



# Tespa1 is a novel component of mitochondria-associated endoplasmic reticulum membranes and affects mitochondrial calcium flux

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## ABSTRACT

Regulation of intracellular Ca<sup>2+</sup> concentration is critical in numerous biological processes. Inositol 1,4,5-trisphosphate receptor (IP<sub>3</sub>R) functions as the Ca<sup>2+</sup> release channel on endoplasmic reticulum (ER) membranes. Much attention has been dedicated to mitochondrial Ca<sup>2+</sup> uptake via mitochondria-associated ER membranes (MAM) which is involved in intracellular Ca<sup>2+</sup> homeostasis; however, the molecular mechanisms that link the MAM to mitochondria still remain elusive. We previously reported that Tespa1 (thymocyte-expressed, positive selection-associated gene 1) expressed in lymphocytes physically interacts with IP<sub>3</sub>R. In this study, we first performed double-immunocytochemical staining of Tespa1 with a mitochondrial marker or an ER marker on an acute T lymphoblastic leukemia cell line, Jurkat cells, by using anti-ATP synthase or anti-calnexin antibody, respectively, and demonstrated that Tespa1 was localized very close to mitochondria and the Tespa1 localization was overlapped with restricted portion of ER. Next, we examined the effects of Tespa1 on the T cell receptor (TCR) stimulation-induced Ca<sup>2+</sup> flux by using Ca<sup>2+</sup> imaging in Jurkat cells. Reduction of Tespa1 protein by *Tespa1*-specific siRNA diminished TCR stimulation-induced Ca<sup>2+</sup> flux into both mitochondria and cytoplasm through the analyses of the mitochondrial Ca<sup>2+</sup> indicator (Rhod-2) and the cytoplasmic Ca<sup>2+</sup> indicator (Fluo-4), respectively. Furthermore, co-immunoprecipitation assay in HEK293 cells revealed that exogenous Tespa1 protein physically interacted with a MAM-associated protein, GRP75 (glucose-regulated protein 75), but not with an outer mitochondrial membrane protein, VDAC1 (voltage-dependent anion channel 1). All these results suggested that Tespa1 will participate in the molecular link between IP<sub>3</sub>R-mediated Ca<sup>2+</sup> release and mitochondrial Ca<sup>2+</sup> uptake in the MAM compartment.

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## 1. Introduction

*Tespa1* (thymocyte-expressed, positive selection-associated gene 1) was firstly identified as a critical gene responsible for T cell development in the thymus [1]. We recently reported that *Tespa1* was highly expressed in T- and B-lymphocytes, and physically interacted with the NH<sub>2</sub>-terminal region of IP<sub>3</sub>R (inositol 1,4,5-trisphosphate receptor) [2]. However, the precise molecular mechanisms and functional relevance of the interaction between *Tespa1* and IP<sub>3</sub>R remain elusive.

Intracellular Ca<sup>2+</sup> is a universal second messenger controlling numerous biological processes [3,4]. Three IP<sub>3</sub>R subtypes including IP<sub>3</sub>R1, IP<sub>3</sub>R2, and IP<sub>3</sub>R3, are differentially expressed among tissues [5–9] and function as the Ca<sup>2+</sup> release channel on endoplasmic

reticulum (ER) membranes [10–14]. IP<sub>3</sub>R is regulated by many intracellular modulators, phosphorylation and associated proteins [15–19].

Although ER is considered to be the main intracellular Ca<sup>2+</sup> store [20], other organelles including mitochondria also play a particular role in Ca<sup>2+</sup> homeostasis [21–24]. ER and mitochondria are known to communicate via contact sites called mitochondria-associated ER membranes (MAM) [25]. Several studies have provided that Ca<sup>2+</sup> channels including IP<sub>3</sub>R are enriched within the MAM [21–24,26], suggesting the importance of the Ca<sup>2+</sup> signaling from ER store into the mitochondria through the MAM. Although a MAM-associated protein, GRP75 (glucose-regulated protein 75), was reported to function as a molecular link between IP<sub>3</sub>R and an outer mitochondrial membrane protein, VDAC1 (voltage-dependent anion channel 1) [27], the precise molecular mechanisms of calcium transfer via MAM remain to be elucidated.

In this study, we found that *Tespa1* was localized very close to mitochondria in an acute T lymphoblastic leukemia cell line, Jurkat cells. Knockdown of *Tespa1* expression caused remarkable

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reduction in T cell receptor (TCR) stimulation-induced mitochondrial  $\text{Ca}^{2+}$  flux in Jurkat cells. Furthermore, Tespa1 protein physically interacted with GRP75. Thus, this is the first report demonstrating that Tespa1 is involved in the regulation of mitochondrial  $\text{Ca}^{2+}$  flux via MAM.

## 2. Materials and methods

### 2.1. Antibodies

The antibodies used were: mouse monoclonal anti-calnexin-1 antibody (MAB3126) from Millipore, mouse monoclonal ATP synthase antibody (3D5) from Abcam, mouse monoclonal anti-IP<sub>3</sub>R3 antibody (610313) from BD Transduction Laboratories, rabbit polyclonal anti-IP<sub>3</sub>R2 antibody (AB3000) from Millipore, rabbit polyclonal anti-IP<sub>3</sub>R1 antibody (ab5840) from Abcam, rabbit polyclonal anti-actin antibody (A2066) from Sigma, rat monoclonal anti-HA antibody (3F10) from Roche, rabbit polyclonal anti-FLAG antibody (F7425) from Sigma, and rabbit polyclonal anti-Tespa1 antibody prepared as described before [2].

### 2.2. Immunocytochemical staining

Immunocytochemical staining was performed as described before [28,29]. The primary antibodies were visualized with proper combinations of secondary antibodies: goat anti-rabbit IgG conjugated to Alexa Fluor 488, goat anti-rabbit IgG conjugated to Alexa Fluor 555, goat anti-mouse IgG conjugated to Alexa Fluor 488, and goat anti-mouse IgG conjugated to Alexa Fluor 555.

### 2.3. Western blotting and immunoprecipitations

Western blotting and immunoprecipitations were performed as described before [28,29].

### 2.4. $\text{Ca}^{2+}$ imaging

Jurkat cells were cultured at 37 °C with 5%  $\text{CO}_2$  in the growth medium (RPMI 1640 containing 10% fetal calf serum and penicillin–streptomycin–glutamine) and transfected with a siRNA using MicroPatorator MP-100 (Digital Bio) according to the manufacturer's instruction. Two distinct siRNAs were designed to target the coding region of human *Tespa1* gene. Scrambled RNAs containing the same number of each nucleotide as the siRNAs targeting the *Tespa1* gene were used as controls. The following siRNA duplexes (Invitrogen) were used in this study: Tespa1#1, 5'-G AGG AAG CAG GAC AGU UUA UCU ACA-3' and 5'-U GUA GAU AAA CUG UCC UGC UUC CUC-3'; scramble#1, 5'-G AGA CGA CAG GUU GAC UAU UAG ACA-3' and 5'-U GUC UAA UAG UCA ACC UGU CGU CUC-3'; Tespa1#2, 5'-C CAA GAG GAU CAC AAA GAC ACU UCU-3' and 5'-A GAA GUG UUG UGA UCC UCU UGG-3'; scramble#2, 5'-C CAG GAC UAA CAG AAA CAU CAG UCU-3' and 5'-A GAC UGA UGU UUC UGU UAG UCC UGG-3'. Two days after transfection, the Jurkat cells were labeled with 4  $\mu\text{M}$  Rhod-2 acetoxymethyl ester (Invitrogen) or with 4  $\mu\text{M}$  Fluo-4 acetoxymethyl ester (Invitrogen) in the growth medium for 50 min at 37 °C. After washing, cells were suspended in the growth medium and prewarmed for 5 min at 37 °C. Immediately after TCR-stimulation by addition of anti-human CD3 antibody (10  $\mu\text{g}/\text{ml}$ ) (clone: OKT3, BioLegend),  $\text{Ca}^{2+}$  flux data was acquired on a BD FACSCalibur with CellQuest software (BD Biosciences) and analyzed with FlowJo software (Tree Star).

### 2.5. Construction and transfection

The cDNAs encoding full-length human GRP75 and VDAC1 were prepared from Jurkat cells by reverse transcription-PCR and were cloned into the pFLAG-CMV4 vector (Sigma) to generate FLAG-tag fusion proteins. The HA-tagged full-length human Tespa1 fusion protein was described before [2]. HEK293 cells were cultured and transfected with plasmids as described before [29].

### 2.6. Statistical analysis

Data are presented as the means  $\pm$  standard errors of the means (SEM) and statistical analysis was performed using an unpaired Student's *t*-test when comparing the means of two groups. Differences of *P* values of less than 0.05 were considered to be statistically significant.

## 3. Results

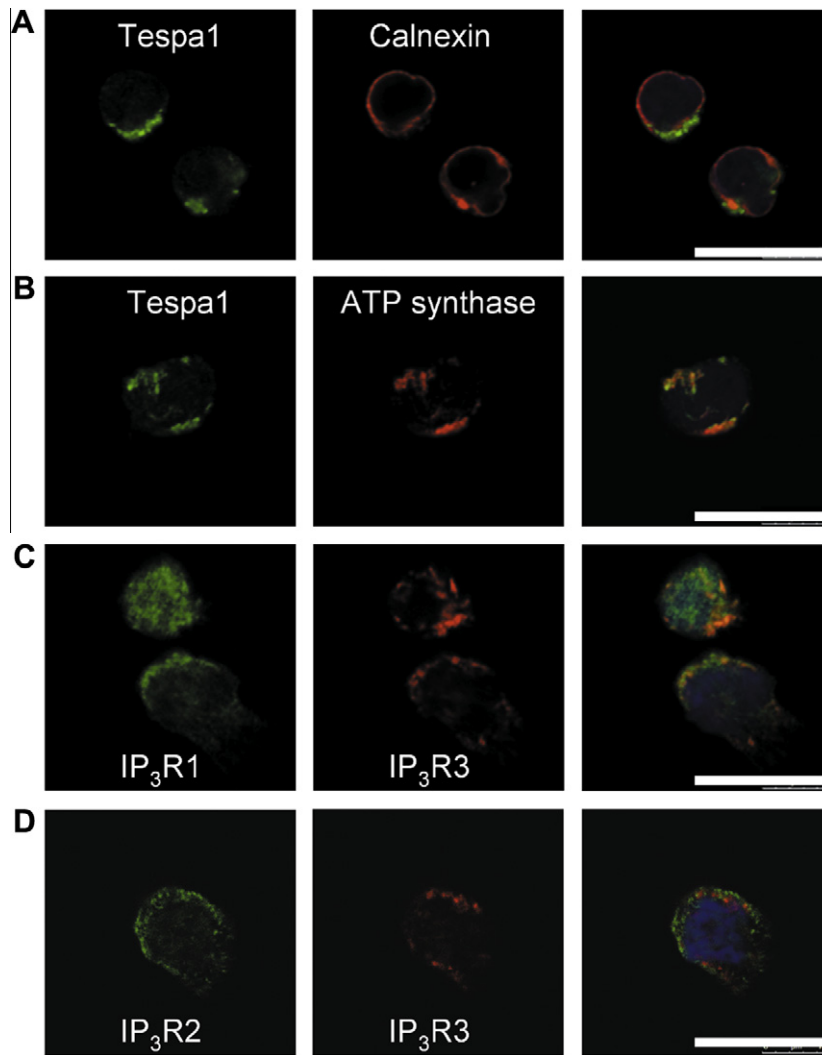
### 3.1. Tespa1 is localized very close to mitochondria in Jurkat cells.

Since we previously reported that Tespa1 physically interacted with the NH<sub>2</sub>-terminal region of IP<sub>3</sub>R [2], we herein examined the subcellular localization of Tespa1 in Jurkat cells. Double-immunocytochemical staining of Tespa1 with ER by using anti-calnexin antibody revealed that Tespa1 was not overlapped with majority of ER, but slightly overlapped with ER in the restricted regions (Fig. 1A), suggesting that Tespa1 resides in a specialized ER but not a typical reticular ER. In contrast, double-immunocytochemical staining of Tespa1 with mitochondria by using anti-ATP synthase antibody revealed that Tespa1 was localized very close to mitochondria (Fig. 1B). As Tespa1 spatially well-colocalized with IP<sub>3</sub>R3 in Jurkat cells [2], present finding led us to hypothesize that Tespa1 may localize in the MAM compartment and function as a regulator of calcium flux from ER calcium store into mitochondria through the regulation of IP<sub>3</sub>R3.

To address spatial distribution of IP<sub>3</sub>R subtypes in Jurkat cells, we performed immunocytochemical staining of IP<sub>3</sub>R subtypes, showing that IP<sub>3</sub>R1 was broadly distributed within the cells, whereas IP<sub>3</sub>R3 was partially colocalized with IP<sub>3</sub>R1 and highly restricted in particular regions like Tespa1 (Fig. 1C). Furthermore, IP<sub>3</sub>R2 and IP<sub>3</sub>R3 were detected in a mutually exclusive manner (Fig. 1D). These results indicated that each IP<sub>3</sub>R subtypes resided in distinct compartments within the cells and might participate in different cellular processes.

### 3.2. Tespa1-knockdown diminishes both mitochondrial and cytoplasmic calcium flux

To clarify whether Tespa1 was involved in intracellular calcium flux, we examined the knockdown effect of Tespa1 on TCR stimulation-induced calcium flux in Jurkat cells by  $\text{Ca}^{2+}$  imaging. *Tespa1*-siRNAs, but not scramble-siRNAs, suppressed Tespa1 protein (Fig. 2A). Two days after the treatment of cells with the siRNA, cells were stimulated with anti-CD3 antibody, which evoked TCR stimulation-induced IP<sub>3</sub>R-mediated calcium efflux from intracellular  $\text{Ca}^{2+}$  store. We measured calcium flux into mitochondria and cytoplasm by using the mitochondrial calcium indicator (Rhod-2) and the cytoplasmic calcium indicator (Fluo4), respectively. When cells were loaded with Rhod-2 and stimulated with anti-CD3 antibody, the total amount of  $\text{Ca}^{2+}$  flux after the stimulation were decreased by 60% (Tespa1#1) and 73% (Tespa1#2) in the *Tespa1*-knockdown cells compared with those of the control scramble-siRNA-treated cells (Fig. 2B and C). Furthermore, when cells were loaded with Fluo-4 and stimulated with anti-CD3 antibody, the total amount



**Fig. 1.** Tespa1 is localized very close to mitochondria in Jurkat cells. (A) Fluorescent confocal images of Jurkat cells for Tespa1 (green), calnexin (red), and the merged photo. (B) Fluorescent confocal images of Jurkat cell for Tespa1 (green), ATP synthase (red), and the merged photo. (C) Fluorescent confocal images of Jurkat cells for IP<sub>3</sub>R1 (green), IP<sub>3</sub>R3 (red), and the merged photo. (D) Fluorescent confocal images of Jurkat cell for IP<sub>3</sub>R2 (green), IP<sub>3</sub>R3 (red), and the merged photo. Blue, 4',6-Diamidino-2-phenylindole (DAPI) staining; scale bar, 15  $\mu$ m. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

of Ca<sup>2+</sup> flux after the stimulation were also decreased by 82% (Tespa1#1) and 83% (Tespa1#2) in the *Tespa1*-knockdown cells compared with those of the control scramble-siRNA-treated cells (Fig. 2D and E). These results suggested that Tespa1 was required for proper TCR stimulation-induced Ca<sup>2+</sup> fluxes from ER stores into both mitochondria and cytoplasm, and that Tespa1 preferentially influenced the mitochondrial Ca<sup>2+</sup> flux.

### 3.3. *Tespa1* protein interacts with a MAM-associated protein, GRP75

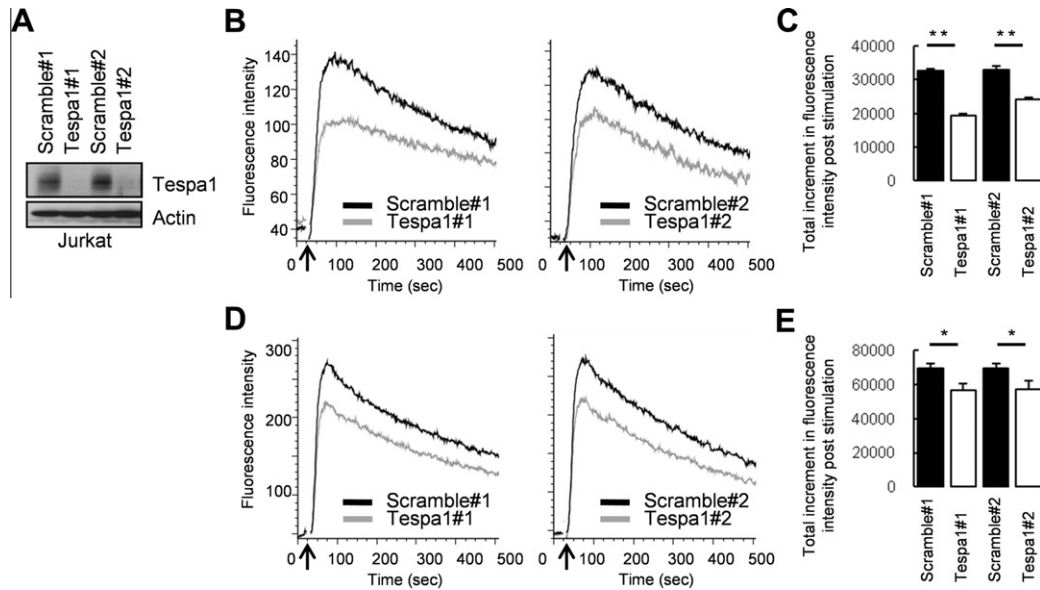
To examine the possibility that Tespa1 participates in the molecular components of MAM, co-immunoprecipitation assay of Tespa1 with a MAM-associated protein, GRP-75, or an outer mitochondrial membrane protein, VDAC1, was performed in HEK293 cells double-transfected with HA-tagged Tespa1 and FLAG-tagged GRP75 or FLAG-tagged VDAC1. Anti-HA immunoprecipitations revealed the association of HA-tagged Tespa1 protein with FLAG-tagged GRP75 protein but not with FLAG-tagged VDAC1 protein (Fig. 3A). Consistent with this result, anti-FLAG immunoprecipitations revealed the association of HA-tagged Tespa1 with FLAG-tagged GRP75 but not with FLAG-tagged VDAC1 (Fig. 3B). Furthermore, subcellular localizations of HA-tagged Tespa1 and

FLAG-tagged GRP75 proteins were detected in a reticular fashion and Tespa1 was partially co-localized with GRP75 (Fig. 3C). These results suggested that Tespa1 protein physically interacts with GRP75 and may be a molecular component of the MAM.

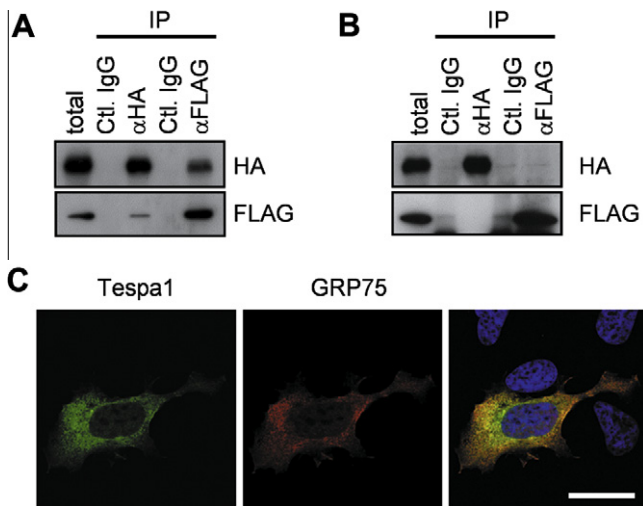
## 4. Discussion

Much attention has been dedicated to the involvement of mitochondria in Ca<sup>2+</sup> homeostasis. Mitochondrial Ca<sup>2+</sup> homeostasis regulates cell bioenergetics, cytoplasmic Ca<sup>2+</sup> signals and activation of cell death pathways [30–32]. As mitochondria structurally and functionally communicates with ER through the contact sites referred as MAM, in which Ca<sup>2+</sup> channels including IP<sub>3</sub>R are enriched, molecular mechanism for the regulation of Ca<sup>2+</sup> signaling through the MAM is a topic in physiology and pathology [21–24,30–32]. Recently, GRP75 protein was reported to be involved in the IP<sub>3</sub>R-mediated mitochondrial Ca<sup>2+</sup> signaling through the interactions with both IP<sub>3</sub>R and VDAC1 proteins [27]; however, the exact molecular components of the MAM and its function remain to be elucidated.

In this study, we found that Tespa1 is localized very close to mitochondria and Tespa1 expression is involved in TCR



**Fig. 2.** Tespa1-knockdown diminishes both mitochondrial and cytoplasmic calcium flux. (A) The Western blot analysis of Tespa1 and actin expression levels in Jurkat cells 48 h after the transfection of siRNAs (specific Tespa1-siRNA: Tespa1#1, Tespa1#2). Scramble-siRNAs (Scramble#1, Scramble#2) were used as controls. (B) Representative  $\text{Ca}^{2+}$  flux detected by using the  $\text{Ca}^{2+}$  indicator Rhod-2 in Jurkat cells pre-treated with siRNA. Anti-CD3 antibody was added to induce TCR-stimulation (arrows). Data are representative of three independent experiments. (C) Quantification of the total increment in fluorescence intensity post-stimulation in (B). (D) Representative  $\text{Ca}^{2+}$  flux detected by using the  $\text{Ca}^{2+}$  indicator Fluo-4 in Jurkat cells pre-treated with siRNA. Anti-CD3 antibody was added to induce TCR-stimulation (arrows). Data are representative of three independent experiments. (E) Quantification of the total increment in fluorescence intensity post-stimulation in (D). Data were presented as the means  $\pm$  SEM,  $n = 3$ . Independent  $t$ -tests were used for statistical comparisons. \* $P < 0.05$ ; \*\* $P < 0.01$  relative to the scramble siRNA-treated control.



**Fig. 3.** Tespa1 protein interacts with a MAM-associated protein, GRP75. (A) Immunoprecipitations with anti-HA ( $\alpha$ HA), anti-FLAG ( $\alpha$ FLAG), or control (Ctl. IgG) antibodies were performed using HEK293 cells co-transfected with HA-tagged Tespa1 and FLAG-tagged GRP75, followed by Western blotting with anti-HA or anti-FLAG antibodies. Total, total lysate; IP, immunoprecipitation. (B) Immunoprecipitations with  $\alpha$ HA,  $\alpha$ FLAG, or Ctl. IgG antibodies were performed using HEK293 cells co-transfected with HA-tagged Tespa1 and FLAG-tagged VDAC1, followed by Western blotting with anti-HA or anti-FLAG antibodies. (C) Fluorescent confocal images of HEK293 cells co-transfected with HA-tagged Tespa1 (green) and FLAG-tagged GRP75 (red) with anti-HA and anti-FLAG antibodies. Blue, DAPI staining; scale bar, 20  $\mu\text{m}$ . (For interpretation of color in this figure, the reader is referred to the web version of this article.)

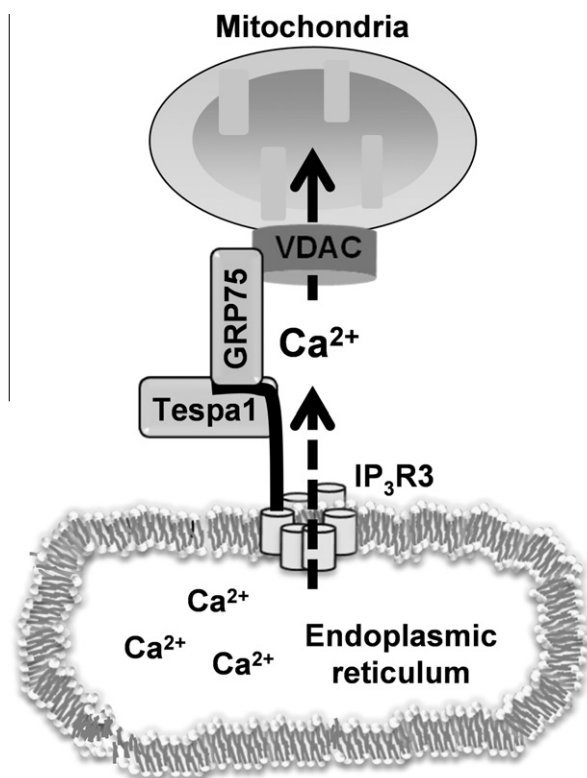
stimulation-induced mitochondrial  $\text{Ca}^{2+}$  flux in Jurkat cells. Notably, Tespa1 physically interacts with a MAM-associated protein, GRP75. Additionally, IP<sub>3</sub>R3, which is previously found to be well-colocalized and physically associated with Tespa1 in Jurkat cells [2], was found to be differentially localized from those of IP<sub>3</sub>R1 and IP<sub>3</sub>R2, indicating that each IP<sub>3</sub>R subtypes resides in distinct

subcellular compartments and may participate in different  $\text{Ca}^{2+}$  fluxes through multiple pathways.

Considering the present data that Tespa1 is localized very close to mitochondria and the physical interaction of Tespa1 with IP<sub>3</sub>R [2], a hypothetical molecular mechanism explaining the relation between Tespa1 and mitochondrial  $\text{Ca}^{2+}$  flux is that Tespa1 is involved in the  $\text{Ca}^{2+}$  transfer from ER to mitochondria through the regulation of IP<sub>3</sub>R in the MAM compartment (Fig. 4). Consistent with this idea, Tespa1 associates with GRP75, thereby suggesting the functional relevance of Tespa1 to the molecular link between IP<sub>3</sub>R on ER membranes and VDAC1 on outer mitochondrial membranes. Considering the fact that the NH<sub>2</sub>-terminal region of IP<sub>3</sub>R is responsible for the interaction not only with Tespa1 [2] but also with GRP75 [27], these three proteins would form a macromolecular complex and function cooperatively in the MAM. Additionally, Tespa1 is spatially well-associated with IP<sub>3</sub>R3 over IP<sub>3</sub>R1 in Jurkat cells [2]. IP<sub>3</sub>R3 may preferentially associate with the mitochondrial  $\text{Ca}^{2+}$  flux via the MAM. Although we did not detect the association between Tespa1 and VDAC1, the possibility that Tespa1 interacts with the other outer mitochondrial membrane proteins will not be excluded.

We also observed reduced IP<sub>3</sub>R-mediated cytoplasmic  $\text{Ca}^{2+}$  flux after TCR-stimulation in the Tespa1-knockdown Jurkat cells, suggesting that Tespa1 is required for proper T-cell signaling pathway: TCR-stimulation triggers IP<sub>3</sub>R-mediated  $\text{Ca}^{2+}$  efflux from intracellular store, which results in activation of  $\text{Ca}^{2+}$ -dependent enzymes and transcription factors. This observation is consistent with the previous report that *Tespa1*-deficiency results in impaired TCR stimulation-induced  $\text{Ca}^{2+}$  flux and less activation of NFAT in thymocytes [1]. Taking account of the difference in the subcellular localization of IP<sub>3</sub>R subtypes in Jurkat cells (Fig. 1C and D), IP<sub>3</sub>R1 and/or IP<sub>3</sub>R2 rather than IP<sub>3</sub>R3 may contribute to the regulation of TCR stimulation-induced cytoplasmic  $\text{Ca}^{2+}$  flux in the Jurkat cells.

In conclusion, this is the first report demonstrating that Tespa1 is a novel molecular component of the MAM and regulates



**Fig. 4.** A schematic representation of Tespa1 protein function as a regulator of mitochondrial  $\text{Ca}^{2+}$  flux from endoplasmic reticulum through the physical association with  $\text{IP}_3\text{R3}$  and GRP75.

mitochondrial  $\text{Ca}^{2+}$  flux from ER. Thus, precise understanding of the full spectrum of the activities of Tespa1 will lead to better understanding of cellular programs and immune-related diseases.

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