Identification of EFHD1 as a novel Ca^{2+} sensor for mitoflash activation

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Abstract

Mitochondrial flashes (mitoflashes) represent stochastic and discrete mitochondrial events that each comprises a burst of superoxide production accompanied by transient depolarization and matrix alkalization in a respiratory mitochondrion. While mitochondrial Ca^{2+} is shown to be an important regulator of mitoflash activity, little is known about its specific mechanism of action. Here we sought to determine possible molecular players that mediate the Ca^{2+} regulation of mitoflashes by screening mitochondrial proteins containing the Ca^{2+} -binding motifs. *In silico* analysis and targeted siRNA screening identified four mitoflash activators (MICU1, EFHD1, SLC25A23, SLC25A25) and one mitoflash inhibitor (LETM1) in terms of their ability to modulate mitoflash response to hyperosmotic stress. In particular, overexpression or down-regulation of EFHD1 enhanced or depressed mitoflash activation, respectively, under various conditions of mitochondrial Ca^{2+} elevations. Yet, it did not alter mitochondrial Ca^{2+} handling, mitochondrial respiration, or ROS-induced mitoflash production. Further, disruption of the two EF-hand motifs of EFHD1 abolished its potentiating effect on the mitoflash responses. These results indicate that EFHD1 functions as a novel mitochondrial Ca^{2+} sensor underlying Ca^{2+} -dependent activation of mitoflashes.

Abbreviations: mPTP, mitochondrial permeability transition pore; fMPT, mitoflash-related mitochondrial permeability transition; ROS, reactive oxygen species; cpYFP, circularly permuted yellow fluorescent protein; RNAi, RNA interference.

Keywords: Mitochondria; Mitochondrial flash (mitoflash); Ca^{2+} -binding proteins; Mitochondrial Ca^{2+} signaling; EFHD1

1. Introduction

Mitochondria are not only the cellular powerhouse, but also the hub of intracellular signaling by modulating and integrating diverse signal transduction pathways such as those mediated by intracellular Ca^{2+} . Often coupled to Ca^{2+} release from the endoplasmic reticulum, mitochondria can rapidly take up a large quantity of Ca^{2+} through the mitochondrial Ca^{2+} uniporter, resulting in transient and/or sustained elevations of mitochondrial Ca^{2+} . This mitochondrial Ca^{2+} signalling mechanism modifies the spatiotemporal dynamics of cytosolic Ca^{2+} signalling and, more importantly, regulates multifaceted mitochondrial activities, including activation of several key enzymes in mitochondrial metabolism, opening of mitochondrial permeability transition pores (mPTPs), and production of reactive oxygen species (ROS) and even mitochondrial fission - fusion dynamics [1].

Recently, we and others have demonstrated a novel type of dynamic mitochondrial activity - mitochondrial flashes (mitoflashes) - which represent stochastic and quantal signaling events occurring under physiological conditions. Individual mitoflashes consist of bursting superoxide production [2-4], transient matrix alkalization [5-7], and oscillation of the inner membrane potential ($\Delta\Psi_m$) at the single-mitochondrion level [2, 7, 8]. They are widely found in different types of cells and tissues across species from worms to humans, indicating that mitoflashes are highly conserved activity of the organelle [2, 3, 9-16]. As expected for their roles in signaling, the rate of mitoflash occurrence is richly modulated by factors including metabolic state, oxidative stress, developmental stage, and aging [3, 9, 11-20]. Interestingly, mitoflash activity is intimately integrated with mitochondrial functions and other core mitochondrial signals. For instance, mitoflash activity is activated by mitochondrial Ca^{2+} elevations and there is a positive correlation between mitoflash frequency and the steady mitochondrial Ca^{2+} concentrations in HeLa cells [13, 21]. Moreover, there is a synergism between Ca^{2+} and ROS signals, such that an otherwise subthreshold mitochondrial Ca^{2+} can stimulate vigorous mitoflash activity in the coexistence of mild increase of mitochondrial basal ROS [13]. In cardiac myocytes, the mitoflash frequency is significantly increased after sustained electric stimulation [22], although acute alterations of intracellular

Ca^{2+} transients and Ca^{2+} sparks appear to be ineffective [2]. However, it remains unclear as to the underlying Ca^{2+} -sensing proteins and their mechanisms of actions in mediating the Ca^{2+} -dependent regulation of mitoflashes.

In the present study, we aimed to identify the key proteins mediating the Ca^{2+} -dependent activation of mitoflashes by screening the EF-hand or C2 motif-containing proteins in MitoCarta. Five proteins from fourteen candidates were shown to participate in regulating Ca^{2+} -dependent activation of mitoflashes via different mechanisms. Particularly, EFHD1 activates mitoflashes without altering mitochondrial Ca^{2+} and mitochondrial functions tested and its EF-hand domains are required for this effect, indicating that EFHD1 acts as a novel mitochondrial Ca^{2+} sensor for mitoflash activation.

2. Methods

2.1 Reagents

D-glucose, histamine, ionomycin, H₂O₂ and menadione were from Sigma-Aldrich. Rhod-2 AM, Fluo-4 AM and MitoSOX were from Molecular Probes (Eugene, OR). Lipofectamine RNAiMax was from Invitrogen. Anti- β -tubulin antibody was from Sigma-Aldrich. Anti-SOD1 and anti-SOD2 antibody were from Abcam. The anti-EFHD1 or anti-MICU1 polyclonal antibodies were generated by immunizing rabbits with human EFHD1 or MICU1 peptides which were expressed and purified from *E.coli*.

2.2 *In silico* analysis of Ca²⁺ binding proteins

The Entrez IDs of human mitochondrial proteins were obtained from MitoCarta database (MitoCarta 1.0) [23] and the corresponding protein sequences were retrieved from UniProt/Swiss-Port database [24]. The motif features of EF-hand (Accession: PS00018 and PS50222) and C2 (Accession: PS50004) annotated by ScanProsite [25] were used as the query to search for Ca²⁺ binding proteins.

2.3 Cell culture and RNA interference assay

Stable expression of mitochondria-targeted cpYFP (mt-cpYFP) in HeLa cells was established as previously described [10]. The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin and streptomycin (Invitrogen) at 37 °C under 5% CO₂. The double-stranded RNA sequences for knockdown of target genes and their negative control are shown in Table S2. For transfection, 100 nM siRNA was transiently transfected into the mt-cpYFP-expressing HeLa cells with Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. Knockdown efficiency was tested by Western-blot or realtime PCR (RT-PCR). The primers used for RT-PCR were: GACCTCTACCTCTGTGGGTTT (forward) and CTCTCCTCCTTGAGTGACTGA (reverse) (LETM1); AACAGGGTATCTCCTCTGAG (forward) and CTGGTTCCGGTCAAGACT (reverse) (SLC25A23); TGGAAGCATTCACGATCT (forward) and TGAACATCCGGGACCGTTA (reverse) (SLC25A25).

2.4 Mitochondria isolation from HeLa cells

Mitochondria were isolated from 2×10^7 HeLa cells using the Mitochondria Isolation Kit (ThermoFisher) according to manufacturer's guidelines. The Dounce homogenizer (Sigma-Aldrich) was used to lyse the cells. The total cell lysate, mitochondrial pellet lysate and the corresponding cytosolic fractions were used for Western-blot.

2.5 Ca^{2+} measurement

For mitochondrial Ca^{2+} measurement, HeLa cells were incubated with Rhod-2 AM (5 μM) in 37°C for 30 min or transfected with mitochondrial targeted GCaMP5 (mt-GCaMP5) plasmid. For cytosolic Ca^{2+} measurement, HeLa cells were incubated with Fluo-4 AM (5 μM) in 37°C for 10 min. After washing with Tyrode's solution consisting of 137 mM NaCl, 5.4 mM KCl, 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 1.8 mM CaCl_2 , 10 mM glucose, and 20 mM HEPES (pH 7.35, adjusted with NaOH), the fluorescence of Rhod-2, Fluo-4, or GCaMP5 was measured with a Zeiss LSM 710 confocal microscope.

2.6 Mitochondrial basal ROS measurement

HeLa cells were incubated with mitoSOX (5 μM) in 37°C for 20 min and then washed with Tyrode's solution. MitoSOX fluorescence was measured with a Zeiss LSM 710 confocal microscope.

2.7 Measurement of mitochondrial respiration

Real-time monitoring of mitochondrial respiration was performed using XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MD), according to the manufacturer's instructions. Cellular oxygen consumption rate (OCR) was monitored in basal condition or after addition of oligomycin (1 μM), FCCP (1 μM) and rotenone (1 μM) / antimycin A (AA, 1 μM) sequentially, all dissolved in unbuffered assay media (Sigma D5030) supplemented with 2 mM GlutaMAX (Gibco), 2.5 mM sodium pyruvate and 25 mM glucose (pH 7.4 at 37°C). After each drug addition, the OCR was measured three times. The ATP coupled OCR, maximal OCR and proton leak were achieved by calculating the percentage of oligomycin sensitive part to basal OCR, the difference between FCCP

accelerated OCR and rotenone / AA to basal OCR, and the oligomycin insensitive part to basal OCR, respectively.

2.8 Control of cytosolic Ca^{2+} in permeabilized cells

HeLa cells were washed in Ca^{2+} -free Tyrode's solution consisting of (in mM) 137 NaCl, 5.4 KCl, 1.2 MgCl_2 , 1.2 NaH_2PO_4 , 10 glucose, and 20 HEPES (pH 7.35, adjusted with NaOH) for 3 min and then permeabilized in the internal solution containing (in mM) 100 potassium aspartate, 20 KCl, 0.8 MgCl_2 , 3 MgATP, 0.5 EGTA, 10 phosphocreatine, 5 U/ml creatine phosphokinase, 10 glutathione, 20 HEPES, 8% dextran (MW 35,000-45,000), and 50 $\mu\text{g/ml}$ saponin (pH 7.2) for 90 sec [26]. After permeabilization, cells were incubated in an intracellular solution containing (in mM) 100 potassium aspartate, 1.0 MgCl_2 , 20 KCl, 0.5 EGTA, 0, 0.16, 0.35, 0.40, 0.41 CaCl_2 (to reach free Ca^{2+} concentration of 0, 100, 500, and 800 nM and 1 μM), 10 glutathione, 20 HEPES, 8% dextran (MW 35,000-45,000), 10 KH_2PO_4 and 5 succinate (pH 7.2).

2.9 Confocal imaging

Mitoflashes were detected in intact or permeabilized HeLa Cells using an inverted confocal microscope (Zeiss LSM 710) with a 40 $\times$, 1.3 NA oil-immersion objective. The biosensor mt-cpYFP was used to report mitoflash biosensor with its dual sensitivity for both ROS and pH. When acquiring the mt-cpYFP signal, the images were acquired by exciting alternately at 488 and 405 nm and collecting the emission at > 505 nm. For Fluo-4 fluorescence, the images were acquired by exciting at 488 nm and collecting the emission at 505-530 nm. For Rhod-2 fluorescence, the images were acquired by exciting at 543 nm and collecting the emission at >560 nm. For GCaMP5 fluorescence, the images were acquired by exciting at 488 nm and collecting the emission at 505-530 nm. For mitoSOX fluorescence, the images were acquired by exciting at 514 nm and collecting the emission at 580–740 nm. For mitoflash detection, time-lapse two-dimensional (xy) images were captured and usually 100 frames of 512 $\times$ 512 (xy) pixels were collected at 1 s/frame in bidirectional scanning mode in a given region. The low laser illumination (<10% at 488 nm, 2% at 405 nm) is negligible for photobleaching during imaging. The axial resolution was set to 1.0 μm , and the size of the

imaging region was $70.97 \times 70.97 \mu\text{m}$. The cells were randomly collected from 10-20 regions of a certain dish within 30 minutes unless otherwise stated. For Ca^{2+} imaging, time-lapse two-dimensional (xy) images were captured and usually 300-450 frames of 512×512 (xy) pixels were collected at 2 s/frame in bidirectional scanning mode in a given region. All experiments were performed at room temperature (22-26 °C) unless specified otherwise.

Time-lapse confocal images were analyzed using custom-developed programs written in interactive data language (IDL Research Systems). Cell-motion artefacts and background fluorescence changes were corrected by image processing and individual mitoflashes were located with the aid of FlashSniper [27].

2.10 Statistics

The data are expressed as mean $\pm$ SEM, and Student's t test was applied to determine statistical significance. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Identification of mitoflash regulators among mitochondrial Ca^{2+} -binding proteins

To identify possible protein regulators underlying the Ca^{2+} -dependent mitoflash activation, we first performed *in silico* screening of 1013 human proteins in MitoCarta [23] using the ScanProsite software. A short list of 14 candidates was generated based on two screening criteria, the presence of a Ca^{2+} -binding motif (EF-hand or C2 domain) and localization in the mitochondrial inner membrane or matrix (Fig. 1A, Table S1). Next, we implemented experimental screening of these candidates by taking advantage of our recent finding that hyperosmotic stress (270mM glucose in Tyrode's solution) stimulates vigorous mitoflash activity through synergistic effect of elevated mitochondrial Ca^{2+} and basal ROS [13]. Specifically, hyperosmotic stress induced by either 270 mM glucose or 270 mM metabolically inactive mannitol in Tyrode's solution increases mitoflash frequency by 10~20 folds, with no significant changes of mitochondrial network morphology [13]. The hyperactivity of mitoflashes develops within the first a few minutes of hyperosmotic challenge and persists stably during the observation period (about 30 minutes), and is reversible upon switching off the hyperosmotic challenge [13]. In HeLa cells stably expressing mt-cpYFP [10], which measures both superoxide flash and pH flash at once and is therefore a robust mitoflash biosensor [2, 7], each candidate gene was targeted with two different siRNAs, and alteration in mitoflash activity responding to the hyperosmotic stimulation was assessed. Of the 14 candidates, we identified five mitoflash regulators in terms of their ability to significantly modify the hyperosmotic stress-induced mitoflash response. In particular, MICU1, SLC25A23, SLC25A25, EFHD1 act as mitoflash activators, knockdown of which decreased mitoflash frequency by 43-62% in hyperosmotic stress; and LETM1 as a mitoflash inhibitor as its knockdown increased mitoflashes by 91% (Figs. 1B, S1). The other nine mitochondrial proteins, however, did not apparently participate in the regulation of mitoflash activity in the present experimental conditions (Fig. 1B).

Interestingly, previous studies have shown that three of the five mitoflash regulators are

directly involved in mitochondrial Ca^{2+} regulation. Specifically, MICU1 has been shown to be an important regulator of the mitochondrial Ca^{2+} uniporter through interacting with its pore-forming subunit MCU [28-30], and its knockdown attenuates mitochondrial Ca^{2+} elevations induced by either hyperosmotic stress or histamine receptor stimulation [13]. LETM1 has been suggested to modulate mitochondrial Ca^{2+} , though the underlying mechanism is controversial [31, 32]. SLC25A23 has also been suggested to regulate mitochondrial Ca^{2+} uptake [33]. In addition, SLC25A25 exerts an influence on mitochondrial respiration and ATP content [34]. Therefore, their effects on mitoflash activity could be a secondary manifestation of altered mitochondrial Ca^{2+} responses or mitochondrial functions. In contrast, little is known about the role of EFHD1 in mitochondrial Ca^{2+} handling.

To dissect possible mechanisms of these mitoflash regulators, we examined their effects on mitochondrial and cytosolic Ca^{2+} transients induced by hyperosmotic stress. We found that MICU1 or SLC25A23 knockdown decreased the mitochondrial Ca^{2+} transient amplitude and LETM1 knockdown showed the opposite effect (Fig. 1C-D), consistent with previous reports [31, 33, 35]. However, cytosolic Ca^{2+} transient amplitude was unaltered after knocking down each of them (Fig. S2A-B). Similar effects were also found in histamine receptor-stimulated Ca^{2+} transient responses (Fig. 1E-F). These results indicate that potentiation or depression of mitoflash activity by these three mitochondrial Ca^{2+} proteins is mediated, at least in part, by enhancing or decreasing mitochondrial Ca^{2+} elevation. Moreover, only a trend of decreasing mitochondrial Ca^{2+} elevation was found with SLC25A25 knockdown (Fig. 1C-F), suggesting that its mitoflash regulatory effect is unlikely accounted for by altered mitochondrial Ca^{2+} (see Discussion). Interestingly, EFHD1 knockdown exerted no significant change in the mitochondrial and cytosolic Ca^{2+} transients (Figs. 1C-F, Fig. S2A-B), suggesting that EFHD1 regulates mitoflash activity by a mechanism other than altering mitochondrial Ca^{2+} . These results indicate that the five mitochondrial Ca^{2+} binding proteins identified above modulate mitoflash activity through multiple mechanisms.

3.2 EFHD1 regulates mitoflashes independently of mitochondrial Ca^{2+} , ROS and

respiratory functions

Next, we sought to further investigate possible regulatory mechanisms of the mitoflash activator EFHD1. In principle, a mitochondrial Ca^{2+} binding protein can alter mitoflash activity by directly modulating mitochondrial Ca^{2+} handling (see above) or impacting other processes that are related to mitoflash generation, such as ROS production and respiratory function. Alternatively, it can also act as a Ca^{2+} sensor to mediate Ca^{2+} -dependent mitoflash response without affecting other mitochondrial functions. To discriminate among these possibilities, we first examined the effects of bidirectional manipulations of EFHD1 on hyperosmotic stress-induced mitochondrial Ca^{2+} elevations. We found that neither EFHD1 overexpression nor its knockdown with two different siRNAs (Fig. S1A and S3) exerted any significant changes on the amplitude of hyperosmotic stress induced mitochondrial Ca^{2+} transients measured with either Rhod-2 or mitochondrial targeted GCaMP5 (Figs. 2A and S4). Concomitantly, mitoflash frequency response was decreased by 39% with EFHD1 knockdown and increased by 29% with EFHD1 overexpression (Fig. 2B), while the unitary properties of mitoflashes remained unaltered (Fig. 2C). Together with the results reported above (Fig. 1B-F), this finding substantiates the notion that regulation of mitoflashes by EFHD1 is not mediated by altering mitochondrial Ca^{2+} elevations.

We next determined whether EFHD1 regulates mitoflashes through modulating mitochondrial production of ROS, which are also known as mitoflash activators [13, 36]. We measured mitochondrial basal ROS with mitoSOX and demonstrated that the ROS level was kept constant after bidirectional manipulations of EFHD1 (Fig. 2D-E), indicating that EFHD1 is dispensable for mitochondrial ROS metabolism. Further, we tested whether EFHD1 alters the sensitivity of mitoflashes to ROS stimulation. Robust mitoflash enhancements were elicited by menadione (200 μM) treatment or exogenous H_2O_2 (300 μM) (Fig. 2F). Importantly, nearly identical mitoflash responses to both menadione and H_2O_2 treatments were detected in either EFHD1 knockdown or overexpression cells (Fig. 2F). Thus, EFHD1 alters neither mitochondrial ROS production nor ROS-dependent mitoflash generation.

It has been well-established that the genesis of mitoflashes is intimately linked to

mitochondrial respiration and energy states [2, 9, 11]. Since multiple metabolic enzymes in mitochondria are sensitive to Ca^{2+} regulation as well [37], it is possible that EFHD1 enhances mitoflashes through augmenting mitochondrial respiratory functions. We therefore measured key parameters of mitochondrial respiration, including mitochondria membrane potential ($\Delta\Psi_m$) and oxygen consumption rates. TMRM measurement of $\Delta\Psi_m$ revealed no significant changes with either siRNA knockdown or overexpression of EFHD1 (Fig. 3A-B). Further, the ATP synthesis-coupled, maximal, and proton leak-associated oxygen consumption rates were all nearly identical among EFHD1 overexpression, EFHD1 knockdown and control cells (Fig. 3C-D). These data indicate that EFHD1 mediates mitoflash activation independently of mitochondrial respiration. Collectively, data thus far show that EFHD1 selectively regulates Ca^{2+} but not ROS-induced mitoflash activation, without affecting mitochondrial Ca^{2+} elevations, mitochondrial basal ROS production, and mitochondrial respiration.

3.3 EFHD1 acts as a mitochondrial Ca^{2+} sensor for mitoflash activation

The results above suggest the possibility that EFHD1 activates mitoflashes by acting as a mitochondrial Ca^{2+} sensor. To validate this hypothesis, we examined the effects of EFHD1 manipulation on mitoflash responses to different types of mitochondrial Ca^{2+} elevations over a broad dynamic range. In the presence of varying extracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_{\text{ex}}$), treatment of cells with the Ca^{2+} ionophore ionomycin (1 μM) elicited a mitochondrial Ca^{2+} transient of a stereotypical amplitude, followed by a steady-state elevation which varied in a $[\text{Ca}^{2+}]_{\text{ex}}$ -dependent manner (Fig. 4A-B). Concomitantly, ionomycin increased mitoflash frequency and, in control cells, the maximal mitoflash response was attained at 5-10 mM $[\text{Ca}^{2+}]_{\text{ex}}$ (Fig. 4C). Strikingly, EFHD1 knockdown decreased mitoflash frequency at all $[\text{Ca}^{2+}]_{\text{ex}}$ tested, and halved the maximal mitoflash response (Fig. 4C). In EFHD1-overexpressing cells, ionomycin-induced mitoflash activity was enhanced by 1.7-folds at 1.8 mM $[\text{Ca}^{2+}]_{\text{ex}}$, while the maximal response was unaltered (Fig. 4C). Consistently, in saponin-permeabilized cells in which cytosolic Ca^{2+} concentrations were under experimental control, EFHD1 overexpression augmented mitoflash frequency at

cytosolic Ca^{2+} concentrations from 100 nM to 800 nM without altering the maximal mitoflash response with Ca^{2+} at 1 μM Ca^{2+} . However, EFHD1 downregulation markedly decreased mitoflash frequency at cytosolic Ca^{2+} concentrations of 800 nM or 1 μM (Fig. 4D). These results indicate that EFHD1 sensitizes mitoflash response to mitochondrial Ca^{2+} elevations, while it appears to be dispensable for basal mitoflash activity.

To verify that the Ca^{2+} sensor role of EFHD1 in the regulation of mitoflashes, we generated a EFHD1 mutant (mtEFHD1) with both of its two EF-hand domains disrupted (EF1: D104A/E115K and EF2: D140A/E151K) (Fig. 5A). Interestingly, mtEFHD1, at a level similar to the overexpression of its wild-type form (Fig. 5B), completely lost the ability to enhance mitoflash activity stimulated by either hyperosmotic stress or ionomycin treatment (Fig. 5C-D). This result indicates that the EF-hand domains are necessary for EFHD1 to activate Ca^{2+} -dependent mitoflashes and thus substantiates the idea on EFHD1 as a mitochondrial Ca^{2+} sensor for Ca^{2+} -dependent mitoflash activation.

4. Discussion

Mitoflashes are increasingly recognized as a universal and fundamental mitochondrial activity. It is important to determine whether the mitoflash activity interacts and is integrated with other core mitochondrial signals and functions, and if so, the underlying molecular mechanisms. In this scenario, major findings of this study are twofold. First, we have identified five mitochondrial Ca^{2+} binding proteins, MICU1, LETM1, EFHD1, SLC25A23 and SLC25A25, as mitoflash regulators according to their ability to potentiate or depress the mitoflash response to hyperosmotic stress. Second, we have shown that EFHD1, one of the five newly identified mitoflash regulators, acts as a Ca^{2+} sensor to enhance mitoflash responses under various conditions of mitochondrial Ca^{2+} elevations, without altering mitochondrial Ca^{2+} handling and other mitochondrial functions.

Of the five Ca^{2+} -binding protein regulators of mitoflashes, MICU1 has been shown to interact with the pore-forming subunit MCU of the mitochondrial Ca^{2+} uniporter and thereby potentiates mitochondrial Ca^{2+} uptake [28, 35, 38, 39]. Our present and previous results [13,

21] support this notion and suggest that its mitoflash-regulatory effect is mediated, at least in part, through directly modulating mitochondrial Ca^{2+} elevations. Likewise, LETM1 has been suggested to alter mitochondrial Ca^{2+} handling, although it remains controversial whether it acts as a K^+/H^+ exchanger to impact mitochondrial Ca^{2+} indirectly [40-43] or as a $\text{H}^+/\text{Ca}^{2+}$ exchanger [31, 44, 45]. That downregulation of LETM1 significantly elevates mitochondrial Ca^{2+} supports a role of LETM1 in modulating mitochondrial Ca^{2+} handling and provides a possible mechanistic basis for the concomitantly enhanced mitoflash activity. Furthermore, SLC25A23 and SLC25A25 are reported to function as Ca^{2+} -sensitive $\text{ATP-Mg}^{2+}/\text{P}_i$ carriers [46, 47], and SLC25A23 has recently been shown to positively regulate mitochondrial Ca^{2+} uptake through interactions with MCU and MICU1 [33]. The latter is consistent with our finding that knockdown of SLC25A23 decreased mitochondrial Ca^{2+} response to both hyperosmotic stress and histamine receptor stimulation. While such decreased mitochondrial Ca^{2+} would partly account for the alteration in the mitoflash response, SLC25A23 has also been shown to promote mitochondrial superoxide production [33], and it is possible that both declines in mitochondrial Ca^{2+} and ROS after SLC25A23 downregulation underlie the reduction of mitoflash activity during our screening test. Consistent with previous report [33], we found only a trend of decreased mitochondrial Ca^{2+} response in SLC25A25 knockdown cells, which may be too small to fully explain the altered mitoflash response. To this end, it has been shown that SLC25A25 knockout in MEF cells decreases basal mitochondrial respiration and ATP content [34]. Since mitoflashes are closely interlinked with mitochondrial metabolism [3, 9, 11, 18], the SLC25A25 effect on mitoflashes might have arisen mainly from compound alterations in mitochondrial respiration and energy production.

For EFHD1, it has been first described as mitocalcin with Ca^{2+} binding property but its functionality is poorly understood thus far, except for a few limited studies. It is implicated in the regulation of neuronal differentiation through altering mitochondrial function and, depending on cell type and environmental context, in the control of cell survival or differentiation [48, 49]. In this study, we have provided several lines of evidence that EFHD1 acts as a novel Ca^{2+} sensor in mediating Ca^{2+} -dependent mitoflash activation. Of particular

interest, EFHD1 specifically and positively regulates mitoflashes induced by mitochondrial Ca^{2+} , but not those by mitochondrial ROS, nor does it alter mitochondrial Ca^{2+} handling, ROS production, and mitochondrial respiration. This potentiation effect is demonstrable regardless of the means used to elevate mitochondrial Ca^{2+} (hyperosmotic stress, ionomycin treatment, and high bathing Ca^{2+} in permeabilized cells). A direct binding of Ca^{2+} with EFHD1, as demonstrated previously by *in vitro* assay [50], at its two EF-hand domains is perhaps a prerequisite of EFHD1 action for its mutant with Ca^{2+} -binding disruptive EF-hands is unable to support its mitoflash-activator role. It is noteworthy that the maximal mitoflash activity at high mitochondrial Ca^{2+} was halved by knockdown of EFHD1 but was unaltered with EFHD1 overexpression. A simple possible explanation of this observation is that endogenous EFHD1, when fully bound to Ca^{2+} , might be sufficient to support a full EFHD1-mediated mitoflash stimulation. This interpretation is consistent with the sensitization of mitoflash activation at submaximal Ca^{2+} concentrations by EFHD1 overexpression. Since transient opening of mitochondrial permeability transition pore (mPTP) is thought to trigger mitoflashes [2] and Ca^{2+} at high concentrations constitutes a robust mPTP activator [51-53], it is tempting to speculate that EFHD1 might directly interact with mPTP or its function analog for mitoflash-related mitochondrial permeability transition (fMPT) [8]. Further studies are required to determine the precise mechanisms of EFHD1 in sensitizing Ca^{2+} -dependent mitoflash activation.

With *in silico* approaches and RNAi screening, we have identified five mitoflash regulators which mediate Ca^{2+} -dependent mitoflash regulation via different mechanisms. In particular, EFHD1 acts as a novel mitochondrial Ca^{2+} sensor without altering mitochondrial Ca^{2+} , ROS production and respiration. Our findings not only reveal key molecular players in regulating Ca^{2+} -dependent mitoflashes, but also identify a novel functional role for EFHD1 in mitochondrial Ca^{2+} and mitoflash signaling. By exploiting EFHD1, future study may devise new means for the investigation of roles of mitoflashes in physiology and pathophysiology.

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Disclosures

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Figure legends

Fig. 1. Screening for Ca^{2+} -dependent mitoflash regulators. (A) Flowchart of our screening protocol. Numbers at each step represent the quantity of candidates identified. (B) Alterations of mitoflash response to hyperosmotic stress after knockdown of the candidate gene expression. The data shown are the combined results of two siRNAs and are normalized by those in scramble siRNA-transfected negative control group (NC, mitoflash frequency was 4.64 ± 0.17 events/100s/cell, $n=314$ cells). The dashed line marks the level of 1.0. Data are expressed as mean $\pm$ SEM, $n=3-14$ independent experiments collected from 57-314 cells, *: $p<0.05$; **: $p<0.01$ versus NC. (C) Mitochondrial Ca^{2+} transients stimulated by hyperosmotic stress (270 mM glucose in Tyrode's solution). The five mitoflash regulatory genes shown in B were down-regulated by RNAi. F_0 : basal Rhod-2 fluorescence; F/F_0 : normalized Rhod-2 fluorescence signal. (D) Analysis of the amplitude of mitochondrial Ca^{2+} transients in (C). Data are expressed as mean $\pm$ SEM, $n=5-7$ independent experiments, 30-50 cells per experiment. *: $p<0.05$; **: $p<0.01$ versus NC. (E) Mitochondrial Ca^{2+} transients stimulated by histamine (100 μM). (F) Analysis of the amplitude of mitochondrial Ca^{2+} transients in (E). Data are expressed as mean $\pm$ SEM, $n=3-10$ independent experiments, 115-200 cells per experiment. *: $p<0.05$; **: $p<0.01$ versus NC.

Fig. 2. EFHD1 regulates mitoflashes independently of mitochondrial Ca^{2+} handling and ROS production. (A) EFHD1 knockdown by siRNA or overexpression (OE) did not alter mitochondrial Ca^{2+} transients induced by hyperosmotic stress. NC: negative control. Data are expressed as mean $\pm$ SEM, $n=80-110$ cells from 3 independent experiments per group. F_0 : basal Rhod-2 fluorescence; F/F_0 : normalized fluorescence signal. NS: no significance versus Rhod-2 F/F_0 of NC at the 2 minutes after high glucose adding. (B) Bidirectional regulation of mitoflashes with EFHD1 knockdown and overexpression (OE). Data are expressed as mean $\pm$ SEM, $n=19-110$ cells from 3-4 independent experiments per group, **: $p<0.01$ versus NC in high glucose. (C) Time courses of mitoflashes in NC, EFHD1-knockdown and overexpression cells. Data are expressed as mean $\pm$ SEM, $n=34-47$ events from 3-4 independent experiments per group. (D) Representative images of mitochondrial basal ROS visualized by mitoSOX.

Scale bars: 20 μm . (E) Statistics of D. a.u., arbitrary units. Data are expressed as mean $\pm$ SEM, n=15-150 cells from 3 independent experiments per group, NS: no significance versus NC. (F) Effects of EFHD1 manipulations on ROS-induced mitoflashes. Mitochondrial ROS were elevated by either 200 μM menadione or 300 μM H_2O_2 treatments. Data are expressed as mean $\pm$ SEM, n=24-125 cells from 3 independent experiments per group, NS: no significance versus NC.

Fig. 3. EFHD1 is dispensable for mitochondrial respiration. (A) Representative images of mitochondrial membrane potential measured with TMRM in HeLa cells with EFHD1 knockdown or overexpression (OE). NC, negative control. Scale bars: 20 μm . (B) Statistics of A. Data are expressed as mean $\pm$ SEM, n=3-5 independent experiments per group, 55-95 cells per experiment. NS: no significance versus NC. (C) Representative traces of measurement of oxygen consumption rate (OCR) in intact HeLa cells. The OCR was normalized to basal conditions. Arrowheads indicate the time points of adding oligomycin (Oligo, 1 μM), FCCP (1 μM), and rotenone (Rot, 1 μM) and antimycin (AA, 1 μM). Data are expressed as mean $\pm$ SEM, n=6-9 measurements from 3 independent experiments per group. (D) Statistics on ATP coupled, maximal, and proton leak coupled OCR in each group. Data are expressed as mean $\pm$ SEM, n=6-9 measurements from 3 independent experiments per group.

Fig. 4. EFHD1 acts as a mitochondrial Ca^{2+} sensor mediating Ca^{2+} -dependent mitoflash activation. (A-B) Ca^{2+} transients (A) and steady state Ca^{2+} elevations (B) induced by ionomycin treatments with 1.8, 5 or 10 mM extracellular Ca^{2+} in control HeLa cells. F_0 : basal Rhod-2 fluorescence; F/F_0 : normalized fluorescence signal. Data are expressed as mean $\pm$ SEM, n=70-97 cells from 3 independent experiments per group. **: $p<0.01$ versus basal Rhod-2 F/F_0 . #: $p<0.05$, and ##: $p<0.01$ versus steady-state Rhod-2 F/F_0 in 1.8mM extracellular Ca^{2+} Tyrode's solution. (C) Effects of EFHD1 knockdown and overexpression on mitoflashes induced by ionomycin stimulated Ca^{2+} elevations. 1 μM ionomycin was used in the presence of 1.8, 5 or 10 mM extracellular Ca^{2+} containing Tyrode's solution ($[\text{Ca}^{2+}]_{\text{ex}}$). Data are expressed as mean $\pm$ SEM, n=15-54 cells from 3 independent experiments per group. *: $p<0.05$, **: $p<0.01$ versus NC cells in corresponding extracellular Ca^{2+} conditions. (D)

Effects of EFHD1 knockdown or overexpression on mitoflash frequency in permeabilized HeLa cells at increasing bathing Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyto}}$). Data are expressed as mean $\pm$ SEM, n=24-65 cells from 3 independent experiments per group. **: $p < 0.01$ versus control cells in the corresponding $[\text{Ca}^{2+}]_{\text{cyto}}$.

Fig. 5. The EF-hand domains are required for EFHD1 to sensitize Ca^{2+} -dependent activation of mitoflashes. (A) Diagram for mutating EFHD1 in EF-hand domains to disable its Ca^{2+} binding property. Arrows indicate the point mutation sites: EF1 (D104A, E115K) and EF2 (D140A, E151K). (B) Expression of wildtype and mutant EFHD1 (mtEFHD1). Up panel: representative Western-blot. Tubulin was used as the internal control. Low panel: statistics for EFHD1 expression. Data are expressed as mean $\pm$ SEM, n=4 independent experiments. NS: no significance versus control. (C, D) mtEFHD1 was unable to potentiate mitoflash response to either hyperosmotic stress (C) or ionomycin-induced mitochondrial Ca^{2+} elevations (D). For ionomycin treatment, 1.8 mM extracellular Ca^{2+} was used. Data are expressed as mean $\pm$ SEM, n=35-102 cells from 3 independent experiments per group. **: $p < 0.01$ versus control. NS: no significance versus control.

Supplementary figure legends:

Fig. S1. Analysis of knockdown efficiency of EFHD1, MICU1, LETM1, SLC25A23, and SLC25A25. For each gene, two different siRNAs were used. (A, B) Western-blot analysis for knockdown efficiency of EFHD1 (A) or MICU1 (B). The inset in each panel shows a representative Western-blot. n=4 experiments for EFHD1 and n=3 experiments for MICU1. (C) Real-time PCR analysis for knockdown efficiency of LETM1, SLC25A23 and SLC25A25. n=4-7 experiments. *: p<0.05; **: p<0.01 versus scramble siRNA-transfected negative control (NC) group.

Fig. S2. Cytosolic Ca^{2+} response induced by hyperosmotic stress. (A) Cytosolic Ca^{2+} transients stimulated by high glucose (270 mM glucose in Tyrode's solution). F_0 : basal Fluo-4 fluorescence; F/F_0 : normalized Fluo-4 fluorescence signal. (B) Analysis of the amplitude of cytosolic Ca^{2+} transients. Data are expressed as mean $\pm$ SEM, n=3-4 independent experiments, 30-50 cells per experiment.

Fig. S3. Western-blot analysis for overexpression and localization of EFHD1. Mitochondria were isolated from control or EFHD1 overexpressed HeLa cells. Then the lysates of total cells (Total), isolated mitochondria (Mito), or the corresponding cytosolic fractions (Cyto) were used for Western-blot analysis. SOD2 and SOD1 were used as mitochondrial and cytosolic internal controls, respectively. Con: control, OE: EFHD1 overexpression. Mito: mitochondria, Cyto: cytosol. Note that EFHD1 is highly enriched to mitochondria.

Fig. S4. Mitochondrial Ca^{2+} response measured by GCaMP5 upon hyperosmotic stress. (A) Mitochondrial Ca^{2+} transients induced by high glucose (270 mM in Tyrode's solution) measured by mitochondrial targeted GCaMP5 (mt-GCaMP5) in EFHD1 knockdown or overexpression (OE) HeLa cells. (B) Analysis of mt-GCaMP5 signal at the 8 minutes after high glucose adding. NC: negative control. Data are expressed as mean $\pm$ SEM, n=28-62 cells from 3 independent experiments per group. F_0 : basal GCaMP5 fluorescence; F/F_0 : normalized fluorescence signal. NS: no significance versus NC group.

Table S 1. Ca²⁺-binding motif containing mitochondrial proteins used for target screening

Gene ID	Gene Symbol	Gene Name
3954	LETM1	leucine zipper-EF-hand containing transmembrane protein 1
55288	RHOT1	ras homolog gene family member T1
89941	RHOT2	ras homolog gene family member T2
8604	SLC25A12	solute carrier family 25 (aspartate/glutamate carrier), member 12
10165	SLC25A13	solute carrier family 25 (aspartate/glutamate carrier), member 13
79085	SLC25A23	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23
29957	SLC25A24	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24
114789	SLC25A25	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25
221154	EFHA1 (MICU2)	EF-hand domain family member A1 (also known as mitochondrial calcium uptake 2)
80303	EFHD1	EF-hand domain family member D1
3313	HSPA9	heat shock 70kDa protein 9 (mortalin)
2820	GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
10367	MICU1	mitochondrial calcium uptake 1
26056	RAB11FIP5	RAB11 family interacting protein 5 (class I)

Table S 2. siRNA sequences of target RNAi screening

siRNA name	Sense	Anti-sense
Negative control (NC)	UUCUCCGAACGUGUCACG UdTdT	ACGUGACACGUUCGGAGAA dTdT
LETM1-1	UCCACAUUUGAGACUCAG UdTdT	ACUGAGUCUCAA AUGUGGAdTdT
LETM1-2	AUGUCCAUUUGGCUGCUG dTdT	CAGCAGCCAAAUGGAACAU dTdT
RHOT1-1	GCAGGCUGCCUUUAAU AUU dTdT	AAUAUAAAAGGCAGCCUGC dTdT
RHOT1-2	CCUGGAAU AUUUGGGCUAU dTdT	AUAGCCCCAAU AUUCCAGG dTdT
RHOT2-1	CCAAGAACCUGAGGAACAU dTdT	AUGUCCUCAGGUUCUUGG dTdT
RHOT2-2	CCAAAGUCCUUUGCACA AUU dTdT	AAUGUGCAAAGGACU UUGG dTdT
SLC25A12-1	GCUAUCUUGGACUGUAUAA dTdT	UUUAUACAGUCCAAGAUAGC dTdT
SLC25A12-2	GCUUGACUUUGGCAGAU AUU dTdT	AUAUCUGCCAAAGUCAAGC dTdT
SLC25A13-1	GCCACAGUU AUUUGGGAGU UdTdT	AACUCCCAAUAACUGUGGC dTdT
SLC25A13-2	GCGGAGUGAUAGACUGCU UdTdT	AAGCAGUCUAUCACUCCGC dTdT
SLC25A23-1	GCUCAUGUUUCACAGUCU UdTdT	AAGACUGUGAAACAUGAGC dTdT
SLC25A23-2	GCGCGCAAUGGU AUUAAU dTdT	AUUAUACCAUUGCCGCGC dTdT
SLC25A24-1	GGGCAUAUGAACAGUACAAdTdT	UUGUACUGUUAU AUGCCC dTdT
SLC25A24-2	GCAUCAGUUAUGUGGUU AdTdT	UAAACCACAU AACUGAUGC dTdT
SLC25A25-1	GGGAGUCAAGAU AUCUGAA dTdT	UUCAGAU AUCUUGACUCCC dTdT
SLC25A25-2	GCAUCGACCUUGCAGUCU AdTdT	UAGACUGCAAGGUCGAUGC dTdT
EFHA1-1	CCUCUUCUCAGUGAUGUU UdTdT	AAACAUCACUGAGAAGAGG dTdT
EFHA1-2	CCAUGCAGAU GUUCAGUU UdTdT	AAACUGAACAUCUGCAUGG dTdT
EFHD1-1	GCCUGAAGAGCAUGAUCAAdTdT	UUGAUC AUGCUCUUCAGGC dTdT
EFHD1-2	CCUUGUCAUCGGCCAGUAA dTdT	UUACUGGCCGAUGACAAGG dTdT
HSPA9-1	CCACCAGGAUAGCUGGAAU dTdT	AUUC CAGCUAUCCUGGUGG dTdT
HSPA9-2	GCCCUAUCUUACAAUGGAU dTdT	AUCCA UUGUAAGAUAGGGC dTdT
GPD2-1	GCCUAUAAUGCUUCCAGU UdTdT	AACUGGAAGCAUUAUAGGC dTdT
GPD2-2	GGAACUGAAUUGGGAUGAU dTdT	AUCAUCCCAAUUCAGU UCC dTdT
MICU1-1	GCAGCUCAAGAAGCACUUCAAdTdT	UUGAAGUGCUUCUUGAGCUGC dTdT
MICU1-2	GCAAUGGCGAACUGAGCAAU AdTdT	UAUUGCUCAGUUCGCCAUUGC dTdT
RAB11FIP5-1	GGUACAAGCUGCACUCCAAdTdT	UUGGAGUGCAGCUUGUACCC dTdT
RAB11FIP5-2	GCUGAGUGCUCAGGCUAAAdTdT	UUUAGCCUGAGCACUCAGC dTdT

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5