalization at t=0 s. Error bars represent SEM.

To exclude the possibility that the lack of response to EGF/ionomycin was somehow a result of the multicolor imaging experiments, we repeated these experiments in single sensor experiments. In these experiments the classical cerulean/citrine-based probes can be used for cytosolic imaging. The higher dynamic range of these sensors compared to the redCALWY probes should make it easier to detect small changes in cytosolic Zn<sup>2+</sup> levels. However, no changes in emission ratio were observed following addition of EGF and ionomycin in MCF-7 cells (Figure 7A) or TamR cells (Figure 7B) transfected with either eZinCh-2 or eCALWY-4. Similarly, EGF/ionomycin addition did not provoke any sensor response in either MCF7 or TamR cells expressing ER-eZinCh-2 or ER-eCALWY-4 (Figure 7C/D).



**Figure 7:** Responses of MCF-7 cells expressing cytosolic eCALWY-4 (A) and TamR cells expressing cytosolic eZinCh-2 (B) to the addition of 10 ng/mL EGF together with 500 nM of the calcium ionophore ionomycin. Responses of MCF-7 cells expressing ER-eZinCh-2 (C) and TamR cells expressing ER-eCALWY-4 (D) to the addition of 10 ng/mL EGF together with 500 nM of the calcium ionophore ionomycin. All traces represent the average of three cells after normalization at t=0 s. Error bars represent SEM.

Discussion and Conclusion

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The previously developed FRET-based Zn<sup>2+</sup> sensors eZinCh-2 and eCALWY-4 were successfully used to test the hypothesis that exposure of wild-type MCF-7 and TamR cells to external stimuli results in ZIP7-dependent Zn<sup>2+</sup> release from the ER stores into the cytosol<sup>[21]</sup>. Our work provided the first accurate determination of the free Zn<sup>2+</sup> concentrations in these breast cancer cell lines and revealed that free Zn<sup>2+</sup> concentrations are similar to those reported previously in other cell lines, both in the cytosol and the ER<sup>[6]</sup>. In contrast to previous reports using a synthetic fluorescent sensor and fixed cells, the genetically encoded sensors showed that addition of external Zn<sup>2+</sup> and the Zn<sup>2+</sup>-specific ionophore pyrithione results in an immediate increase in both cytosolic and ER Zn<sup>2+</sup>. This result is important, because external Zn<sup>2+</sup>/pyrithione was previously hypothesized to induce an intracellular signaling cascade resulting in ZIP7-mediated release of Zn<sup>2+</sup> from the ER to the cytosol. Another treatment that had been implicated to trigger specific release of Zn<sup>2+</sup> from the ER to the cytosol, EGF and ionomycin, was extensively tested in live cell imaging experiments in cells expressing a cytosolic sensor, an ER-targeted sensor or a combination of both. No changes in emission ratio were observed in either the cytosol or the ER in either MCF7 cells or TamR cells. These results suggest that EGF/ionomycin treatment does not trigger Zn<sup>2+</sup> release from the ER into the cytosol.

An important difference between this work and previous reports is the use of genetically encoded sensors instead of small-molecule dyes. Using the Zn<sup>2+</sup>-specific fluorescent dye FluoZin-3, Taylor and coworkers did not observe an immediate increase in fluorescence after the addition of Zn<sup>2+</sup>/pyrithione, which is surprising because pyrithione is a Zn<sup>2+</sup>-specific ionophore that transfers Zn<sup>2+</sup> across the plasma membrane. One possible explanation is that the fixation protocol that was used previously interfered with accurate and early detection of increases in Zn<sup>2+</sup> concentration. E.g. fixation does not allow continuous monitoring of the free Zn<sup>2+</sup> concentration in the same cell and cells on different coverslips were required for each time point after Zn<sup>2+</sup> treatment. Finally, unlike genetically encoded FRET sensors that can easily be targeted to a specific place in the cell, FluoZin-3 has been shown to not be exclusively localized in the cytosol, but also present in other subcellular compartments<sup>[23]</sup>.

In contrast to previous studies using Zinquin, no detectable changes in emission ratio were observed using our genetically encoded sensors in either MCF-7 or TamR cells following addition of EGF/ionomycin, neither in the cytosol nor when the sensors were targeted to the ER. Because the affinity of Zinquin that was used in previous studies is relatively low (*K<sub>d</sub>* = 7 μM), increasing levels of cytosolic Zn<sup>2+</sup> upon EGF/ionomycin treatment should have been easily detectable by the sensors used in this study, eZinCh-2 (*K<sub>d</sub>* = 1 nM at pH 7.1) and eCALWY-4 (*K<sub>d</sub>* = 0.63 pM at pH 7.1)(Table 1). We therefore conclude that addition of external EGF together with ionomycin does not trigger release of Zn<sup>2+</sup> from the ER stores into the cytosol. Previous experiments with EGF/ionomycin were also

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performed on fixed cells, which holds the same limitations as discussed above. It may therefore be worthwhile to apply genetically encoded sensors also in other cell systems in which treatment with EGF/ionomycin has been reported to result in a transient increase in cytosolic  $\text{Zn}^{2+}$  levels such as the  $\text{Zn}^{2+}$  waves reported by Yamasaki and coworkers in mast cells.<sup>[3]</sup>

Having shown that addition of  $\text{Zn}^{2+}$  together with pyrithione results in an immediate increase in cytosolic and ER free  $\text{Zn}^{2+}$  concentrations, our results do not support the suggested  $\text{Zn}^{2+}$  release from the ER several minutes after this treatment. In addition, no changes in overall cytosolic or ER free  $\text{Zn}^{2+}$  levels were observed upon addition of EGF/ionomycin using our genetically encoded sensors. Other observations that were originally linked to the release of  $\text{Zn}^{2+}$  from the ER may therefore also need to be reconsidered. For example, the association of protein kinase CK2 with ZIP7, and the subsequent phosphorylation of ZIP7, could be a downstream consequence of the pyrithione-mediated increase in cytosolic  $\text{Zn}^{2+}$ . Similarly, the reported activation of downstream signaling pathways may occur through phosphatase inhibition<sup>[20]</sup>. Addition of 20  $\mu\text{M}$   $\text{Zn}^{2+}$  together with 10  $\mu\text{M}$  pyrithione will increase the level of cytosolic free  $\text{Zn}^{2+}$  level to at least several nanomolar, which is sufficient to directly inhibit phosphatases<sup>[25]</sup>. Since no changes in overall intracellular  $\text{Zn}^{2+}$  levels were observed upon treatment with EGF/ionomycin, another mechanism may need to be invoked to explain the increased levels of tyrosine phosphorylation observed after treatment with this trigger. Alternatively, phosphatase inhibition may result from transient increases in local  $\text{Zn}^{2+}$  concentrations that cannot be detected using the current sensor technology.

In conclusion, the present study using genetically encoded FRET sensors does not provide supportive evidence for ZIP7-mediated release of  $\text{Zn}^{2+}$  from ER stores into the cytosol in TamR cells. These unexpected results suggest that it may be worthwhile to apply the specific properties of genetically encoded FRET sensors to carefully reassess other examples of triggered  $\text{Zn}^{2+}$  release from intracellular stores.

## Experimental Section

### Mammalian cell culture and transfection

MCF-7 and TamR cells were cultured in Roswell Park Memorial Institute media (RPMI), supplemented with 5% (v/v) fetal calf serum (FCS), 2 mM glutamine, 1 mM fungizone, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin (all from Life Technologies) at 37 °C in a humidified atmosphere containing 5%  $\text{CO}_2$ . For TamR cells stripped fetal calf serum (SFCS) was used instead of FBS and 0.1  $\mu\text{M}$  4-hydroxytamoxifen (4-OH tamoxifen) was added to the media. Cells were seeded on glass coverslips ( $\varnothing$  30 mm, VWR) one day before transfection. About 200 000 cells were seeded to reach a confluency of ~80% on the day of transfection. Lipofectamine 2000 (Life Technologies) was used to carry out transfections, following the manufacturer's instructions. Cells were imaged either one day (single sensor experiments) or two days (two sensor

experiments) after transfection in a HEPES buffer (Live Cell Imaging Buffer, Life Technologies) at 37 °C. DOI: 10.1039/C5MT00257E

### Intracellular FRET imaging

Imaging on MCF-7 and TamR cells was performed with a confocal microscope (Leica, TCS SP5X) equipped with a 63x water immersion objective, acousto-optical beamsplitters (AOBS), a white light laser, and a 405 nm laser. FluoZin-3 AM was excited at 494 nm, followed by monitoring its emission between 505–530 nm. For all eCALWY-4 and eZinCh-2 constructs, cerulean was excited using the 405 nm laser. For the redCALWY-4 the emission of a white light laser was set to 550 nm (5% of full power) to excite mOrange2. Emission was monitored using the AOBS and avalanche photo diode/photomultiplier tubes hybrid detectors (HyD, Leica): cerulean (450–500), citrine (515–595 nm), mOrange2 (565–600), and mCherry (600–630). Images were recorded at either 7.5 s intervals (two sensor experiments) or at 5 s intervals (single sensor experiments). For the *in situ* calibration experiments, cells were imaged for a few minutes with HEPES buffer without additives. Next HEPES buffer containing 50  $\mu\text{M}$  N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, Sigma) was added, followed by addition of HEPES buffer containing 100  $\mu\text{M}$   $\text{ZnCl}_2$  and 5  $\mu\text{M}$  of the  $\text{Zn}^{2+}$ -specific ionophore 2-mercaptopyridine N-oxide (pyrithione, Sigma), with a final concentration of 36  $\mu\text{M}$  TPEN, 100  $\mu\text{M}$   $\text{ZnCl}_2$  and 5  $\mu\text{M}$  pyrithione.  $\text{Zn}^{2+}$  stimulation was performed by perfusion with plain HEPES buffer for a few minutes, followed by perfusion with HEPES buffer supplemented with 20  $\mu\text{M}$   $\text{ZnCl}_2$  and 10  $\mu\text{M}$  pyrithione (Sigma). For EGF/ionomycin stimulation, cells were perfused with HEPES buffer without additives for a few minutes, followed by perfusion with HEPES buffer containing 10 ng/mL EGF (Sigma, E9644) and 500 nM ionomycin (Sigma, 56092-82-1). Image analysis was performed using ImageJ software as described before<sup>[24, 26]</sup>. Steady-state fluorescence intensity ratio of acceptor over donor was measured, followed by the determination of the minimum and maximum ratios to calculate the free  $\text{Zn}^{2+}$  concentration using equation 1:

$$[\text{Zn}^{2+}] = \frac{R_d \cdot (R - R_{0\%})}{(R_{100\%} - R)} \quad (1)$$

In which  $R_{0\%}$  is the ratio in the  $\text{Zn}^{2+}$  depleted state, after addition of 50  $\mu\text{M}$  TPEN, and  $R_{100\%}$  was obtained upon  $\text{Zn}^{2+}$  saturation with 100  $\mu\text{M}$   $\text{ZnCl}_2$  in the presence of 5  $\mu\text{M}$  pyrithione<sup>[6, 24]</sup>.

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