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A Method for Identifying The Topology of Etoposide-induced Protein 2.4 Using Split mNeonGreen2*

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Abstract Convenient, reliable detection of transmembrane protein topology, especially the orientation of the amino (N-) and carboxyl (C-) termini of a membrane-spanning segment, may aid in identifying protein-protein interactions and clarifying the important biological functions of proteins. Self-complementing split fluorescent proteins have been widely used to image protein-protein interactions, label endogenous proteins and visualize mRNA localization. Here, we expand this toolset and develop an efficient method combining a self-complementing split mNeonGreen2 with site-directed labeling (SSDL) to identify the topology of transmembrane proteins. With SSDL, for the first time, we clearly demonstrate that both the N- and C-termini of etoposide-induced protein 2.4, which localizes in the endoplasmic reticulum, have a cytosolic orientation. This method can be useful for determining the topology of other organelle-based transmembrane proteins that have insufficient structural information.

Key words mNeonGreen2, site-directed-labeling, transmembrane protein, EI24
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1 INTRODUCTION

Obtaining the structural information of transmembrane proteins is very useful for understanding their functions. A fundamental aspect of the structure of transmembrane proteins in living cells is the membrane topology, which refers to the number of transmembrane segments, the orientations of the N- and C-termini of the membrane-spanning segment, and the position of the transmembrane segments in the protein sequence [1]. Although the number of transmembrane protein structures solved by X-ray crystallography or cryo-electron microscopy (cryo-EM) is increasing [2], crystallization of all transmembrane protein is difficult, and achieving near-atomic resolution for small proteins and structure determination of large proteins is challenging by nuclear magnetic resonance spectroscopy. Thus, thousands of transmembrane protein's topologies are still unknown. Furthermore, to analyze the protein structure, proteins are first extracted from the membrane and solubilized in detergent; thus, the orientation information of transmembrane proteins is lost during the X-ray crystallographic process, which may hinder further study of the protein function. Nondestructive and reliable identification of the characteristics of different parts of a polypeptide chain relative to the membrane in living cells is important for in-depth understanding of the biological functions of transmembrane proteins.

Despite the essential role of topology in protein

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function, the techniques available for characterizing the orientation of the N- and C-termini of membrane-spanning segments within the diverse microenvironments of the cell are limited. Computational tools with different algorithms for predicting the topology of α-helical and β-barrel transmembrane proteins exist. However, since multiple factors decide the orientation of the transmembrane domain and the topology is either not conserved in a protein superfamily[1, 3], the predictions are not completely accurate. Other approaches for determining the N- and C-termini of membrane-spanning domains are based on changes in the molecular mass of the protein, such as the changes that occur with proteolysis[4], cysteine modification by the Maleimide [5-6], and protease digestion protection [7]. A disadvantage of these methods is the requirement to isolate the post nuclear supernatant (PNS) of cells and disrupt the cell membrane with mild detergent; substantial investments of time and effort are needed for these steps, which may easily produce artificial results. Another strategy uses a fluorescent protein combined with protection against protease digestion protection (fluorescence protease protection, FPP) [8]. Although useful and no need to isolate PNS, this technique still requires to disrupt the cell membrane[8]. Moreover, in some cases, the addition of a GFP tag interferes with the proper localization and function of the protein[9-11].

Here, we developed an efficient technique that combines a self-complementing split mNeonGreen2 (mNG2) with site-directed labeling (SSDL) to determine the topology of transmembrane proteins in living mammalian cells. This method does not involve "poking holes" in a cell membrane or separating PNS fractions, and the orientation of the N- and C-termini of membrane-spanning segments can be detected reliably and relatively rapidly. Using this method, we demonstrated that etoposide-induced protein 2.4 (EI24), a protein whose predicted topology still varies depending on the structural prediction program, has an even number of transmembrane segments and that both termini have a cytosolic orientation.

2 MATERIALS AND METHODS

2.1 Cell culture and transfection

HEK293T cells (obtained from American Type Culture Collection, ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% heat-inactivated bovine calf serum (HyClone) in an incubator at 37℃ with 5% CO_2. U2OS cells (ATCC) were cultured in McCoy's modified 5A medium (MCMM, Gibco) containing 10% heat-inactivated bovine calf serum in an atmosphere of 5% CO_2 and 95% air at 37 ℃. We performed transient transfections of cells using Lipofectamine® 2000 reagent (Thermo) according to the manufacturer's instructions. For imaging, the cells were grown on glass bottom dishes (Cellvis) and analyzed 24 h after transfection.

2.2 Plasmid construction

The DNA sequence of mNG2 [12] was directly synthesized (IDO Biotechnology Company, Beijing, China). The mNG2 sequence was split between the tenth and eleventh β-strands at amino acid 214 to obtain the $mNG2_{1-10}$ and $mNG2_{11}$ fragments. The $mNG2_{1-10}$ DNA was PCR amplified and cloned into the *Age* Ⅰ and *Bgl* Ⅱ sites of EGFP-C1 (Clontech, California, USA) and the same restriction enzyme cutting sites of DsRed2-ER (Clontech) to replace the *EGFP* or *DsRed2* gene, thus generating $mNG2_{1-10}$-Cyto and $mNG2_{1-10}$-ER, respectively. The full-length *Homo sapiens P450* (CYP2C9, NM_000771.3) cDNAs with *Age* Ⅰ and *Bgl* Ⅱ sites were PCR amplified and cloned into EGFP-C1 to replace the *EGFP* gene to generate CYP-C1 vector. To generate CYP-$mNG2_{11}$, $mNG2_{11}$ cDNA containing *Eco*R Ⅰ and *Bam*H Ⅰ sites were PCR amplified and inserted into the CYP-C1 vector. The cDNA of Calnexin (CNX) (*Homo sapiens*, NM_001363993.1) was cloned into an EGFP-N1 vector using the *Bam*H Ⅰ and *Not* Ⅰ sites to replace *EGFP* to generate CNX-N1 vector. The $mNG2_{11}$ sequence was cloned into the CNX-N1 plasmid with *Nhe* Ⅰ and *Bgl* Ⅱ sites to generate CNX-$mNG2_{11}$. To label the N- and C- termini of EI24 (*Homo sapiens*, NM_004879.4) with $mNG2_{11}$ separately, the gene encoding *Ei24* was first PCR amplified and inserted into the *Hin*d Ⅲ and *Sal* Ⅰ sites of mEmerald-C1 and the *Nhe* Ⅰ and *Bgl* Ⅱ sites of mEmerald-N1(Addgene), and mEmerald was then replaced with $mNG2_{11}$ to generate the $mNG2_{11}$-Ei24 and Ei24-$mNG2_{11}$ plasmids.

All of the fast digest restriction enzymes were purchased from Thermo Fisher Scientific (Waltham, MA). Q5 high-fidelity DNA polymerase and T4 ligase were purchased from New England Biolabs (Ipswich, MA).

2.3 Confocal microscopy and cell imaging

Confocal microscopy images were obtained by an FV1200 laser-scanning confocal microscope

(Olympus). To verify that the plasmids were constructed correctly, images were taken using a 100×, 1.4 numerical aperture (NA) oil-immersion objective. To identify the topology of EI24, images were taken with a 10× objective. The maximum power near the rear pupil of the objective was 0.017 mw for the 488-nm laser. All of the images were analyzed and processed by ImageJ software.

3 RESULTS AND DISCUSSION

3.1 Establishment of the SSDL assay

The ER is a single, continuous membrane system, and as the largest organelle in eukaryotic cells, the ER is the major site for synthesizing proteins and lipids, maintaining calcium homeostasis, and folding and modifying proteins in the ER lumen [13−15]. Identifying the topology of an ER transmembrane protein is a prerequisite for studying its functions and finding new interacting proteins. In developing a self-complementing split fluorescent protein-based assay for determining ER transmembrane protein topology within cells, we split mNG2, which has good photostablility, acid tolerance and monomeric quality, and higher brightness than that of super-folder GFP[16], into two parts from the loop region between the tenth and eleventh β-strands to obtain the $mNG2_{1-10}$ and $mNG2_{11}$ fragments as reported[12]. These two fragments associate with each other spontaneously to form fluorescent mNG2. Next, $mNG2_{1-10}$, which is almost nonfluorescent until complementation, was constructed as $mNG2_{1-10}$-Cyto and $mNG2_{1-10}$-ER, which localize to the cytosol and ER lumen, respectively (Figure 1). The $mNG2_{11}$ tag (16-amino acid short peptide) was fused to the N- or C- terminus of a protein of interest (POI) and named $mNG2_{11}$-POI or POI-$mNG2_{11}$. Subsequently, cells were cotransfected with (a) $mNG2_{1-10}$-ER and $mNG2_{11}$-POI, (b) $mNG2_{1-10}$-ER and POI-$mNG2_{11}$, (c) $mNG2_{1-10}$-Cyto and POI-$mNG2_{11}$, or (d) $mNG2_{1-10}$-Cyto and $mNG2_{11}$-POI, as shown in Figure 1. Due to the integrity of the ER, if

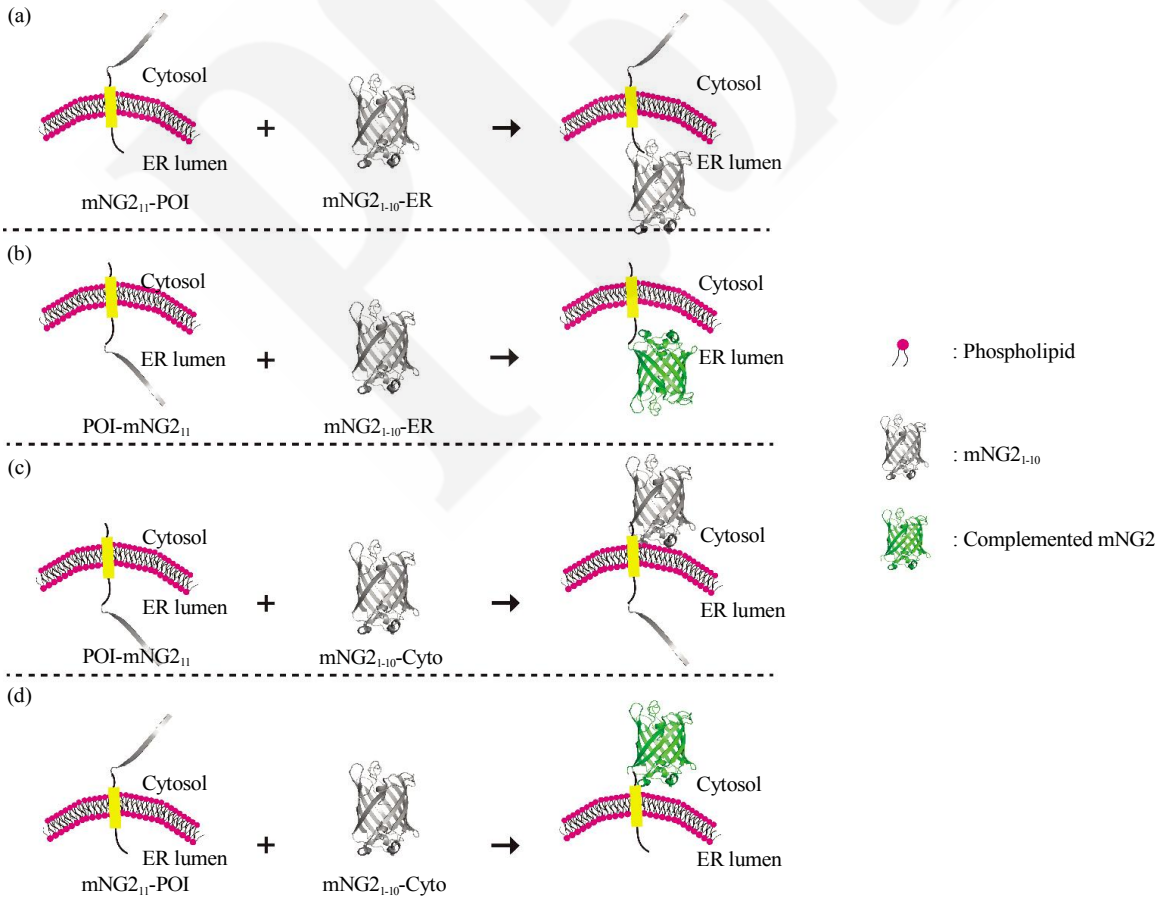

Fig. 1 Schematic of the SSDL assay for determining the topology of ER transmembrane protein

(a) and (b) Coexpression of $mNG2_{1-10}$-ER with $mNG2_{11}$-POI (a) or POI-$mNG2_{11}$(b). (c) and (d) Coexpression of $mNG2_{1-10}$-Cyto with POI-$mNG2_{11}$ (c) or $mNG2_{11}$-POI (d). When $mNG2_{11}$ and $mNG2_{1-10}$ have the same orientation (b and d), the fully functional mNG2 can form, and green fluorescence can be observed.

the orientation of the terminus of the POI with $mNG2_{11}$ is different from the orientation of $mNG2_{1-10}$-ER, the two fragments, $mNG2_{11}$ and $mNG2_{1-10}$, cannot form a fully functional mNG2, and there is no fluorescence signal (Figure 1a). Conversely, if one terminus of the POI with $mNG2_{11}$ faces the ER lumen, the two parts of mNG2 will assemble into a fluorescent protein, and fluorescence can occur (Figure 1b). Thus, by tagging $mNG2_{11}$ at different termini of a POI and using ER-localized $mNG2_{1-10}$-ER or cytosol-localized $mNG2_{1-10}$-Cyto, the topology of the protein can easily be determined by a fluorescence readout.

3.2 Verification the SSDL-based system for protein topology identification

For a proof of principle, we first applied the SSDL-based method to determine SSDL could reveal the membrane topology of the ER proteins CYPs, which are membrane-bound hemoproteins that play a pivotal role in the detoxification of xenobiotics, cellular metabolism and homeostasis[17]. Alterations in CYP topology have been observed recently in which the paralog with one transmembrane helix had the C terminus facing the cytoplasm and the N terminus anchored at ER membrane, while the paralog with two transmembrane helices showed a dual topology facing either the cytosol or the ER lumen [18]. Here, the $mNG2_{11}$ fragment was attached to the C-terminal end of CYP2C9 (Figure 2a), which is a member of CYP superfamily and has a single-pass transmembrane protein with its C terminus facing the cytoplasm. CYP2C9-$mNG2_{11}$ was coexpressed with $mNG2_{1-10}$-ER or $mNG2_{1-10}$-Cyto. The results showed that the fluorescence was observed only in the CYP2C9-$mNG2_{11}$/$mNG2_{1-10}$-Cyto group (Figure 2b), while not in the CYP2C9-$mNG2_{11}$/$mNG2_{1-10}$-ER group (Figure 2c). High-resolution images were obtained to further confirm the localization of the C terminus of CYP2C9 facing the cytosol (Figure 3a), which is consistent with the reported structure of CYP2C9[19].

We further evaluated the applicability of the SSDL-based assay by testing whether this technique could properly define the topology of other transmembrane proteins localized to the ER. CNX, a well-characterized transmembrane chaperone, consists of an N-terminal calcium-binding ER luminal domain that facilitates protein folding and assembly and a short, acidic cytoplasmic tail[20–21]. We attached $mNG2_{11}$ to the N-terminal end of CNX. In cells coexpressing

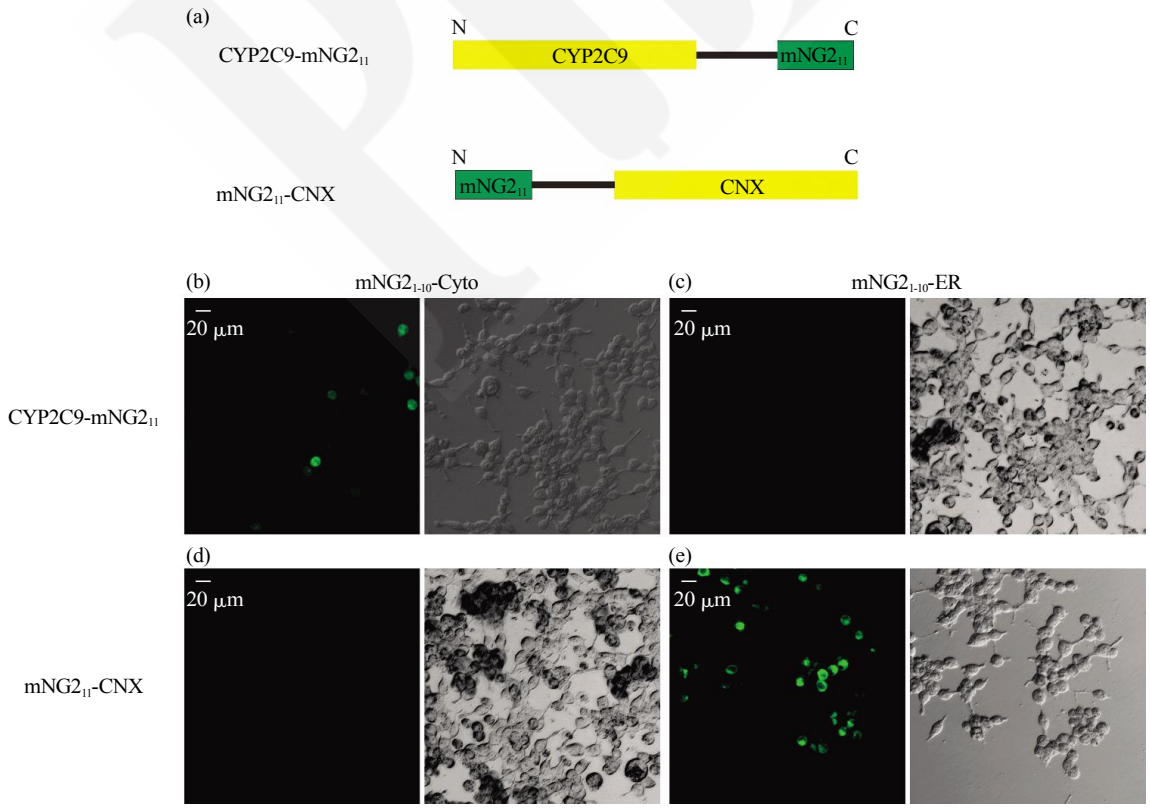

Fig. 2 Verification of the SSDL assay

(a) Schematic of CYP2C9-$mNG2_{11}$ and $mNG2_{11}$-CNX constructions. HEK293T cells coexpressing CYP2C9-$mNG2_{11}$ with $mNG2_{1-10}$-Cyto (b) or $mNG2_{1-10}$-ER (c). HEK293T cells coexpressing $mNG2_{11}$-CNX with $mNG2_{1-10}$-Cyto (d) or $mNG2_{1-10}$-ER (e).

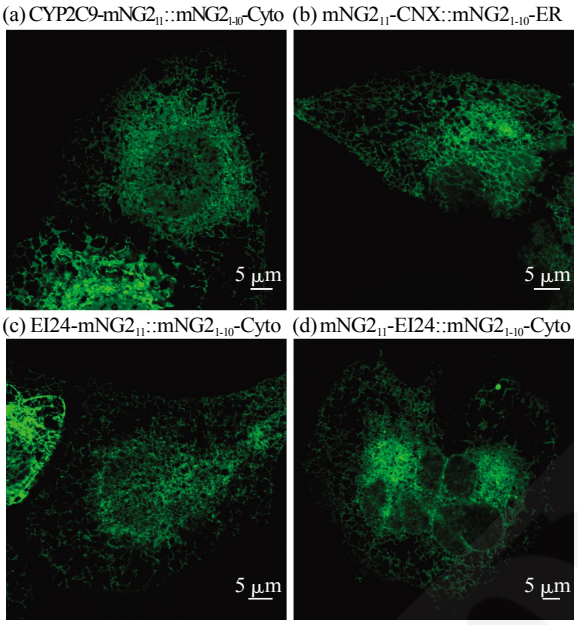

Fig. 3 Protein localization in high resolution images

U2OS cells coexpressing (a) CYP2C9-mNG2$_{11}$ with mNG2$_{1-10}$-Cyto, (b) mNG2$_{11}$-CNX with mNG2$_{1-10}$-ER, (c) EI24-mNG2$_{11}$ with mNG2$_{1-10}$-Cyto, and (d) mNG2$_{11}$-EI24 with mNG2$_{1-10}$-Cyto.

mNG2$_{11}$-CNX and mNG2$_{1-10}$-ER, the fully functional and fluorescent mNG2 was formed (Figure 2e and Figure 3b), while fluorescence was not observed in the mNG211-CNX/mNG2$_{1-10}$-Cyto transfected group (Figure 2d), which further demonstrated that CNX mediates the folding of newly synthesized proteins in the lumen of the ER.

3.3 SSDL applied to identify EI24 topology

Ei24 is a DNA damage response gene induced by the tumor suppressor p53 [22]. It has been reported that the protein encoded by *Ei24* localizes to the ER membrane [23–24] and has proapoptosis [25], cancer suppression [23], autophagy maintenance [24], and ER calcium homeostasis [26] functions. Despite the important roles of EI24 in biological function, the crystal structure of EI24 is still unknown, and the predicted topology of EI24 varies among different protein structure prediction programs. The predicted number of transmembrane segments is five, according to the UniProt database(http://www.uniprot.org/uniprot/O14681), which indicated that proteins with an uneven number of membrane-spanning domains assuming a final topology with an ER luminal N-terminus and a cytoplasmic C-terminus or with the opposite orientation. In contrast, the SMART database indicates that the number of transmembrane segments is six (http://smart.embl-heidelberg.de/). A protein with an even number of transmembrane helixes may have two possible topologies with two locations for the N- and C-termini, either the cytosol or the ER lumen. Using the protease digestion protection method, Lieu *et al* [7]. showed the N-terminus of EI24 faces the cytosol. However, the C-terminal orientation cannot be determined since the numbers of transmembrane segments differ between the two protein structure prediction programs. To identify the topology of EI24, we fused the fragment of mNG2$_{11}$ to the N- or C-terminal ends of EI24 (Figure 4a) and coexpressed the fusion product with ER-localized mNG2$_{1-10}$-ER or cytosol-localized mNG2$_{1-10}$-Cyto. The results showed fluorescence for either the N- or C-terminal portions of mNG2$_{11}$-labeled EI24 coexpressed with mNG2$_{1-10}$-Cyto but not with mNG2$_{1-10}$-ER, which indicated that both the N- and C-termini of EI24 face the cytosol (Figure 4c-f and Figure 3c, d) and that the number of transmembrane segments of EI24 is even (Figure 4b).

The correct topological identification is essential for proper functioning of transmembrane proteins. EI24 interacts with BCL-2 through N- terminal residues 52–115 to mediate tumor invasiveness [23]. Since the N-terminal end of EI24 is exposed to the cytosol, the interaction between EI24 and Importin family members in the cytoplasm to inhibit p53 nuclear import may occur under normal physiological conditions [7]. We found that EI24 interacts with ATP2A2 through C-terminal residues 293–299 to regulate ATP2A2 activity and calcium homeostasis, further determining the destiny of pancreatic β cells[26]. The cytosolic orientation of the C-terminus of EI24 may facilitate the interaction with ATP2A2 to sense, respond to or participate in feedback with changes in the cellular environment.

The SSDL assay described here provides a new approach for determining protein topology in cells. Compared with previous methods, SSDL offers several advantages. First, the mNG2$_{11}$ fragment is very short and does not interfere with the proper folding, localization, and function of the fusion protein. Second, unlike other biochemical methods, the SSDL assay does not require the isolation of cell PNS for Western blot detection, which means that only a relatively small number of cells expressing the mNG2$_{11}$ fragment are needed to determine the protein topology.

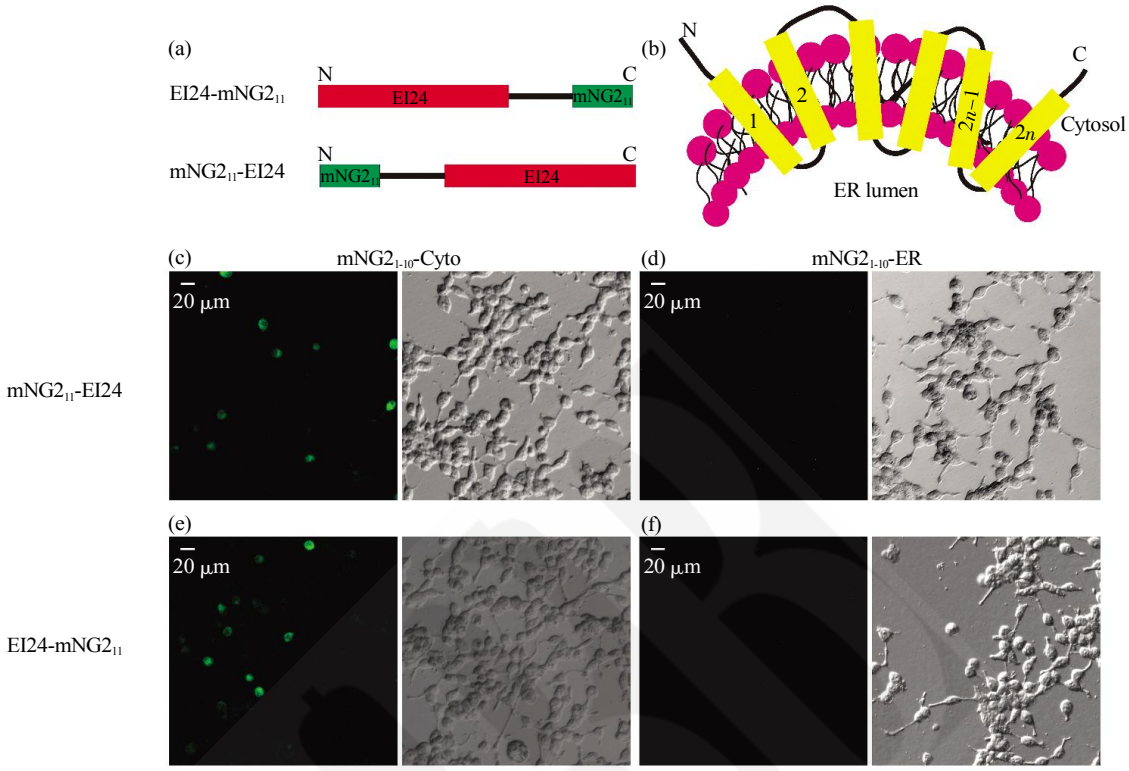

Fig. 4 The SSDL assay reveals the topology of the ER transmembrane protein EI24

(a) Schematic of the EI24-mNG2$_{11}$ and mNG2$_{11}$-EI24 constructs. (b) Cartoon of the topology of EI24 with both termini oriented to the cytosol, n represents for natural number ⩾ 1. HEK293T cells expressing mNG2$_{11}$-EI24 with mNG2$_{1-10}$-Cyto (c) or mNG2$_{1-10}$-ER (d) were subjected to the SSDL assay. The cells coexpressing EI24-mNG2$_{11}$ with mNG2$_{1-10}$-Cyto or ER mNG2$_{1-10}$-ER are shown in (e) and (f).

Third, the use of the SSDL assay eliminates the need to modify a specific amino acid or permeabilize the cell membrane, a process that can easily introduce false negative or false positive results. The SSDL assay is limited in its ability to determine the topologies of a protein whose terminus is embedded in a lipid bilayer. Therefore, combining computational approaches examining the relatively hydrophobic regions of a protein with the SSDL assay may facilitate the determination of protein topology.

In addition to resolve the topologies of transmembrane proteins in the ER, the SSDL assay is applicable to other cell systems, such as mitochondria, the Golgi and endosomes. Since the red-colored self-complementing split fluorescent protein sfCherry2$_{1-10/11}$ has already been reported, the SSDL assay can be used to resolve the topologies of two proteins at the same time *via* two colors. It has been reported that transmembrane helices can dynamically reorient across the membrane in response to drastic changes in lipid composition [27]. Accordingly, the SSDL assay may be used to determine the change in topology in response to stimuli.

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一种利用荧光蛋白 mNeonGreen2 鉴定 EI24 蛋白拓扑结构的方法 *

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摘要 方便且精准地检测跨膜蛋白拓扑结构，尤其是跨膜片段的氨基(N-)和羧基(C-)端的朝向，有利于发现新的蛋白质与蛋白质之间的相互作用，并进一步揭示蛋白质重要的生物学功能．自组装荧光蛋白已被广泛用于观察蛋白质与蛋白质之间的相互作用、标记细胞内源蛋白质并实现 mRNA 定位的可视化．本文扩展了自组装荧光蛋白的应用，将自组装荧光蛋白 mNeonGreen2 与定点标记技术相结合，以确定跨膜蛋白的拓扑结构．通过该方法，第一次清楚地证明了 EI24 的 N 端和 C 端均朝向细胞质方向．此外，该方法可用于确定定位于其他细胞器且结构尚未解析的跨膜蛋白的拓扑结构．

关键词 mNeonGreen2，定点标记，跨膜蛋白，EI24

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