Development of a genetically encoded sensor for endogenous CaMKII activity

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ABSTRACT

CaMKII is a crucial oligomeric enzyme in neuronal and cardiac signaling, fertilization and immunity. Here, we report the construction of a novel, substrate-based, genetically-encoded sensor for CaMKII activity, FRESCA (**F**RET-based **S**ensor for **C**aMKII **A**ctivity). Currently, there is one biosensor for CaMKII activity, Camui, which contains CaMKII. FRESCA allows us to measure all endogenous CaMKII variants, while Camui can track a single variant. Since there are ~40 CaMKII variants, using FRESCA to measure aggregate activity allows a fresh perspective on CaMKII activity. We show, using live-cell imaging, FRESCA response is concurrent with Ca²⁺ rises in HEK293T cells and mouse eggs. In eggs, we stimulate oscillatory patterns of Ca²⁺ and observe the differential responses of FRESCA and Camui. Our results implicate an important role for the variable linker region in CaMKII, which tunes its activation. FRESCA will be a transformative tool for studies in neurons, cardiomyocytes and other CaMKII-containing cells.

INTRODUCTION

Calcium-calmodulin dependent protein kinase II (CaMKII) is a serine/threonine kinase that plays critical signaling roles in multiple mammalian tissues (Bucks et al., 2010; Rokita & Anderson, 2012; Shonesy, Jalan-Sakrikar, Cavener, & Colbran, 2014) and is

implicated in a number of diseases (Mollova, Katus, & Backs, 2015; Robison, 2014;
 2 Steinkellner et al., 2012; Tu, Okamoto, Lipton, & Xu, 2014). CaMKII plays a key role in
 all electrically coupled cells, such as neurons and cardiomyocytes, and even cells that are
 4 not – such as lymphocytes and eggs – all of which communicate using Ca^{2+} . Depending
 on the stimulus, the Ca^{2+} response leads is either a single Ca^{2+} rise or a more complex
 6 responses such as oscillations (Cuthbertson, Whittingham, & Cobbold, 1981; Eisner,
 Caldwell, Kistamas, & Trafford, 2017; Rutecki, 1992; Swann & Lai, 2013). Absence of
 8 Ca^{2+} signals causes severe defects in cell functionality, such as memory deficits in the
 case of neurons (Herring & Nicoll, 2016), or in the case of fertilization, failure to
 10 conceive (Escoffier et al., 2016; Yoon et al., 2008). CaMKII is responsible for reacting to
 Ca^{2+} oscillations and transducing this signal to downstream molecules. Indeed, it has been
 12 shown that neuronal CaMKII has a threshold frequency for activation (Chao et al., 2011;
 De Koninck & Schulman, 1998).

14
 CaMKII has a unique oligomeric structure among the protein kinase family (Fig. 1A).
 16 Each subunit of CaMKII is comprised of a kinase domain, regulatory segment, variable
 linker region, and hub domain (Fig. 1B). The hub domain is responsible for
 18 oligomerization, which organizes into two stacked hexameric (or heptameric) rings to
 form a dodecameric (or tetradecameric) holoenzyme (Bhattacharyya et al., 2016; Chao et
 20 al., 2011; Rosenberg et al., 2006). In the absence of Ca^{2+} , the regulatory segment binds to
 and blocks the substrate-binding pocket. Ca^{2+} /calmodulin (Ca^{2+} /CaM) turns CaMKII on
 22 by competitively binding the regulatory segment and exposing the substrate-binding
 pocket (Fig. 1C).

- 2 It has been demonstrated that CaMKII has a threshold frequency for activation (Chao et
al., 2011; De Koninck & Schulman, 1998). There are four human CaMKII genes;
- 4 CaMKII α and β are predominantly expressed in neurons, CaMKII δ is predominantly
expressed in the heart and CaMKII γ is found in multiple organ systems, including the
- 6 reproductive organs. The kinase and hub domains of all four genes are highly conserved
(~90% on average), however, the linker domain connecting the kinase and hub domains

Figure 1. CaMKII: an essential enzyme. A) CaMKII is an oligomeric complex turned on by $\text{Ca}^{2+}/\text{CaM}$ binding, which facilitates phosphorylation at Thr 286. B) Each CaMKII subunit is comprised of a kinase domain, regulatory segment which houses the CaM binding domain, a variable linker region, and a hub domain. C) The regulatory segment of CaMKII maintains its off state in the absence of calcium by blocking its substrate binding pocket. $\text{Ca}^{2+}/\text{CaM}$ competes with the regulatory segment, thereby activating the kinase and allowing for Thr286 phosphorylation, which yields autonomous activity. Even when the calcium stimulus diminishes, CaMKII stays on as long as Thr 286 is phosphorylated. D) In mammalian eggs, addition of PLC ζ or Sr^{2+} leads to stimulation of calcium oscillations and CaMKII activation. CaMKII is expected to be “off” in the absence of Ca^{2+} and turn “on” after Ca^{2+} levels rise.

is variable in length and composition. Details elucidating the importance of the variable linker region remain to be uncovered, but there are >30 different splice variants of each of the four genes, which mostly vary in the linker region only. It has been shown that CaMKII activity is tuned by the length of the variable linker *in vitro* (Bayer, De Koninck, & Schulman, 2002; Chao et al., 2011). Specifically, as the variable linker is lengthened, less Ca^{2+} is needed for activation (*i.e.*, activation of CaMKII is easier). Thus, it is important for us to consider the complexity of endogenous CaMKII expressed in various cell types.

Camui is currently the only biosensor for CaMKII activity (Takao et al., 2005). Camui is a Förster resonance energy transfer (FRET)-based biosensor for CaMKII activity, which exploits the conformational change that CaMKII undergoes when it binds to Ca^{2+} /CaM (Fig. 2A). To date, Camui has been a very useful tool to study and understand CaMKII activity in various cell types (mainly neurons and cardiomyocytes) and under various conditions (Erickson, Patel, Ferguson, Bossuyt, & Bers, 2011; Kwok et al., 2008; Takao et al., 2005). However, a major limitation is that the Camui sensor is constructed of a CaMKII variant itself, and thus will only report on this particular variant. To enhance our understanding of this complex protein, we need a way to measure endogenous CaMKII activity. One option is to re-engineer Camui with the appropriate CaMKII isoform to be studied, however this becomes limiting when there are multiple isoforms expressed in a single cell type, such as during the development of the female gamete, the egg. We now report the development of a novel biosensor that detects endogenous CaMKII activity. Herein, we show the efficacy of this new sensor in mouse eggs.

RESULTS AND DISCUSSION

2 Development of a novel biosensor for endogenous CaMKII activity

We developed a novel substrate-based sensor for CaMKII activity, FRESCA (**FRET**
 based **S**ensor for **C**aMKII **A**ctivity, Fig. 2A). We monitored CaMKII activity using
 FRESCA in real-time following the induction of Ca^{2+} responses to several agonists that
 are capable of initiating egg activation and embryogenesis. Building on a previous design
 for an Aurora kinase biosensor (Liu, Vader, Vromans, Lampson, & Lens, 2009), we
 replaced the sequence encoding the Aurora kinase substrate for the CaMKII substrate
 (syntide). The design also employs FHA2, a phosphate-binding domain, to facilitate a
 conformational change once the adjacent CaMKII substrate is phosphorylated (Durocher
 et al., 2000). FHA2 will bind to this phosphorylated Thr residue and produce a decrease
 in FRET between the terminal CFP/YFP pair.

14 *Measuring the FRESCA response in HEK293T cells*

We first tested the selectivity of FRESCA in HEK293T cells, which express negligible
 levels of CaMKII. We transfected HEK293T cells with either (i) CaMKII, calmodulin
 and FRESCA, or (ii) calmodulin and FRESCA. Ionomycin was added to the HEK293T
 cells to induce Ca^{2+} release and simultaneously monitored FRET (CFP/YFP ratio). We
 observed that with CaMKII present, the addition of ionomycin causes a reduction in
 FRET, indicating that CaMKII is active and phosphorylating FRESCA (Fig. 2B, red
 lines). Importantly, we did not observe a FRET change when CaMKII was not co-
 transfected, demonstrating that FRESCA is selective for CaMKII and not being
 phosphorylated by other HEK cell kinases (Fig. 2B, blue lines).

A FRESKA: FRET sensor for CaMKII Activity

C FRESKA in MII eggs

Figure 2. Monitoring endogenous CaMKII activity using FRESKA. A) A cartoon of a substrate-based CaMKII biosensor is shown: FRET Sensor for CaMKII Activity (FRESKA). Active CaMKII phosphorylates its substrate (syntide), which then acts as a substrate for FHA2 (phosphate binding domain). This induces a conformational change in the sensor as a consequence of CaMKII activity. B) FRESKA is transfected into HEK293 cells with and without CaMKII. Ionomycin (2.5 μ M) is added to induce Ca^{2+} entry and FRET is monitored as YFP/CFP ratio. Without CaMKII transfected, there is no FRET change visualized. C) FRESKA expression in mouse MII eggs shows a widespread cytoplasmic distribution. D) Ca^{2+} is monitored using rhod-2 (black) and CaMKII activity is monitored using FRESKA (red). Multiple traces are shown after 0.5 μ M ionomycin is added. E) One representative trace is shown after addition of 0.5 μ M ionomycin. F) Multiple traces are shown after 2.5 μ M ionomycin is added. G) One representative trace is shown after addition of 2.5 μ M ionomycin. H) Multiple traces are shown after 5 μ M ionomycin is added. I) One representative trace is shown after addition of 5 μ M ionomycin.

Figure 2 – figure supplement 1. FRESKA expression in GV oocytes. Expression is mostly cytoplasmic, as well as some limited nuclear expression.

Figure 2 – figure supplement 2. Quantification of FRESKA response to ionomycin addition. Time to FRET peak indicates how long it takes FRESKA to reach maximum Δ FRET signal after addition of ionomycin and increase in Ca^{2+} . Δ FRET amplitude

indicates the overall change in FRET during the duration of the Ca^{2+} signal. Statistics are reported in the tables below, differences were considered significant at $P < 0.05$ (*).

Figure 2 – figure supplement 3. Pharmacological tests of FRESKA specificity in mouse eggs. Various compounds were added to mouse eggs expressing FRESKA. Ca^{2+} was monitored using Rhod-2 and FRET was monitored by YFP/CFP ratio. A) CaMKII inhibitor: KN93 (0.5 μM), PKC inhibitors: GO6983 (3 μM) and Bim1 (5 μM), and a PKC activator: PMA (1 μM) were added to mouse eggs and stimulated with 0.5 μM ionomycin. These were directly compared to FRESKA alone and a non-injected control with 0.5 μM ionomycin. Colors correspond to the bar graph in (B). B) Quantification of the amplitude of FRET signal in (A). C) CaMKII inhibitor: AS105 (5 μM) and inactive analog of this inhibitor: AS461 (5 μM) were added to mouse eggs and stimulated with 2.5 μM ionomycin. These were directly compared to FRESKA alone and a non-injected control with 2.5 μM ionomycin. Colors correspond to the bar graph in (D). D) Quantification of the amplitude of FRET signal in (C). Differences were considered significant at $P < 0.05$ (*).

Using FRESKA to monitor CaMKII activity in mouse eggs

HEK293T cells provided a good model for a highly controlled evaluation of CaMKII

activity and the ability of FRESKA to specifically report on CaMKII in the presence of

other cellular kinases. However, we wanted to test FRESKA in a more complex and native system, importantly, with endogenous CaMKII and where the Ca^{2+} response has a clear physiological function. To accomplish this, we expressed FRESKA in mouse eggs to measure endogenous CaMKII γ activity following increases in intracellular Ca^{2+} induced by a variety of agonists.

In all mammals, Ca^{2+} oscillations are required for initiation of embryogenesis (Deguchi, Shirakawa, Oda, Mohri, & Miyazaki, 2000; Fissore, Dobrinsky, Balise, Duby, & Robl, 1992). Mammalian eggs are arrested at metaphase II of meiosis; both Ca^{2+} oscillations and consequent CaMKII activity are required for release from this arrest (Fig. 1D) (Banks et al., 2010; Chang, Minahan, Merriman, & Jones, 2009; Miao, Stein, Jefferson, Padilla-Banks, & Williams, 2012; Miyazaki et al., 1992; Presler et al., 2017). Ca^{2+} oscillations are induced following gamete fusion when the sperm releases into the egg a sperm specific protein (PLCzeta; ζ), which triggers the Ca^{2+} responses (Ducibella et al., 2002; Saunders et al., 2002). On average, there is one Ca^{2+} rise every 20 minutes and oscillations in mouse zygotes last for ~4 hours, which coincides with the formation of the pronuclei (PN) (Jones, Carroll, Merriman, Whittingham, & Kono, 1995). The source of this Ca^{2+} is from internal stores, which are replenished by Ca^{2+} influx from the extracellular media. CaMKII is activated simultaneously with the initiation of Ca^{2+} oscillations and female mice that are CaMKII γ null are sterile (Banks et al., 2009).

Despite the role of CaMKII γ in the initiation of development, the complete profile of CaMKII activity during fertilization in mammals is not known. Further, CaMKII activity also seems to play a role in preventing apoptosis in *Xenopus* and mouse eggs, although

the pattern and degree of activation for this activity are even less studied (Nutt et al.,
2005).

To date, CaMKII activity has only been assessed based on a few Ca^{2+} rises using *in vitro*
kinase assays and during only the first hour of oscillations, which is considerably shorter
than the time scale for normal oscillations in the mouse. Therefore, there is a need to
monitor CaMKII activity in live cells and for an extended time, which is what we address
here.

FRESCA and ionomycin-induced Ca^{2+} oscillations in mouse eggs

FRESCA expression and distribution in germinal vesicle (GV) oocytes and MII stage
oocytes, henceforth referred to as eggs, was widespread and cytoplasmic (Fig. 2C).
However, a small amount of FRESCA appeared to enter the nucleus of GV oocytes (Fig.
2, supplement 1).

Given the immediate and large Ca^{2+} rise caused by the addition of ionomycin, we first
tested FRESCA responses in eggs using this ionophore. We analyzed the effect of 3
concentrations of ionomycin: 0.5 μM , 2.5 μM and 5 μM . Upon addition of ionomycin to
eggs expressing FRESCA, we observed a FRET decrease, indicating CaMKII activity
(Fig. 2D-I). At the lowest ionomycin concentration (0.5 μM), CaMKII activity appears to
perfectly track the Ca^{2+} pulse (Fig. 2D, E). Conversely, at higher ionomycin
concentrations, CaMKII activity is unstable during the duration of the Ca^{2+} pulse,
although higher concentrations appeared to prolong and increase the FRET response of

FRESCA (Fig. 2, supplement 2). The time to FRET peak was faster with addition of higher ionomycin concentrations (Fig. 2, supplement 2).

We tested the specificity of FRESCA for CaMKII in mouse eggs. We first used CaMKII inhibitors, which should eliminate the FRET response if CaMKII is the only kinase phosphorylating FRESCA in eggs (Fig. 2, supplement 3). We show that the addition of KN93, a commonly used allosteric inhibitor for CaMKII, significantly reduces FRET (Madgwick, Levasseur, & Jones, 2005; Smyth et al., 2002). Addition of AS105, an ATP competitive CaMKII specific inhibitor, also significantly reduces FRET, while its inactive analog (AS461) does not affect FRET (Neef et al., 2018). We also tested inhibition and activation of protein kinase C (PKC), since PKC is the other major Ca^{2+} sensitive kinase in mouse eggs (Medvedev, Stein, & Schultz, 2014; Wang et al., 2010). Addition of PKC inhibitors Bim1 (Halet, 2004) and GO6983 (Gou, Wang, Zou, Qi, & Xu, 2018) do not affect the FRESCA signal, indicating that PKC is not phosphorylating FRESCA. Eggs do not express conventional PKC isoforms, so the results of the broad-spectrum PKC inhibitor (GO6983) reinforced the results of Bim1. Finally, addition of PMA, a PKC activator shown to stimulate this enzyme in mouse eggs (Halet, 2004), also does not induce FRET (Fig. 2, supplement 3). Taken together, we report that FRESCA is a specific reporter of CaMKII activity in mouse eggs.

Figure 3. Endogenous CaMKII activity tracks Ca²⁺ oscillations in mouse eggs. A) Ca²⁺ oscillations in eggs are induced by addition of Sr²⁺ to the extracellular media. Ca²⁺ is monitored by Rhod-2 (black line) and endogenous CaMKII activity is tracked by FRESKA (red line). B) One representative trace from Sr²⁺ oscillations is shown. Inset focuses on the first Ca²⁺ rises.

Figure 3 – figure supplement 1. Quantification of Rhod-2 and FRET signals for FRESKA during Sr^{2+} induced oscillations. Three Ca^{2+} rises were quantified. The “1st rise” is that which induced the first FRET response, and then the subsequent 2 rises were measured. Statistics are reported in the tables below, differences were considered significant at $P < 0.05$ (*).

Figure 3 – figure supplement 2. Increased time course of FRESCA response to Sr^{2+} . Sr^{2+} (10 mM) was added to mouse eggs expressing FRESCA. Ca^{2+} is monitored by Rhod-2 (black line) and endogenous CaMKII activity is tracked by FRESCA (red line) for 6 hours. The progressive decreasing amplitude of the Rhod-2 AM fluorescence signal (especially after 180 min) is an artifact due to compartmentalization of the dye due to prolonged monitoring at $>35^\circ\text{C}$.

FRESCA and Sr^{2+} -induced Ca^{2+} oscillations

Addition of 10 mM Sr^{2+} to the extracellular media in place of external Ca^{2+} is a common method of parthenogenetic activation in mouse eggs, and it induces highly consistent oscillations in these cells (Fig. 3, black lines); these oscillations initiate all events of egg activation (Bosmikich & Whittingham, 1995; Carvacho, Lee, Fissore, & Clapham, 2013; Kline & Kline, 1992). Further, the TRPV3 channel has recently been identified as the channel responsible for Sr^{2+} influx in mouse eggs (Carvacho et al., 2013). We therefore examined the response of endogenous CaMKII to Sr^{2+} -induced oscillations. We observed endogenous CaMKII activity (monitored by FRESCA) almost simultaneously with the initiation of oscillations. Indeed, nearly all eggs (11/13) showed CaMKII activity within the first two rises. Importantly, CaMKII activity is reproduced over time, as FRESCA continues to track each Ca^{2+} rise for >2 hours (Fig. 3B).

We propose a molecular model to describe this data. The Ca^{2+} rises progressively decrease in duration/amplitude over time (Deguchi et al., 2000), however, the FRESCA responses are not diminished and the peak kinase activity seems to outlast the peak elevation of $\text{Ca}^{2+}/\text{Sr}^{2+}$. This suggests autophosphorylation of CaMKII at Thr 286, which facilitates activation at subsequent Ca^{2+} pulses by increasing the affinity for $\text{Ca}^{2+}/\text{CaM}$ (see cartoons in Fig. 3A) (Meyer, Hanson, Stryer, & Schulman, 1992). Additionally, a

prolonged time course of FRESKA response to Sr^{2+} indicates that FRESKA continues to faithfully track endogenous CaMKII up to 6 hours (Fig. 3, supplement 2).

Using Camui to measure CaMKII activity in mouse eggs

As described, the highly used Camui biosensor is comprised of CaMKII itself (see Fig. 4A), specifically CaMKII α , which has a 30-residue variable linker region (Fig. 4C).

Despite its widespread use, Camui has not yet been used to monitor CaMKII activity in mouse eggs. Given that it has been demonstrated that CaMKII activity is tuned by the length of the variable linker (Bayer et al., 2002; Chao et al., 2011), specifically, as the variable linker is lengthened, less Ca^{2+} is needed for activation, we hypothesized that FRESKA may report CaMKII activity in mouse eggs more faithfully than Camui. This assumption is based on the knowledge that mouse eggs express equimolar concentrations of the two versions of CaMKII γ ($\gamma 3$ and γJ), which have 69 and 90 residue variable linkers, respectively (Fig. 4C) (Hatch & Capco, 2001; Suzuki, Hara, Takagi, Yamamoto, & Ueno, 2011), considerably longer than the 30-residue linker of CaMKII α . We therefore tested the response of Camui compared to FRESKA.

We expressed the Camui reporter in mouse eggs using mRNA injection, and similar to FRESKA, expression was robust within ~30 minutes and we began FRET measurements ~4 hours post injection to attain stable Camui levels. As shown by confocal microscopy, Camui attained a widespread cytoplasmic expression in eggs, although in GVs it was excluded from the nucleus, which is consistent with the reported expression of CaMKII in the cytosol of mouse eggs (Fig. 4B and Fig 4., supplement 1) (Hatch & Capco, 2001).

Figure 4. Simultaneously monitoring Ca^{2+} influx and CaMKII activity using Camui.

A) Camui is an existing biosensor for CaMKII activity, which exploits the conformational change of CaMKII binding to Ca^{2+} /CaM to report on activity using FRET. B) Camui expression in mouse MII eggs shows a widespread cytoplasmic distribution. C) Linear sequences of Camui (CaMKII α), and the two CaMKII isoforms reported in eggs (CaMKII γ 3 and γ J). D) Changes in Ca^{2+} are monitored using rhod-2 (black) and CaMKII activity is monitored using Camui (red). Multiple traces are shown after 0.5 μM ionomycin is added. E) One representative trace is shown after addition of 0.5 μM ionomycin. F) Multiple traces are shown after 2.5 μM ionomycin is added. G) One representative trace is shown after addition of 2.5 μM ionomycin. H) Multiple traces are shown after 5 μM ionomycin is added. I) One representative trace is shown after addition of 5 μM ionomycin.

Camui in GV oocytes

Figure 4 – figure supplement 1. Camui expression in GV oocytes. Expression is mostly cytoplasmic, and is excluded from the nucleus.

	0.5 μM	2.5 μM	5.0 μM
Mean	2.675	1.790	1.625
Std. Deviation	0.6426	0.6088	0.5418
Std. Error	0.1855	0.1477	0.1916

	0.5 μM	2.5 μM	5.0 μM
Mean	0.1382	0.2712	0.2750
Std. Deviation	0.06715	0.04664	0.06573
Std. Error	0.01939	0.01166	0.02324

Figure 4 – figure supplement 2. Quantification of Camui response to ionomycin addition. Time to FRET peak indicates how long it takes FRESKA to reach maximum ΔFRET signal after addition of ionomycin and increase in Ca^{2+} . ΔFRET amplitude indicates the overall change in FRET during the duration of the Ca^{2+} signal. Statistics are reported in the tables below, differences were considered significant at $P < 0.05$ (*).

Camui and ionomycin-induced Ca^{2+} oscillations

We first induced Ca^{2+} release in eggs by adding ionomycin as previously described and simultaneously monitored changes in FRET values (YFP/CFP) (Fig. 4C-H). As with FRETSCA, a decrease in FRET is indicative of an increase in CaMKII activity. It is clear that CaMKII activity increases (red line) coincident with the increase in Ca^{2+} (black line) in all conditions. The Ca^{2+} and Camui responses increased dose-dependently and approximately synchronously, as the large increase in the amount of Ca^{2+} release caused by increasing ionomycin from 0.5 μM to 2.5 μM , results in a 1.9-fold increase in CaMKII activity (mean amplitude of FRET change) (Fig. 4, supplement 2). Further increasing ionomycin from 2.5 μM to 5 μM produces nearly no change in total Ca^{2+} release, although it is very likely that the reporting range of Rhod-2 is saturated at these levels of Ca^{2+} release. The CaMKII activity also appears to remain constant, although this may also represent saturation of the FRET signal (Fig. 4, supplement 2). Notably, addition of 5 μM ionomycin results in a prolonged duration of activity compared to lower concentrations, but it is unclear whether this reflects the extended activation of the enzyme or cellular stress.

The absolute amplitude of the change in the FRET ratio for Camui after the addition of 0.5 μM ionomycin is ~10-fold greater than what is observed for FRETSCA (0.014 for FRETSCA compared to 0.14 for Camui), however, this measurable signal change in FRETSCA is sufficient to monitor endogenous CaMKII γ activity, which we cannot detect with Camui, which reports CaMKII α activity. In addition, it is worth noting that the shape of the FRETSCA traces is slightly different from those of Camui for the same

stimulus. The peak activity of FRESCA is shorter than the corresponding Ca^{2+} peak,

- 2 although the return to basal activity is more protracted, whereas the Camui response more perfectly tracks the shape of the Ca^{2+} peak.

Figure 5. Camui activity tracks Ca^{2+} oscillations in mouse eggs in a delayed manner. A) Ca^{2+} oscillations are induced by addition of Sr^{2+} . Ca^{2+} is monitored by Rhod-2 (black line) and CaMKII activity is tracked by Camui (red line). CaMKII cartoons indicate hypothesized molecular details during the Ca^{2+} pulses. B) One representative trace from Sr^{2+} oscillations is shown. Blue lines indicate baseline CaMKII activity after each rise. Arrow indicates first significant FRET response.

Figure 5 – figure supplement 1. Quantification of Rhod-2 and FRET signals for Camui during Sr^{2+} induced oscillations. Three Ca^{2+} rises were quantified. The “1st rise” is that which induced the first FRET response, and then the subsequent 2 rises were measured. Statistics are reported in the tables below, differences were considered significant at $P < 0.05$ (*).

Camui and Sr^{2+} induced Ca^{2+} oscillations

We next examined the Camui response to Sr^{2+} -induced oscillations (Fig. 5). Remarkably, despite the presence of robust changes in intracellular Ca^{2+} levels, Camui (Fig. 5, red line) did not report any CaMKII activity until the ~6th significant Ca^{2+} rise (Fig. 5B, arrow and inset). In total, only 33% of the eggs expressing Camui showed activity in the first two rises, whereas nearly all FRESKA expressing eggs showed activity within the first two rises. These data suggest that the endogenous CaMKII γ in eggs is more sensitive

to $\text{Ca}^{2+}/\text{CaM}$ than $\text{CaMKII}\alpha$. This finding is in line with previous data showing that

longer linker CaMKII splice variants ($\text{CaMKII}\gamma 3$ and $\text{CaMKII}\gamma J$) are activated at lower concentrations of $\text{Ca}^{2+}/\text{CaM}$ than shorter linker variants ($\text{CaMKII}\alpha$) (see Fig. 4C) (Chao et al., 2011). Additionally, the delayed response seen in the Camui eggs could also be a result of endogenous CaMKII being activated first (lower EC_{50} for $\text{Ca}^{2+}/\text{CaM}$) thereby competing with Camui for the available activating ligand.

Another distinctive feature of the Camui response caused by Sr^{2+} oscillations is that whereas the initial Camui responses were delayed, once they commenced, they displayed an integrated activation with each subsequent pulse. For example, we analyzed the mean amplitude for the first three observable FRET changes. From the first to the second FRET change, there was a 1.6-fold increase in CaMKII activity. From the second to the third FRET change, there was a negligible change, and these changes occurred while the amplitude of the Ca^{2+} peaks progressively decreased and/or remained unchanged (Fig. 5, supplement 1). These data indicate that CaMKII activity, once stimulated, is cooperative with each additional Ca^{2+} pulse. This result is consistent with previous data showing that CaMKII activity is highly cooperative *in vitro* (Chao et al., 2010; Chao et al., 2011). As depicted in Figure 5A, a potential explanation for this is phosphorylation at Thr286 which may persist even in the absence of elevated Ca^{2+} . It has been clearly shown that CaMKII with Thr286 phosphorylated has a significantly higher affinity for $\text{Ca}^{2+}/\text{CaM}$ (Meyer et al., 1992). This would also explain why the FRET level does not return to baseline in between later Ca^{2+} oscillations (Fig. 5B, blue lines).

Measuring CaMKII activity under native fertilization conditions

2 In mammals, fertilization-associated Ca^{2+} oscillations are induced by the release of
sperm's PLC ζ into the ooplasm (Saunders et al., 2002). We tested the response of both
4 FRESCA and Camui in response to the expression of PLC ζ .

6 *FRESCA and PLC ζ -induced Ca^{2+} oscillations*

We accomplished PLC ζ expression by injection of its mRNA into FRESCA expressing
8 eggs, and thereafter began monitoring changes in FRESCA responses (Fig. 6A, B). The
initiation of oscillations stimulated the early activity of the endogenous CaMKII γ , and
10 this activity was detected with each additional rise. Similar to Sr^{2+} induced oscillations,
we observed a relative decrease in the amplitude of the Ca^{2+} pulses over time, yet the
12 FRESCA response was largely maintained (Fig. 6, supplement 1). These observations are
the longest evaluation of CaMKII activity following natural oscillations ever reported
14 following fertilization, as previous studies only reported up to 60 min post-initiation of
oscillations (Markoulaki, Matson, & Ducibella, 2004; Ozil et al., 2005).

16

Camui and PLC ζ -induced Ca^{2+} oscillations

18 We next assessed how Camui would report CaMKII activity induced by Ca^{2+} oscillations,
and compare the response to those induced by Sr^{2+} . To do this, eggs expressing Camui
20 were injected with PLC ζ mRNA and Ca^{2+} and FRET responses were monitored. Similar
to FRESCA, we observed that Ca^{2+} oscillations nearly immediately induced CaMKII
22 activity as monitored by Camui (Fig. 6D, arrow and bottom inset). However, this initial

activity was not detected in subsequent rises, and only the first and second (and to a less
 2 extent, third) Ca^{2+} rises induced Camui responses despite the presence of robust and
 frequent Ca^{2+} oscillations (Fig. 6C, D, supplement 2). Additionally, it is worth pointing
 4 out that the area under the curve for the third Ca^{2+} rise in these experiments was
 significantly reduced. This may be due to the fact that Camui itself is contributing
 6 significantly to the existing CaMKII in the egg, and potentially altering Ca^{2+} dynamics.
 These results raised the possibility that Camui is not well suited to detect CaMKII
 8 activity initiated by sporadic and low magnitude Ca^{2+} rises, which are characteristic of
 mammalian fertilization. Regardless, it remains to be elucidated why Sr^{2+} induced
 10 oscillations are able to protractedly promote robust and persistent Camui responses
 whereas the Camui response to PLC ζ -induced oscillations fades rapidly.

Figure 6. FRESKA, but not Camui, continues to report CaMKII activation by Ca^{2+} oscillations induced by PLCζ injection

Ca²⁺ oscillations are induced by injection of PLCζ mRNA. CaMKII activity is monitored using FRESKA or Camui (FRET, red lines) and Ca²⁺ is monitored using Rhod-2 (black lines). A) An overlay of 3 representative eggs using FRESKA as the reporter of endogenous CaMKII activity. Cartoon depictions of hypothesized states of CaMKII are shown below. Red circles indicate Thr286 phosphorylation. B) One representative trace from PLCζ-induced oscillations and FRESKA reporting is shown. Insets highlight the first and last pulses. C) An overlay of 4 representative eggs using Camui as the reporter of CaMKIIα activity. Cartoon depictions of hypothesized states of CaMKII are shown below. Red circles indicate Thr286 phosphorylation. D) One representative trace from PLCζ-induced oscillations and Camui reporting is shown. Insets highlight the first and last pulses.

Figure 6 – figure supplement 1. Quantification of Rhod-2 and FRET signals for FRESKA during PLC ζ induced Ca^{2+} rises. Three Ca^{2+} rises were quantified. The “1st rise” is that which induced the first FRET response, and then the subsequent 2 rises were measured.

Figure 6 – figure supplement 2. Quantification of Rhod-2 and FRET signals for Camui during PLCζ induced Ca²⁺ rises. Three Ca²⁺ rises were quantified. The “1st rise” is that which induced the first FRET response, and then the subsequent 2 rises were measured. Differences were considered significant at P < 0.05 (*).

CONCLUDING REMARKS

It has been appreciated for decades that both Ca²⁺ oscillations and CaMKII activation in mouse eggs is crucial to fertilization and initiation of embryo development. Here, we provide an analysis of CaMKII activation in real-time in eggs using FRET-based CaMKII biosensors. Importantly, our new biosensor, FRESKA, allowed us to monitor endogenous CaMKII (CaMKIIγ3 and γJ) activation in real-time as a consequence of different

activation stimuli (ionomycin, Sr^{2+} , and PLC ζ). The FRESCA response was noticeably

different from the Camui sensor, which reports on CaMKII α . When different Ca^{2+} oscillation patterns are induced, we observe subsequent differences in CaMKII activity.

From our data, it is clear that both the (i) pattern of Ca^{2+} oscillations as well as the (ii) specific CaMKII isoform responding play a role in CaMKII activation.

The pivotal role of CaMKII activation in causing release of the meiotic arrest and

activation of the embryonic developmental program in vertebrates was recently and more specifically evidenced by careful mass spectrometry experiments (Presler et al., 2017).

This study showed that soon after fertilization, and temporally coinciding with the Ca^{2+} wave, there is a strong increase in protein phosphorylation that far outweighs the

biochemical changes caused by protein degradation that accompanies fertilization.

Remarkably, the study also found that 25% of the phosphorylated sites matched the

minimal phosphorylation motif of CaMKII. It is therefore important to determine how

Ca^{2+} rises turn on CaMKII activity, and what parameter(s) of individual rises within an

oscillatory pattern are necessary for periodic and consistent stimulation of its activity. We

propose that the magnitude of the initial activation of CaMKII depends on the magnitude

of the stimulus and on internal regulation of CaMKII, which is largely based on the

variable linker region. Knowing the minimal Ca^{2+} signal that increases the activity of

CaMKII γ is important as we seek to develop more physiological methods of

parthenogenetic activation to treat some cases of infertility.

More broadly, now that we have demonstrated the utility of FRESKA in mouse eggs, this
 2 opens the door to measuring endogenous CaMKII activity in other cell types, such as
 neurons and cardiomyocytes. CaMKII activation has been heavily studied *in vitro* (Chao
 4 et al., 2010; Chao et al., 2011; Rosenberg, Deindl, Sung, Nairn, & Kuriyan, 2005), and it
 is intriguing to also consider the potential effects of subunit exchange in cellular
 6 conditions (Bhattacharyya et al., 2016; Stratton et al., 2013). It will be necessary to
 increase the signal to noise ratio of the FRESKA sensor in order to achieve a more robust
 8 signal for accurate quantification of kinetics and amplitudes. Once this is accomplished,
 we believe that FRESKA will provide new insights into CaMKII activity in cells and
 10 allow us to unravel the complexity of this unique protein kinase.

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MATERIALS AND METHODS

2 Plasmid design

In order to accommodate the requirements for FHA2 binding (Durocher et al., 2000),

4 syntide was modified from PLARTLSVAGLPGKK to PLARALTVAGLPGKK to create syntide-2. Syntide-2 was generated by annealing

6 GATCCGGCGGCGCCGGCGGCGGCccgctggcgcgccctgaccgtggcgggcctgccgggcaaaaaaGCG and

8 GGCCGCCtttttggccggcaggcccgccacggtcagggcgcgccagcggGCCGCCGGCGGCGCCG CCG (IDT), which produced *Bam*HI site at the 5' end and a *Not*I site on the 3' end. This

10 product was phosphorylated (Ambion Pnk), purified (Thermo Fisher) and then ligated using T4 DNA ligase (Invitrogen) into a plasmid encoding the Aurora kinase FRET

12 sensor (kind gift from Thomas Maresca). The final FRESCA sensor (with syntide-2 in place of the Aurora substrate) was cloned into pCDNA3.1.

14

HEK 293T Cell culture

16 All HEK293T cell cultures were grown in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and maintained at 37°C and 5%

18 carbon dioxide levels. The identity of these cells was authenticated by ATCC (CRL-3216 ATC 293T, Lot #63226319). Cells were transfected using Lipofectamine® 2000 Reagent

20 (Invitrogen) and 150 ng of DNA constructs.

22

Collection of mouse eggs

2 Metaphase II (MII) eggs were collected from the oviducts of 6- to 10-week-old CD-1
female mice 12–14 h after administration of 5 IU of human chorionic gonadotropin
4 (hCG), which was administered 46–48h after the injection of 5 IU of pregnant mare
serum gonadotropin (PMSG; Sigma; Saint Louis, MO). Cumulus cells were removed
6 with 0.1% bovine testes hyaluronidase (Sigma). MII eggs were placed in KSOM with
amino acids (Millipore Sigma) under mineral oil at 37°C in a humidified atmosphere of
8 5% CO₂ until the time of monitoring. All animal procedures were performed according to
research animal protocols approved by the University of Massachusetts Institutional
10 Animal Care and Use Committee.

Preparation of cRNAs and Microinjections

The sequences encoding Camui and FRESCA were subcloned into a pcDNA6 vector
14 (pcDNA6/Myc-His B; Invitrogen, Carlsbad, CA) between the XhoI and PmeI restriction
sites. Mouse PLC ζ was a kind gift from Dr K. Fukami (Tokyo University of Pharmacy
16 and Life Science, Japan) and subcloned into a PCS2+ vector, as previously described by
us (Kurokawa et al., 2007). Plasmids were linearized with a restriction enzyme
18 downstream of the insert to be transcribed and cDNAs were *in vitro* transcribed using the
T7 or SP6 mMESSAGE mMACHINE Kit (Ambion, Austin, TX) according to the
20 promoter present in the construct. A Poly (A)-tail was added to the mRNAs using a
Tailing Kit (Ambion) and poly(A)-tailed RNAs were eluted with RNAase-free water and
22 stored in aliquots at -80 °C. Microinjections were performed as described previously (Lee
et al., 2016). cRNAs were centrifuged, and the top 1–2 μ l was used to prepare micro

drops from which glass micropipettes were loaded by aspiration. cRNA were delivered
 2 into eggs by pneumatic pressure (PLI-100 picoinjector, Harvard Apparatus, Cambridge,
 MA). Each egg received 5–10 pL, which is approximately 1–3% of the total volume of the
 4 egg. Injected MII eggs were allowed for translation up to 4h in KSOM. Group of eggs
 were injected with mouse PLC ζ after 4h of FRET construct injection.

FRET and Calcium imaging

8 To estimate relative changes in the cytoplasmic activity of Camui and/or FRESCA,
 emission ratio imaging of the Camui and FRESCA (YFP/CFP) was performed using a
 10 CFP excitation filter, dichroic beam splitter, CFP and YFP emission filters (Chroma
 technology, Rockingham, VT; ET436/20X, 89007bs, ET480/40m and ET535/30m). To
 12 measure Camui and/or FRESCA activity and $[Ca^{2+}]_i$ simultaneously, eggs that had been
 injected with Camui and/or FRESCA cRNAs were loaded ~ 4 hours post-injection with 1
 14 μ M Rhod-2AM supplemented with 0.02% pluronic acid for 20 minutes at RT. Eggs were
 then attached on glass-bottom dishes (MatTek Corp., Ashland, MA) and placed on the
 16 stage of an inverted microscope. CFP, YFP and Rhod-2 intensities were collected every
 20 second by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson,
 18 AZ). The rotation of excitation and emission filter wheels was controlled using the
 MAC5000 filter wheel/shutter control box (Ludl) and NIS-elements software (Nikon).
 20 Imaging was performed on an inverted epifluorescence microscope (Nikon Eclipse TE
 300, Analis Ghent, Belgium) using a 20x objective. For studies where ionomycin was
 22 used to induce Ca^{2+} responses, eggs were transferred into a 360 μ L Ca^{2+} -free TL-Hepes
 drop on a glass bottom dish, after which and following a brief monitoring period to

determine baseline $[Ca^{2+}]_i$ values, different concentrations of ionomycin were added and

2 Ca^{2+} responses monitored. For Sr^{2+} studies, eggs were transferred into a Ca^{2+} - free TL-Hepes, containing 10mM Sr^{2+} . In cases where $[Ca^{2+}]_i$ oscillations were induced by
4 injection of mPLC ζ cRNA, eggs were placed in TL-Hepes media containing 2 mM Ca^{2+} within 20 minutes of the injection of mPLC ζ which occurred 4 hours post-injection of the
6 FRET constructs (Camui or FRESCA).

8 **Pharmacological tests in mouse eggs**

Mouse eggs were transferred to Ca^{2+} free TL-Hepes containing desired concentrations of
10 pharmacological compounds 5 min prior to Ca^{2+} imaging. FRET (YFP/CFP) was monitored simultaneously with Ca^{2+} (rhodamine signal). First, we determined how much
12 inhibitor could be added without affecting Ca^{2+} entry. Concentrations of inhibitors were chosen based on this information as well as what was used in previous studies, see text
14 for references. All media was Ca^{2+} free with the exception of PMA. The following concentrations were used: KN93 (0.5 μ M), GO6983 (3 μ M), Bim1 (5 μ M), PMA (1 μ M),
16 AS105 (5 μ M), AS461 (5 μ M). AS105 has not been used in mouse eggs, so we adjusted the concentration to a level where the Ca^{2+} entry was not affected. Eggs with the first 4
18 compounds added were stimulated with 0.5 μ M ionomycin 5 min after monitoring started, while the eggs with AS compounds were stimulated with 2.5 μ M ionomycin.
20 Side by side controls were performed under the same conditions (0.5 μ M vs. 2.5 μ M ionomycin).

22

Data processing & statistical analyses

2 Graphs reporting FRET changes and Ca^{2+} responses were prepared using the values of
the YFP (436x535)/CFP (436x480) ratios on the left axis, whereas Rhod-2 values were
4 calculated using the following formula $(F)/F_0$ (actual value at x time/average baseline
values for the first 2 minutes of monitoring) and the scale placed on the right axis. Values
6 from three or more experiments performed on different batches of eggs are presented as
means $\pm$ s.e.m and were analyzed by the Student's t-test. Differences were considered
8 significant at $P < 0.05$.

10 Table 1. Replicates in Camui experiments

Camui	Experiment type	# of replicates	# of mice	# of total eggs
	Iono 0.5 μM	3x	2	12
	Iono 2.5 μM	4x	3	16
	Iono 5.0 μM	3x	2	12
	Sr^{2+}	2x	3	11
	PLC ζ	3x	3	16

Table 2. Replicates in FRESCA experiments

FRESCA	Experiment type	# of replicates	# of mice	# of total eggs
	Iono 0.5 μ M	4x	2	16
	Iono 2.5 μ M	4x	3	16
	Iono 5.0 μ M	3x	2	8
	Sr^{2+}	3x	3	22
	PLC ζ	2x	3	9
Added compounds				
Control	Iono 0.5 μ M	5x	2	27
	Iono 2.5 μ M	2x	2	13
	PMA	2x	2	10
	BIM1	1x	2	5
	KN93	2x	2	10
	GO6983	2x	2	9
	AS105	2x	2	10
	AS461	2x	2	15

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