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# The 14-3-3γ isoform binds to and regulates the localization of endoplasmic reticulum (ER) membrane protein TMCC3 for the reticular network of the ER

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（課程博士関係）

学 位 論 文 の 内 容 要 旨

# The 14-3-3γ isoform binds to and regulates the localization of endoplasmic reticulum (ER) membrane protein TMCC3 for the reticular network of the ER

# 14-3-3γ が TMCC3 に結合し、その局在を制御することで、小胞体ネットワークを形成 する

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## Introduction

The endoplasmic reticulum (ER) is continuous membrane system composed of ER sheets and ER tubules. The ER tubules are interconnected by three-way junctions formed by conserved ER membrane protein, atlastins and lunapark. Atlastin, dynamin-like GTPases, drive homotypic membrane fusion between the two ER tubules, leading to the generation of three-way junctions. Lunapark is then recruited to the nascent three-way junctions for stabilization. Furthermore, we have demonstrated that TMCC3 (transmembrane and coiled-coil domain family 3) is ER membrane protein that specifically localizes to the three-way junctions and binds to atlastins.

Reticular network of ER tubules undergoes constant remodeling through formation and loss of three-way junctions. We had previously demonstrated that TMCC3 acts upstream of atlastins. TMCC3 localizes to three-way junctions independently of atlastins, and promotes the formation of three-way junctions by enhancing atlastins activity. However, it still remains unknown how the TMCC3 activity is regulated for formation of the reticular ER network.

The earlier study identified 14-3-3 proteins as TMCC3-binding proteins. It is well known that 14-3-3 proteins interact with target proteins in phosphorylation-dependent manner, thereby regulating various cellular processes. However, it still remains unknown whether binding of 14-3-3 proteins to TMCC3 is involved in regulation of the ER morphology. In this study, we characterize the interaction between 14-3-3γ and TMCC3 in the context of regulation of the ER morphology and demonstrate that 14-3-3 binding negatively regulates TMCC3 localization to the three-way junctions for the formation of reticular ER network.

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## Results

### 1. Overexpression of 14-3-3γ reduces localization of TMCC3 to three-way junctions

We set out to examine the effect of 14-3-3 proteins on localization of TMCC3 to three-way junctions. HA-tagged 14-3-3γ (HA-14-3-3γ) or mCherry was then transfected into U2OS cells stably expressing GFP-TMCC3 (U2OS-

GFP-TMCC3 cells), followed by immunostaining. The result showed that 60% of cells overexpressing HA-14-3-3γ were lost GFP-TMCC3 puncta indicative of three-way junctions whereas almost all of the cells overexpressing mCherry retained the puncta, indicating that overexpression of HA-14-3-3γ reduces localization of TMCC3 to three-way junctions.

To confirm the result, we examined whether overexpression of HA-14-3-3γ affected the localization of endogenous TMCC3. HA-14-3-3γ or control vector was transfected into U2OS cells, followed by immunostaining with anti-TMCC3 pAb and anti-PDI mAb as an ER marker. Similar to the previous result, peripheral puncta of endogenous TMCC3 were obviously reduced in the cells overexpressing HA-14-3-3γ compared to the cells transfected with control vector.

We next examined whether overexpression of 14-3-3γ affected the protein level of TMCC3. HA-14-3-3γ or the control vector was transfected into U2OS cells. The total cell lysates were subjected to immunoblotting with anti-TMCC3 pAb and anti-GAPDH. The result showed that overexpression of HA-14-3-3γ did not change the protein level of endogenous TMCC3.

**2. Overexpression of 14-3-3γ decreases the number of three-way junctions**

We reasoned that if 14-3-3γ negatively regulated TMCC3 localization, overexpression of 14-3-3γ would affect the ER morphology. HA-14-3-3γ or mCherry was transfected into U2OS cells, followed by immunostaining with anti-PDI mAb as an ER marker. The number of three-way junctions as detected by PDI staining was counted within 10 × 10 $\mu m^2$ of the peripheral ER. In agreement with the reasoning, the cells overexpressing HA-14-3-3γ significantly decreased the number of three-way junctions relative to the cells overexpressing mCherry.

To confirm the result, we also carried out the similar experiment using ER-GFP, GFP fused to the ER signal sequence of calreticulin and KDEL (ER retention signal) as an ER marker. HA-14-3-3γ or control vector was transfected into U2OS cells, followed by transfection with ER-GFP. The cells

were then immunostained. Similar to PDI staining result, overexpression of HA-14-3-3γ decreased the number of three-way junctions detected by ER-GFP.

### 3. TMCC3 binds to 14-3-3γ through the N-terminus

We sought to determine which region of TMCC3 was responsible for binding to 14-3-3γ. We started by confirming that 14-3-3γ binds to TMCC3. U2OS-GFP-TMCC3 cells were extracted with 1% TritonX-100 and subjected to immunoprecipitation with anti-GFP pAb, followed by immunoblotting with anti-14-3-3 mAb. Endogenous 14-3-3 proteins were coimmunoprecipitated with GFP-TMCC3, confirming that TMCC3 bound to 14-3-3 proteins.

We next transfected fragments of TMCC3 with the N-terminal HA tags into HEK293 cells along with FLAG-14-3-3γ, followed by immunoprecipitation with anti-FLAG mAb. The HA-tagged N-terminal fragment encompassing the large cytoplasmic region (HA-TMCC3-N) was coimmunoprecipitated with FLAG-14-3-3γ, indicating that N-terminal cytoplasmic region had the binding site for 14-3-3γ. Similar to HA-TMCC-N, the HA-tagged mutant deleting almost C-terminal half from TMCC3-N (HA-TMCC3-D1) was coimmunoprecipitated with FLAG-14-3-3γ. By contrast, the HA-tagged mutant deleting the first 89 amino acids (aa) from TMCC3-D1 (TMCC3-D2) was not coimmunoprecipitated with FLAG-14-3-3γ. These results indicate that 89 aa of N-terminal is binding site for 14-3-3γ.

### 4. Phosphorylated serine 15 is required for potent binding to 14-3-3γ

We examined whether TMCC3 had the 14-3-3 binding motif in the 89 aa of N-terminal. Full-length aa sequence of mouse TMCC3 was subjected to 14-3-3-Pred, the web server to predict 14-3-3 binding motifs. 14-3-3-Pred showed there were three possible binding motifs in the 89 aa of N-terminal with amino acid positioned at 12-17 has the highest score and meet completely to 14-3-3 binding motif. We then generated the point mutant of TMCC3-D1 substituting alanine for serine 15 (TMCC3-D1-S15A). HA-TMCC3-D1 or HA-tagged TMCC3-D1-S15A (HA-TMCC3-D1-S15A) was transfected into

HEK293 cells along with FLAG-14-3-3γ, followed by immunoprecipitation with anti-FLAG mAb. While HA-TMCC3-D1 was strongly coimmunoprecipitated with FLAG-14-3-3γ, coimmunoprecipitation of HA-TMCC3-D1-S15A was significantly decreased relative to HA-TMCC3-D1. These results indicate that serine 15 was required for potent binding to 14-3-3γ.

Since TMCC3-D1-S15A still showed a weak binding to 14-3-3γ, we further substituted serine 25 and serine 46 to alanines in TMCC3-D1-S15A (TMCC3-D1-S15/25/46A), and performed immunoprecipitation analysis. A small amount of HA-tagged TMCC3-D1-S15/25/46A (HA-TMCC3-D1-S15/25/46A), which was comparable to that of HA-TMCC3-D1-S15A, was coimmunoprecipitated with FLAG-14-3-3γ. These results indicate that the other two deduced binding motifs predicted with low score are not involved in binding to 14-3-3γ.

We next examined whether serine 15 was phosphorylated. HA-TMCC3-D1, HA-TMCC3-D1-S15A, HA-TMCC3-D1-S15/25/46A, or control vector was transfected into HEK293 cells, followed by immunoprecipitation with anti-HA mAb. The samples were subjected to immunoblotting with antibody recognizing phosphorylated serine in the consensus 14-3-3 binding motif (anti-phospho-14-3-3 binding motif pAb). The anti-phospho-14-3-3 binding motif pAb detected HA-TMCC3-D1, but not recognized HA-TMCC3-D1-S15A and HA-TMCC3-D1-S15/25/46A, indicating that serine 15 is phosphorylated. Similar results were obtained in U2OS cells.

**5. TMCC3 mutant subtituting alanine for serine 15 is prone to localize at three-way junction against overexpression of 14-3-3γ**

We generated the full-length TMCC3 mutant by substituting alanine for serine 15 (TMCC3-S15A), and established U2OS cell lines stably expressing GFP tagged TMCC3-S15A (U2OS-GFP-TMCC3-S15A). HA-14-3-3γ or a control vector was then transfected into the U2OS-GFP-TMCC3 cells or U2OS-GFP-TMCC3-S15A cells, followed by immunostaining. Overexpression of 14-3-3γ significantly increased the number of U2OS-GFP-TMCC3 cells and U2OS-GFP-TMCC3-S15A cells losing their puncta relative

to the control vector. However, the number of the cells losing GFP-TMCC3-S15A puncta was significantly lower than that of the cells losing GFP-TMCC3 puncta, indicating that TMCC3-S15A was more resistant to localize at three-way junctions against 14-3-3γ overexpression. Collectively, these result indicate that binding to 14-3-3γ through phosphoserine 15 negatively regulates localization of TMCC3 to three-way junctions.

**6. The negative regulation of TMCC3 by 14-3-3γ is involved in formation of the reticular ER network**

We next examined whether negative regulation of TMCC3 by 14-3-3γ is involved in formation of reticlar ER network. The siRNA targeting TMCC3 was transfected into U2OS cells followed with the transfection of HA-TMCC3, HA-TMCC3-S15A, or control vector. As negative control, the negative control siRNA was transfected followed by transfection with control vector. The samples were subjected for immunostaining with anti-CLIMP-63 pAb as an ER sheet marker and anti-α-tubulin mAb. The ratio of CLIMP-63 staining area to total area determined by α-tubulin staining significantly increased in the TMCC3 knockdown-cells transfected with control vector relative to negative control cells, indicating that, consistent with our previous finding, TMCC3 knockdown caused ER sheet expansion. On the other hand, the TMCC3-knockdown cells expressing HA-TMCC3 showed the ratio of CLIMP-63 staining area to total area were comparable to the negative control cells, indicating that expression of HA-TMCC3 rescued the phenotype of the TMCC3 knockdown. Importantly, the TMCC3-knockdown cells expressing HA-TMCC3-S15A showed the ratio of CLIMP-63 staining area to total cell area significantly higher than the TMCC3-knockdown cells expressing HA-TMCC3, but lower than the TMCC3-knockdown cells transfected with the control vector. These results indicate that expression of HA-TMCC3-S15A partially rescued the phenotype of the TMCC3 knockdown, suggesting that the negative regulation of TMCC3 by 14-3-3γ was involved in formation of the reticular ER network.

## Conclusion

In this study, we demonstrate that 14-3-3γ binding negatively regulates TMCC3 localization for the reticular ER network. Binding of 14-3-3γ to TMCC3 is depend on phosphorylation of serine 15 in the 14-3-3 binding motif.

神戸大学大学院医学(系)研究科（博士課程）

# 論文審査の結果の要旨

| 受付番号 | 甲第 3308 号 | 氏名 | SAIHAS SUHDA |
|---|---|---|---|
| 論文題目<br>Title of Dissertation | The 14-3-3γ isoform binds to and regulates the localization of endoplasmic reticulum (ER) membrane protein TMCC3 for the reticular network of the ER<br><br>14-3-3γが TMCC3 に結合し、その局在を制御することで、小胞体ネットワークを形成する | | |
| 審査委員<br>Examiner | 主査 Chief Examiner 南 康博<br>副査 Vice-examiner 鈴木 聡<br>副査 Vice-examiner 山口 透 | | |

（要旨は１，０００字～２，０００字程度）

（目的）

小胞体は酵母から哺乳動物まで全ての真核生物に保存された最も大きい細胞内小器官である。小胞体はシート構造とチューブ構造が連続した特徴的な形状を持つ。チューブ構造は、three-way junction によって連結されている。近年、我々は小胞体膜タンパク質 TMCC3 が three-way junction に局在し、その形成を促進することを報告した。しかし、この TMCC3 の活性がどのように調節されるかは不明である。TMCC3 結合タンパク質として報告されている 14-3-3 は、リン酸化依存的に標的タンパク質に結合し、様々な細胞機能を調節していることが知られている。そこで、本研究では、TMCC3 の 14-3-3 結合リン酸化アミノ酸を同定し、その結合が TMCC3 の three-way junction への局在化、小胞体チューブネットワーク形成に関与するかを解析した。

（方法）

・14-3-3 による TMCC3 の three-way junction への局在化への効果

GFP-TMCC3 を恒常的に発現している U2OS 細胞(U2OS-GFP-TMCC3 細胞)に HA-14-3-3γ あるいはコントロールとして mCherry をトランスフェクションした。Three-way junction に局在する GFP-TMCC3 の点状構造の数をカウントした。同様に、野生型 U2OS 細胞に HA-14-3-3γ あるいは mCherry をトランスフェクションし、抗 TMCC3 抗体と抗 PDI 抗体で染色した。Three-way junction に局在する内在性 TMCC3 の点状構造の数をカウントした。

・14-3-3 による three-way junction 形成への効果

野生型 U2OS 細胞に HA-14-3-3γ あるいはコントロールとして mCherry をトランスフェクションし、抗 PDI 抗体で染色した。細胞辺縁の面積 $10 \times 10\ \mu m^2$ において、three-way junction の数をカウントした。

・TMCC3 の 14-3-3 結合領域

HEK293 細胞に、HA-TMCC3-N （TMCC3 の N 末細胞質領域全体）、HA-TMCC3-D1（HA-TMCC3-N の C 末半分を欠損した欠損体）あるいは HA-TMCC3-D2（HA-TMCC3-D1 の N 末 1-89 アミノ酸配列を欠損した欠損体）および FLAG-14-3-3γ をそれぞれトランスフェクションした。TritonX-100 抽出液に抗 FLAG 抗体を加えて免疫沈降した。

・TMCC3 の 14-3-3 結合リン酸化アミノ酸

HA-TMCC3-D1-S15A（HA-TMCC3-D1 の 15 番目のセリンをアラニンに置換した変異体）あるいは HA-TMCC3-D1-S15/25/46A（HA-TMCC3-D1 の 15 番目、25 番目、46 番目のセリンをアラニンに置換した変異体）および FLAG-14-3-3γ をそれぞれトランスフェクションした。TritonX-100 抽出液に抗 FLAG 抗体を加えて免疫沈降した。

・TMCC3-S15A による three-way junction への局在化への効果

GFP-TMCC3-S15A（GFP-TMCC3 の 15 番目のセリンをアラニンに置換した変異体）を恒常的に発現している U2OS 細胞(U2OS-GFP-TMCC3-S15A 細胞)あるいは U2OS-GFP-TMCC3 細胞に、HA-14-3-3γ あるいはコントロールベクターをトランスフェクションした。Three-way junction に局在する GFP-TMCC3-S15A あるいは GFP-TMCC3 の点状構造の数をカウントした。

・TMCC3-S15A による小胞体形態形成への効果

U2OS 細胞に TMCC3 siRNA をトランスフェクションして TMCC3 ノックダウン細胞を調製した。TMCC3 ノックダウン細胞に HA-TMCC3 あるいは HA-TMCC3-S15A をトランスフェクションし、抗 CLIMP-63 抗体と抗 α-tubulin 抗体で染色した。CLIMP-63 染色領域（シート構造領域）/α-tubulin 染色領域（細胞質全体）を計測した。

（結果）

1. 14-3-3 による TMCC3 の three-way junction への局在化への効果
   U2OS-GFP-TMCC3 細胞 に HA-14-3-3γ を発現させたところ、GFP-TMCC3 の点状構造の数が減少した。同様に、野生型 U2OS 細胞に HA-14-3-3γ を発現させたところ、内在性の TMCC3 の点状構造の数が減少した。
2. 14-3-3 による three-way junction 形成への効果
   野生型 U2OS 細胞に HA-14-3-3γ を発現させたところ、three-way junction の数が減少した。
3. TMCC3 の 14-3-3 結合領域
   免疫沈降実験により、FLAG-14-3-3γ は、HA-TMCC3-N あるいは HA-TMCC3-D1 と結合するが、HA-TMCC3-D2 と結合しなかった。
4. TMCC3 の 14-3-3 結合リン酸化アミノ酸
   HA-TMCC3-D1-S15A と FLAG-14-3-3γ の結合は、野生型 HA-TMCC3-D1 との結合に比べて、減少した。HA-TMCC3-D1-S15/25/46A と FLAG-14-3-3γ の結合は、HA-TMCC3-D1-S15A との結合と同程度であった。
5. TMCC3-S15A による three-way junction への局在化への効果
   U2OS-GFP-TMCC3-S15A 細胞 に HA-14-3-3γ を発現させたところ、コントロールの U2OS-GFP-TMCC3 細胞と比べて、GFP-TMCC3 の点状構造が減少した細胞の数が少なかった。14-3-3 タンパク質の存在下でも、GFP-TMCC3 に比べて、GFP-TMCC3-S15A が three-way junction へ局在した。
6. TMCC3-S15A による小胞体形態形成への効果
   TMCC3 ノックダウン細胞ではチューブ構造が減少し、CLIMP-63 染色領域（シート構造領域）が増加する。TMCC3 ノックダウン細胞に HA-TMCC3 を発現すると CLIMP-63 染色領域が減少し、チューブ構造が回復した。一方、HA-TMCC3-S15A を発現すると、CLIMP-63 染色領域が HA-TMCC3 程には減少せず、部分的にチューブ構造が回復した。

（結論）

14-3-3 が TMCC3 の 15 番目セリンにリン酸化依存的に結合することにより、TMCC3 の three-way junction への局在化を抑制し、小胞体チューブネットワーク形成を調節していることを明らかにした。

本研究は、TMCC3 の 14-3-3 結合リン酸化アミノ酸の同定とそれによる小胞体チューブネットワーク形成への影響を解析したものである。その結果、14-3-3 が TMCC3 の three-way junction への局在化を抑制、three-way junction の形成ひいては小胞体チューブネットワーク形成を制御していることを証明した点において、価値ある業績であると認める。よって、本研究者は博士（医学）の学位を得る資格があると認める。