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Targeting of inositol 1,4,5-trisphosphate receptor to the endoplasmic reticulum by its first transmembrane domain

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SYNOPSIS

Targeting of inositol 1,4,5-trisphosphate receptors (IP₃R) to membranes of the endoplasmic reticulum (ER) and their retention within ER or trafficking to other membranes underlies their ability to generate spatially organized Ca²⁺ signals. N-terminal fragments of type 1 IP₃R (IP₃R1) were tagged with enhanced green fluorescent protein, expressed in COS-7 cells and their distribution was determined by confocal microscopy and subcellular fractionation. Localization of IP₃R1 in the ER requires translation of between 26 and 34 residues beyond the end of the first transmembrane domain (TMD1), a region that includes TMD2. Replacement of these post-TMD1 residues with unrelated sequences of similar length (24-36 residues) partially mimicked the native residues. We conclude that for IP₃R about 30 residues after TMD1 must be translated to allow a signal sequence within TMD1 to be extruded from the ribosome and mediate co-translational targeting to the ER. Hydrophobic residues within TMD1 and TMD2 then ensure stable association with the ER membrane.

Page heading: ER targeting of IP₃ receptors.

Key words: Ca²⁺ signalling, endoplasmic reticulum, inositol 1,4,5-trisphosphate receptor (IP₃R), protein targeting, signal sequence.

Abbreviations used: BSA, bovine serum albumin; EGFP (EYFP), enhanced green (yellow) fluorescent protein; ER, endoplasmic reticulum; IP₃R, inositol 1,4,5-trisphosphate receptor; PBS, phosphate-buffered saline; SRP, signal recognition particle; TMD, transmembrane domain. The abbreviations used for fragments of IP₃R1 are defined in Figure 1C.

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INTRODUCTION

Inositol 1,4,5-trisphosphate receptors (IP₃R) are the intracellular Ca²⁺ channels that both initiate and regeneratively propagate the cytosolic Ca²⁺ signals evoked by the many receptors that stimulate IP₃ formation [1]. All IP₃R are tetramers, each with an IP₃-binding site lying close to the N-terminal and six transmembrane domains (TMD) lying close to the C-terminal (Figure 1A). The last pair of TMD from each subunit together with their intervening luminal loop form the pore [2, 3]. In most animal cells, IP₃R are expressed mainly within the membranes of the endoplasmic reticulum (ER), but they are also expressed within the nuclear envelope [4], nucleoplasmic reticulum [5], Golgi apparatus [6], plasma membrane [7] and perhaps also in secretory vesicles [8], although the latter is contentious [9]. Within these membranes, IP₃R are not uniformly distributed and different subtypes may differ in their distributions [10, 11]. The subcellular distribution of IP₃R accounts for their ability to generate cytosolic Ca²⁺ signals that are spatially organized, thereby allowing Ca²⁺ to regulate specifically a diverse array of cellular processes [1]. The versatility of Ca²⁺ as a ubiquitous intracellular messenger thus depends upon precise targeting of IP₃R to specific subcellular compartments.

Whatever the final destination of an IP₃R, it must first be directed to the ER, where it may either be retained (the fate of most IP₃R) or be allowed to move on to other membranes via the Golgi apparatus. Targeting of proteins to the ER is mediated by a short stretch of amino acid residues, the signal sequence, which may be either an N-terminal sequence that is later cleaved, or an internal, non-cleavable sequence [12]. The latter, signal-anchor sequences, serve the dual purpose of directing the protein to the ER and anchoring it within the membrane. Signal sequences vary widely in primary sequence, but in both prokaryotes and eukaryotes they share a hydrophobic core of 8-12 residues for cleavable signals and of 20-30 residues for internal signals [13]. The diversity of signal sequences allows them to function with different efficiencies and also provides a mechanism that allows proteins that might become terminally misfolded to be directed away from the ER for degradation during ER stress [14].

The signal sequence, whether N-terminal or internal, is recognized by the signal recognition particle (SRP). For most eukaryotic secretory or membrane proteins, this occurs co-translationally [15], but a minority of proteins (those with a C-terminal signal sequence) are post-translationally targeted [16]. Co-translational targeting is initiated when SRP binds simultaneously to the exposed signal sequence and the ribosome, forming the SRP-ribosome nascent chain complex [15]. SRP may also recognize a conformation of the ribosome within which a signal-anchor sequence is still concealed and so pre-associate with the ribosome before binding tightly to the emerging signal sequence [17]. The SRP-ribosome nascent chain complex then binds to the SRP receptor within the ER membrane [18], SRP dissociates, protein synthesis resumes, and the growing protein is directed into the ER through the open translocon. The latter is a channel formed largely from the Sec61 complex that allows proteins to pass into either the lumen of the ER or laterally into the ER membrane [19, 20].

After incorporation into the ER membrane, proteins may either remain there or move on. Proteins remain because they express signals that prevent them from leaving the ER or promote their retrieval from the Golgi apparatus. Luminal ER proteins are retrieved by a C-terminal KDEL motif, while integral membrane proteins are retrieved from post-ER compartments by cytosolic motifs such as the C-terminal di-lysine or N-terminal di-arginine motif [21, 22]. TMD can also mediate ER retention [23-25]. None of the cytosolic motifs known to mediate ER retrieval are present in IP₃R, but our earlier work demonstrated that any pair of TMD with a linking luminal loop can retain an IP₃R fragment or a plasma membrane protein within the ER [26].

IP₃R lack an N-terminal signal sequence, but as with other ER membrane proteins, hydrophobic residues within the TMD can provide internal signal sequences. The type 1 ryanodine receptor (RyR1), for example, is targeted to the ER by its first TMD [27] and a sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA1) is targeted and retrieved by its first pair of TMD [28]. For IP₃R1, the TMD region is sufficient for ER targeting [29, 30], with later work suggesting that the first pair of TMD is essential [3, 26]. Our analysis of fragments comprising individual TMD or pairs of TMD demonstrated that any pair of TMD linked by their luminal loops is sufficient to localize IP₃R1 to the ER [26]. These results, derived from short fragments of IP₃R1 lacking the N-terminal, suggest that IP₃R1 is targeted to the ER only after translation of the first and second TMD and the following loops. This highlights residues lying between the beginning of TMD1 and the end of the cytosolic loop following TMD2 (Figure 1B) as the most

likely determinants of IP₃R1 targeting to the ER. However, the relative roles of residues within this region when IP₃R1 has its normal large cytosolic N-terminal are not clear. Here we address this issue by systematically examining the subcellular distribution of IP₃R1 fragments progressively truncated from the C-terminal.

EXPERIMENTAL

Expression constructs

All constructs are based on the full-length rat IP₃R1 [31] (GenBank accession number GQ233032) lacking the S1 splice region [32]. Most proteins were N-terminally tagged with enhanced green fluorescent protein, EGFP (

excited with the 488 nm, 514 nm, 561 nm, 594 nm and 633 nm lines, respectively. Emitted signals were collected using emission filters with detection bands of 500-565 nm, 520-600 nm (520-560 nm when imaged together with Mitotracker and 520-540 nm with DsRed), 565-675, 600-650 nm and 640-750 nm, respectively. In all dual-labelling analyses, we confirmed that there was no bleed-through between the two wavelengths. All images were exported as tiff files and processed using Adobe Photoshop.

Most confocal images are shown to highlight a single typical cell. Views of fields of cells are shown in Supplementary Figure S2. Because the cytosol and ER are entwined, the distinction between them is not always immediately obvious in confocal images. The difference between reticulate and cytosolic distributions is more clearly discernible at the cell boundary. The ER extends to the periphery without clear boundaries, while the cytosol clearly defines the cell boundary. Images of the cell periphery are shown at higher magnification in each figure and in Supplementary Figure S3. To allow a more quantitative analysis of the co-localization of IP₃R fragments and calreticulin immunostaining (Table 1), we adopted the method shown in Supplementary Figure S4. Briefly, three lines were drawn across each cell to exclude the nucleus, and the Pearson correlation coefficient (r) was computed for the relationship between the intensities of the two fluorescence channels (green for IP₃R fragments, and red for calreticulin). For an IP₃R construct known to be expressed primarily within membranes of the ER (FL) r was 0.70 ± 0.07 , and for a construct known to be cytosolic (NT) r was 0.37 ± 0.06 (Table 1). These values of r , for an ER and cytosolic protein, are those against which all other fragments are compared statistically in Table 1.

Subcellular fractionation and western blotting

Cells were harvested 24 h after transfection, washed with PBS, scraped into 250 μ l ice-cold PBS containing protease inhibitors (Roche, 1 tablet/10 ml) and disrupted by 30 passages through a 25-gauge needle. After centrifugation (30,000 \times g, 30 min), the supernatant (S1), containing cytosolic proteins, was saved. The pellet was resuspended in 250 μ l Na₂CO₃ (0.1 M, pH 11.5), incubated on ice for 45 min to dissociate peripheral membrane proteins, and after further centrifugation (30,000 \times g, 30 min), the second supernatant (S2) containing peripheral membrane proteins was saved. The pellet (P) containing integral membrane proteins was resuspended in 250 μ l of cold PBS containing protease inhibitors and 1% Triton X-100.

Samples (corresponding to equivalent numbers of cells for each fraction) were loaded onto pre-cast NuPAGE 3-8% Tris-Acetate or 4-12% Bis-Tris gels (Invitrogen). SDS-PAGE (XCell SureLock Mini-Cell, Invitrogen) and transfer to PVDF membrane (XCell II Blot Module or iBlot dry gel system, Invitrogen) were performed according to the manufacturer's instructions. Membranes were blocked overnight in PBS containing 0.1% Tween (PBS-T) supplemented with 1% BSA, incubated for 1 h with a rabbit polyclonal antibody to GFP (AbCam, 1:1000 in PBS-T with 1% BSA), washed (3 x 10 min) with PBS-T, and then incubated with a secondary donkey anti-rabbit antibody coupled to horseradish peroxidase (HRP) (Santa Cruz, 1:5000 in PBS-T with 1% BSA). Membranes were washed (3 x 10 min) with PBS-T, and HRP was detected using Supersignal West Pico Chemiluminescent substrate (Pierce). Immunoreactive bands were quantified using GeneTools software (Syngene).

RESULTS AND DISCUSSION

ER localization of N-terminally truncated IP₃R requires more than TMD1

Full length IP₃R1 tagged at its N-terminal with EYFP (FL, Figure 1C) was localized in the ER of COS-7 cells. It exhibited strong perinuclear fluorescence that extended towards the periphery of the cell in a tubular network and it co-localized with calreticulin, an ER luminal protein (Figure 2A, Table 1 and Supplementary Figure S2). After subcellular fractionation, FL was found mostly ($88 \pm 4\%$) in the P fraction (integral membrane proteins, see Experimental). EYFP (not shown) or EGFP alone was diffusely spread throughout the cell including the nucleus, and did not co-localize with calreticulin (Figure 2B). EGFP was found largely ($77 \pm 5\%$) in the S1 fraction (cytosolic proteins) (Figure 2B). An EYFP-tagged fragment of IP₃R1 (TMD1-2) that includes only the last 58 residues of the N-terminal cytosolic region and extends to the end of the cytosolic loop following TMD2 had a distribution similar to that of FL and it co-localized with calreticulin (Table 1). TMD1-2 was present largely ($84 \pm 5\%$) in the P fraction (Figure 2C). A similar, but shorter, IP₃R1 fragment (TMD1) truncated after 8 of the 12 residues linking TMD1

to TMD2 did not co-localize with calreticulin (Figure 2D), but instead co-localized with Mitotracker Red (Figure 2E) [26]. As expected [26], there was no such co-localization of TMD1-2 with mitochondria (Supplementary Figure S5). TMD1 was also found largely in the P fraction ($82 \pm 6\%$). The presence of TMD1 in the P fraction highlights the limitations of using simple fractionation methods alone to resolve the targeting of IP₃R fragments to the ER. A fragment (TMD2) comprising only the last 8 residues of the loop linking TMD1 to TMD2 and terminating 12 residues after the end of TMD2, was also expressed in mitochondria, but not in the ER (not shown) [26]. Western blotting with an anti-GFP antibody confirmed that the expressed proteins had the expected sizes (Figure 2A-D).

These results confirm earlier work showing that in these minimal C-terminal fragments of the IP₃R1, neither of the first two TMD is alone sufficient to allow expression in the ER, while together they mediate effective ER localization [26]. Subsequent experiments aim firstly to establish whether there is a similar requirement for localization of full-length IP₃R in the ER and secondly to define the role of these residues in ER targeting. To identify the first ER targeting signal in native IP₃R1, we examined the distribution of fragments of IP₃R1 truncated only at the C-terminal (Figure 1C). For simplicity, the truncated IP₃R constructs used to address these issues, all of which have the same N-terminal, are abbreviated by reference to the number of residues after TMD1 (Figure 1C).

Localization of IP₃R1 in the ER requires translation of TMD1 and TMD2

In keeping with previous reports [29, 30], the large N-terminal fragment of the IP₃R1 preceding TMD1 (NT) was cytosolic. It was diffusively distributed throughout the cytosol, excluded from the nucleus, and it did not co-localize with calreticulin (Table 1 and Figure 3A). After subcellular fractionation very little of the NT ($4 \pm 1\%$) was detected in the P fraction (integral membrane proteins) (Figure 3A). The latter is consistent with an earlier conclusion, although in that study there was more contamination of the membrane fraction with the N-terminal fragment [3].

A fragment of IP₃R1 truncated part way through the loop following TMD1 (NT[8]) had a similar distribution to NT. It was diffusely spread from around the nucleus to the plasma membrane, uniformly defined the boundaries of the cell, it was not co-localized with calreticulin, and very little of the protein ($17 \pm 2\%$) was detected in the P fraction after subcellular fractionation (Figure 3B and Table 1). It is noteworthy that while neither the long (NT[8]) nor short (TMD1) fragment truncated after TMD1 are localized to the ER, the former remains cytosolic, while the latter is expressed in mitochondria (Figure 2E) [26]. We speculate that TMD1 is released from the ribosome before the signal sequence can be recognized by SRP (see later discussion) and the basic residues flanking TMD1 then favor post-translational targeting to mitochondria [34].

Addition to NT[8] of the four remaining residues from the loop following the first TMD (to give NT[12], Figure 1C), had no effect on the distribution; NT[12] was cytosolic (Figure 3C and Table 1). Extending the N-terminal fragment further to include TMD2 and the following loop (NT[54+8]), caused the protein to be localized in the ER. The distribution of NT[54+8] was indistinguishable from that of FL, it defined the cell boundary in a reticulate fashion, it co-localized with calreticulin (Table 1), and most protein ($87 \pm 6\%$) was found in the P fraction (Figure 3D). NT[54+8], which includes the entire N-terminal region of IP₃R1, has the same native C-terminal residues as the much smaller fragment (TMD1-2), although NT[54+8] has an additional C-terminal FLAG tag comprising 8 residues. Both fragments were similarly expressed in the ER (Figures 2C and 3D). These results suggest that for both short fragments and native IP₃R1, translation of TMD1 and TMD2 are required for effective localization in the ER.

The minimal requirement for localization of IP₃R in the ER is translation of 26-34 residues after TMD1

To define more specifically the minimal requirements for ER localization, we used constructs truncated within TMD2 or the succeeding loop. IP₃R1 truncated just four residues into TMD2 (NT[16]) was diffusively expressed. It did not obviously co-localize with calreticulin (Figure 4A), with the quantitative analysis ($r = 0.61 \pm 0.16$) suggesting a distribution intermediate between that of an ER protein (FL, $r = 0.70 \pm 0.07$) and cytosolic protein (NT, $r = 0.37 \pm 0.06$) (Table 1). Its definition of the cell boundary was similar to that of cytosolic fragments (Supplementary Figure S3). After subcellular fractionation, however, most NT[16] was in the P fraction ($66 \pm 6\%$).

Results from confocal and fractionation assays concur where proteins are entirely cytosolic (eg, NT, Figure 3A) or entirely localized in ER (eg, FL, Figure 2A), but quantitative analysis of cell fractions seems better able to resolve incomplete targeting than is the more qualitative assessment of confocal images (Figures 3 and 4).

A fragment (NT[26]) that extends 14 residues into TMD2 appeared to be cytosolic in most cells (Figure 4B and Table 1), but in a minority of cells (~20%) the distribution was reticulate and similar to that of FL (Supplementary Figure S6); $84 \pm 3\%$ of NT[26] was in the P fraction. A slightly longer fragment (NT[34]) that includes TMD2 and four residues from the following loop was localized in the ER, as was a fragment extended by a further ten residues (NT[44]). For both fragments, the distribution was reticulate, they co-localized with calreticulin (Figure 4C and D, and Table 1), and they were predominantly found in the P fraction ($81 \pm 4\%$ and $88 \pm 5\%$ for NT[34] and NT[44], respectively). These results define the minimal number of residues beyond TMD1 that must be translated to allow effective localization of IP₃R1 in the ER: 12 residues are not sufficient (Figure 3C), 26 residues are partially effective (Figure 4B and Supplementary Figure S6), and 34 residues after TMD1 allow the protein (Figure 4C) to be targeted to the ER as effectively as FL (Figure 2A and Table 1). We conclude that between 26 and 34 residues beyond the end of TMD1 must be translated for native IP₃R1 to be effectively localized in the ER (Figure 5A).

Another analysis of similar IP₃R1 fragments truncated at the C-terminus but with a C-terminal tag (11 residues) demonstrated that when these were expressed in COS-1 cells, a fragment truncated six residues after TMD1 (NT[6+11]) was equally distributed between cytosolic and membrane fractions, whereas all constructs longer than NT[35+11] were entirely in the membrane fraction (Figure 5A) [3]. Using an *in vitro* translation system, a construct that included 21 residues before TMD1, nine native residues after it, and a C-terminal tag of 42 residues (NT[9+42]) was effectively co-translationally inserted into microsomal membranes [35]. NT[9+42] and NT[35+11] have similar numbers of residues after TMD1 (51 and 46, respectively). Although the results from these analyses *in vitro* (suggesting a requirement for only TMD1) [35] and cells (suggesting a requirement for TMD1 and TMD2) [3] were thought to be contradictory, they are each consistent with a need for ≤ 51 residues after TMD1 to be translated to allow ER localization of IP₃R1. We can now refine that requirement and conclude that between 26 and 34 residues beyond the end of TMD1 must be translated for IP₃R1 to be targeted to the ER.

Residues after TMD1 mediate both exposure of the signal sequence and membrane-anchoring of IP₃R

Signal sequences bind to SRP only after about ten residues have emerged from the ribosomal tunnel [36], which is ~10 nm long and can accommodate 30-40 unfolded residues [37]. The requirement for translation of 26-34 residues after TMD1, which includes TMD2 and some of the following loop (Figure 1B and C), may therefore reflect a need for these specific residues to contribute to ER targeting or they may be required only to allow exposure of a signal sequence in TMD1 (Figure 5A). The latter would be consistent with the analysis *in vitro* showing that TMD1 with the following loop mediates co-translational targeting when it is followed by a sequence of 42 residues unrelated to the IP₃R [35].

If the post-TMD1 residues serve only to expel the signal sequence from the ribosome tunnel, any sequence of 30-40 residues after the signal sequence within TMD1 would be sufficient to allow ER targeting. We therefore expressed IP₃R1 with the loop following TMD1 duplicated to provide 24 residues beyond the end of TMD1 (NT[2x12]) or with three repeats to provide 36 residues (NT[3x12]) (Figure 1C). These proteins are similar in length to the shortest fragments that were partially (NT[26], Figure 4B) or completely (NT[34], Figure 4C) localized in the ER. Both NT[2x12] and NT[3x12] were found largely in the P fraction ($63 \pm 4\%$ and $58 \pm 5\%$, respectively). Their integration into membranes is therefore much greater than for NT[12] ($17 \pm 1\%$), but clearly less than for NT[26] ($84 \pm 3\%$) and NT[34] ($81 \pm 4\%$). These results suggest that a major role of the post-TMD1 residues is to allow a hydrophobic signal sequence within TMD1 to be pushed out of the ribosome tunnel and allow its recognition by SRP.

However, the replicated (hydrophilic) residues from the post-TMD1 loop were not as effective as the native (hydrophobic) residues of TMD2 in causing IP₃R1 fragments to associate with the P fraction (~60% vs ~80%), and nor were the fragments with replicated loops clearly co-localized with the ER in confocal images (Figure 5B and C, and Table 1). We suggest that in addition to providing residues that allow the signal sequence to be extruded from the ribosome,

hydrophobic residues within TMD2 also contribute to anchoring the large IP₃R fragments in the ER membrane.

Conclusions

Co-translational targeting of IP₃R to the ER [35] is the first step in the sequence of events that leads to IP₃R being precisely located within intracellular membranes and thereby placed to generate spatially organized Ca²⁺ signals [1]. The large N-terminal cytosolic region of the IP₃R (2272 residues) is translated and folds to include a functional IP₃-binding site before the protein is directed to the ER. This ensures the final cytosolic disposition of this region and dictates the transmembrane topology of the complete IP₃R. Evidence derived from expression *in vitro* and in cells of IP₃R fragments with and without the complete N-terminal indicates that the sequence that includes TMD1 and TMD2 is sufficient for ER targeting [3, 26, 35] (Figure 2). Effective targeting occurs after translation of between 26 and 34 residues beyond the end of TMD1, a region that includes TMD2 (Figure 5A). Replacement of this post-TMD1 region with a similar number of hydrophilic residues can partially substitute for the native residues in mediating ER targeting (Figure 5B and C), suggesting that a major role is to facilitate extrusion from the ribosome of a signal recognition sequence within TMD1 allowing its recognition by SRP. Once targeted to the ER, the TMD provide the hydrophobic anchors that retain IP₃R within the ER, with TMD1 and TMD2 together sufficient to ensure complete retention within the ER [26].

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Figure 1 Fusion proteins used

(A) Key regions of IP₃R1. (B) Sequence of the TMD1-2 region. Numbers in parentheses denote the number of residues within each region. (C) The proteins used, and their abbreviations, are shown with N-terminal EGFP (black) or EYFP (grey) tags shown as ovals, and the C-terminal FLAG epitope as a flag. TMD are shown by black bars, linking loops by white bars, and the N- and C-termini by hatching.

Figure 2 N-terminally truncated IP₃R1 fragments are targeted to the ER by the first TMD pair

(A-E) COS-7 cells transiently transfected with the indicated constructs are shown in the left column and stained for calreticulin (Mitotracker in panel E) in the second column. The third column shows the first two columns overlaid with the construct in green and calreticulin (or Mitotracker) in red. Bars, 10 μ m. The fourth column shows enlargements of the highlighted boundaries, with green and red borders enclosing the construct and organelle marker, respectively. Here, and in all subsequent figures, images are representative of at least three independent transfections. The fifth column shows Western blots (with an antibody to GFP) of the 3 fractions derived from subcellular fractionation of the cells: S1 (first supernatant, cytosolic proteins), S2 (second supernatant, peripheral membrane proteins) and P (pellet, integral membrane proteins). For each gel, the 3 lanes were loaded with material from an equivalent number of cells. Molecular weight markers are shown in kDa. The final column summarizes the results obtained from the subcellular fractionation (means \pm SEM, $n \geq 3$, see Experimental).

Figure 3 Localization of IP₃R1 in the ER requires translation of the first TMD pair

(A-D) Cells transiently transfected with the indicated constructs are shown in the same format as Figure 2.

Figure 4 Localization of IP₃R1 in the ER requires translation of 26-34 residues after TMD1

(A-D) Cells transiently transfected with the indicated constructs are shown in the same format as Figure 2.

Figure 5 Non-native sequences after TMD1 facilitate ER localization

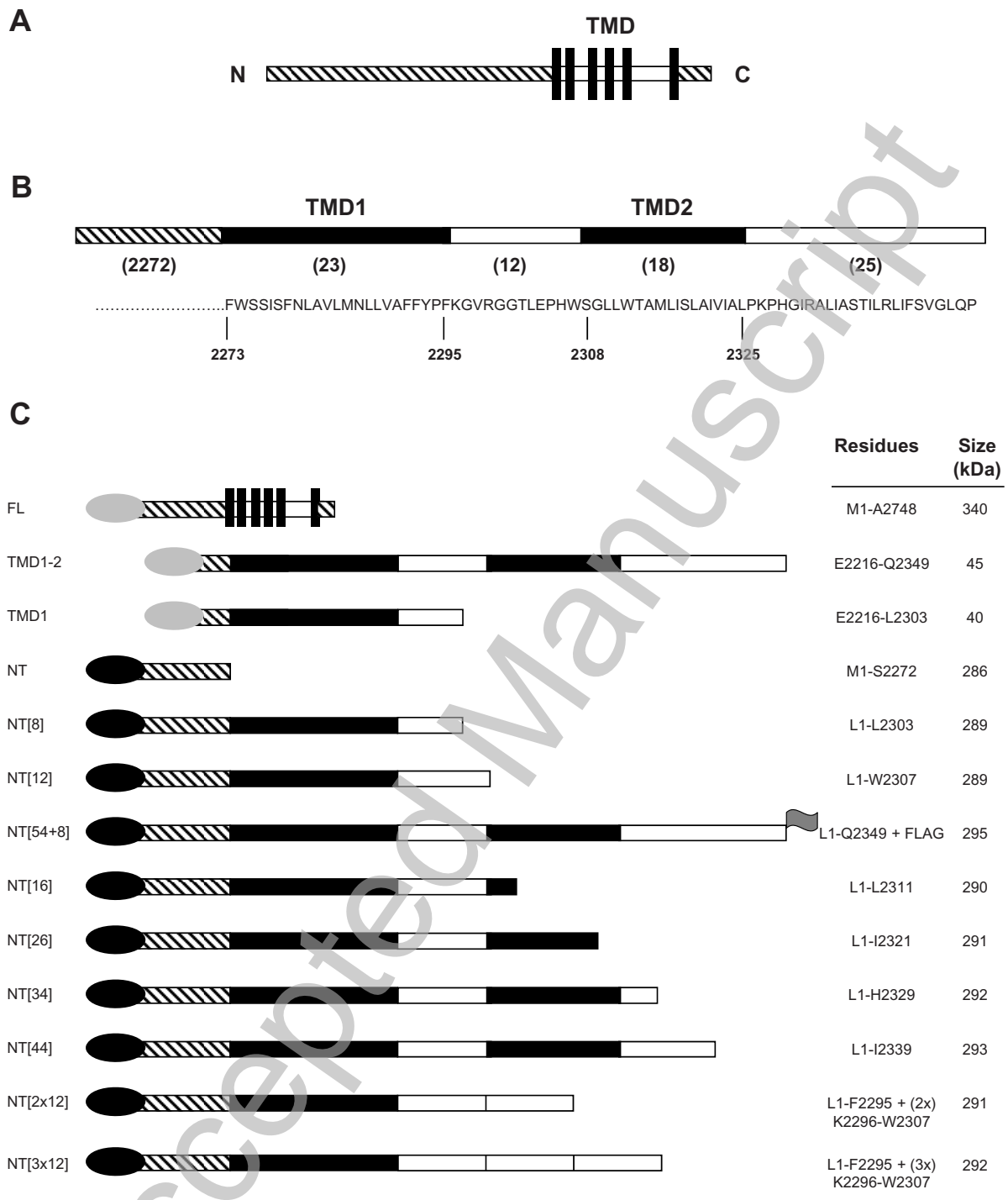
(A) Summary of present results and those from previous analyses of expression in COS-1 cells [3] (open triangles) or *in vitro* [35] (filled triangle). Native IP₃R sequence is shown in red (TMD1), black (TMD2), white (non-TMD) or hatched (N-terminal domain); EGFP/EYFP tags are shown in green, and C-terminal tags or non-native sequence in grey. All sequences from TMD1 towards the C-terminal (including C-terminal tags) are drawn to scale (see scale bar). The boxed area shows the \sim 40 residues concealed within the ribosome tunnel. As more of TMD2 and the following loop are translated, the putative signal sequence in TMD1 emerges from the ribosome, allowing it to bind SRP. (B, C) Cells transiently transfected with the indicated constructs are shown in the same format as Figure 2.

Table 1 Co-localization of IP₃R fragments and calreticulin

Pearson's correlation coefficient (*r*) was calculated for the fluorescence intensities for the wavelengths corresponding to each IP₃R fragment and calreticulin as described in Supplementary Figure S4. Results are means ± SEM, for 3-6 cells (n is shown for each fragment), with 3 lines analyzed in each cell. * and § denote values significantly different (p < 0.05) from NT (*) and FL (§) using one-way anova with Dunnett's post-hoc test.

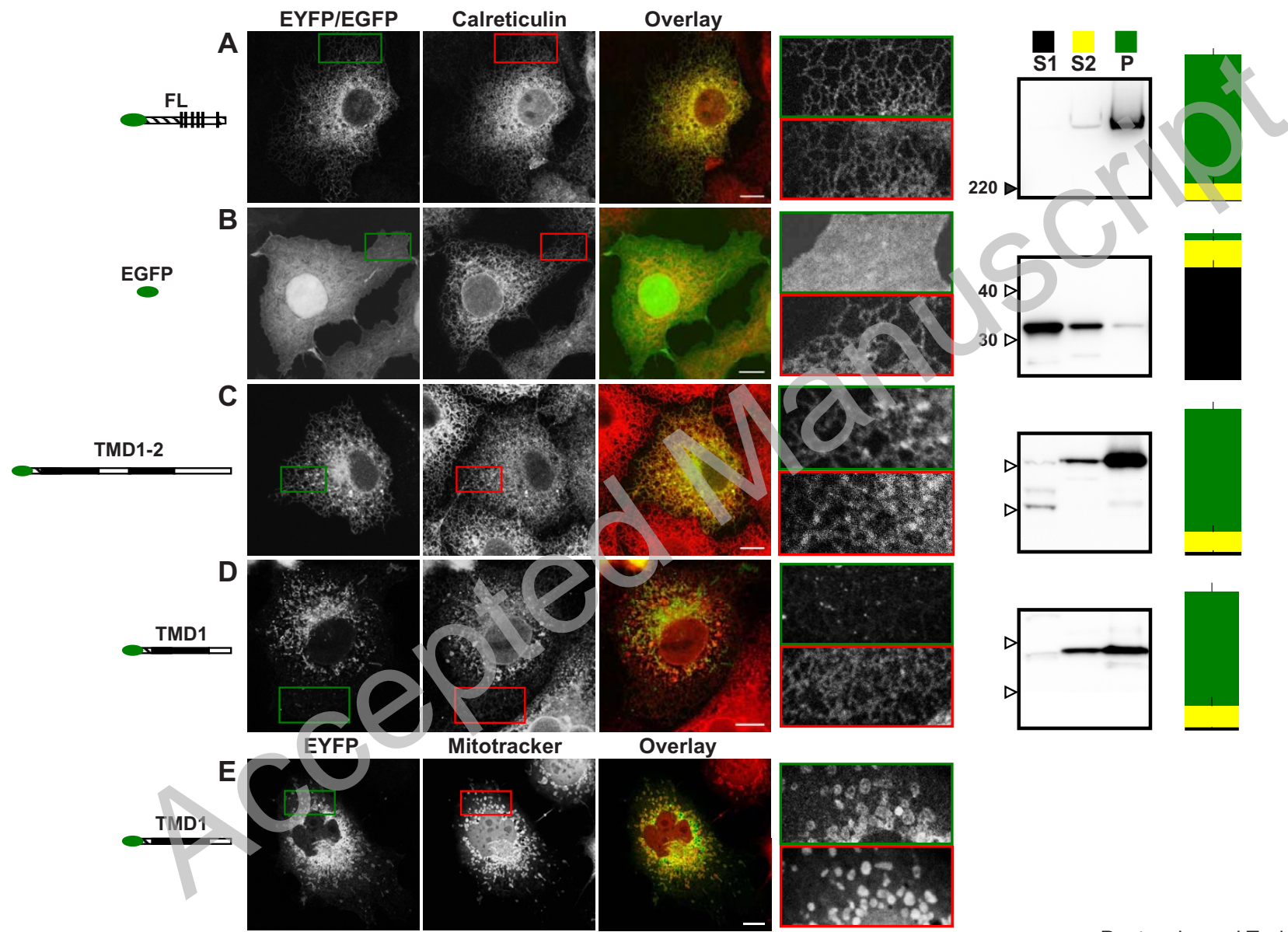
	FL [*]	TMD1-2	NT [§]	NT[8]	NT[12]	NT[54+8] [*]	NT[16] [*]	NT[26]	NT[34] [*]	NT[44] [*]	NT[2x12]	NT[3x12]
<i>r</i>	0.70 ± 0.07	0.64 ± 0.03	0.37 ± 0.06	0.39 ± 0.12	0.46 ± 0.04	0.74 ± 0.07	0.61 ± 0.06	0.61 ± 0.06	0.70 ± 0.05	0.75 ± 0.03	0.50 ± 0.07	0.45 ± 0.08
(n)	(3)	(3)	(6)	(3)	(5)	(3)	(6)	(3)	(3)	(5)	(5)	(6)

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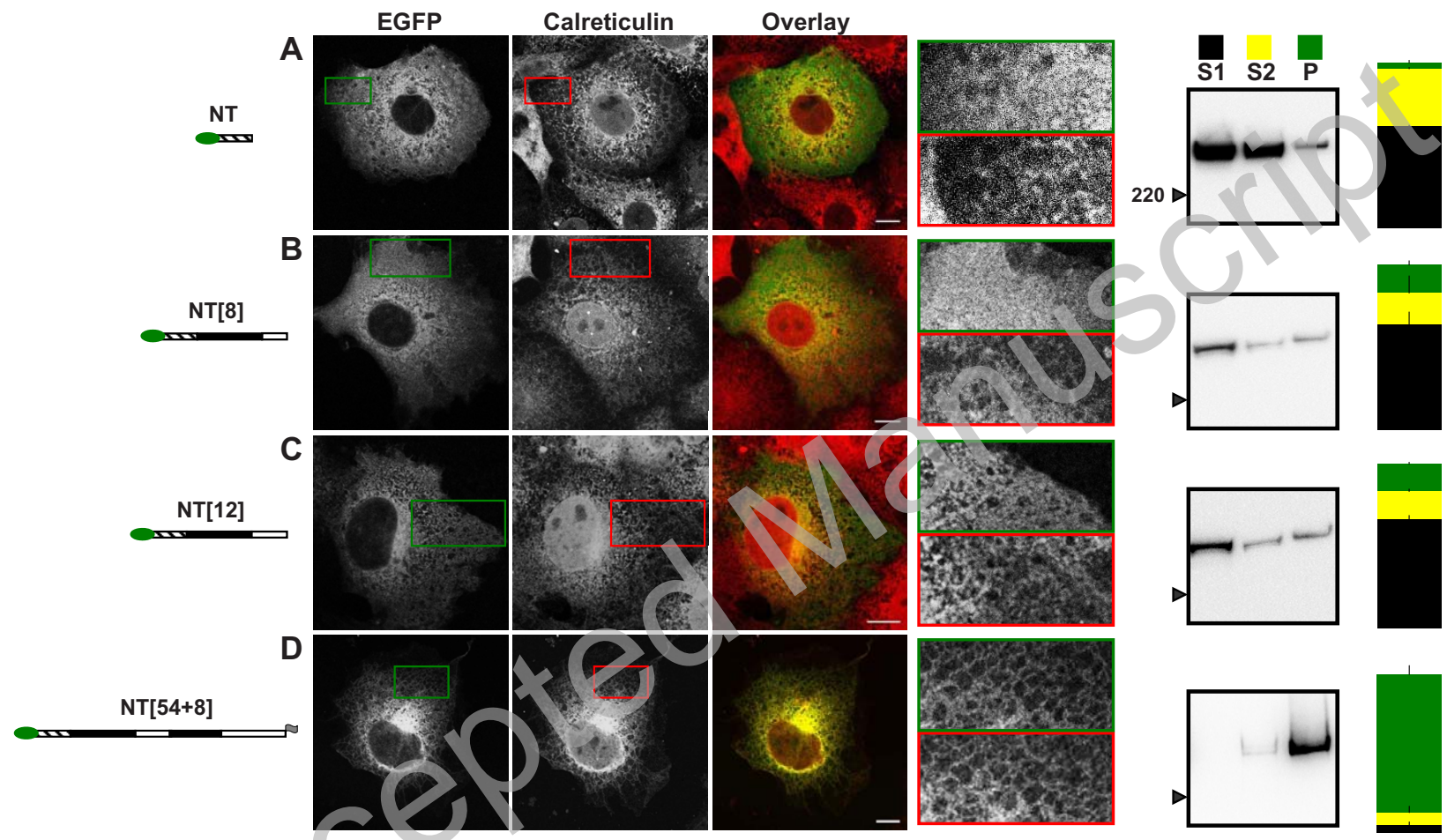


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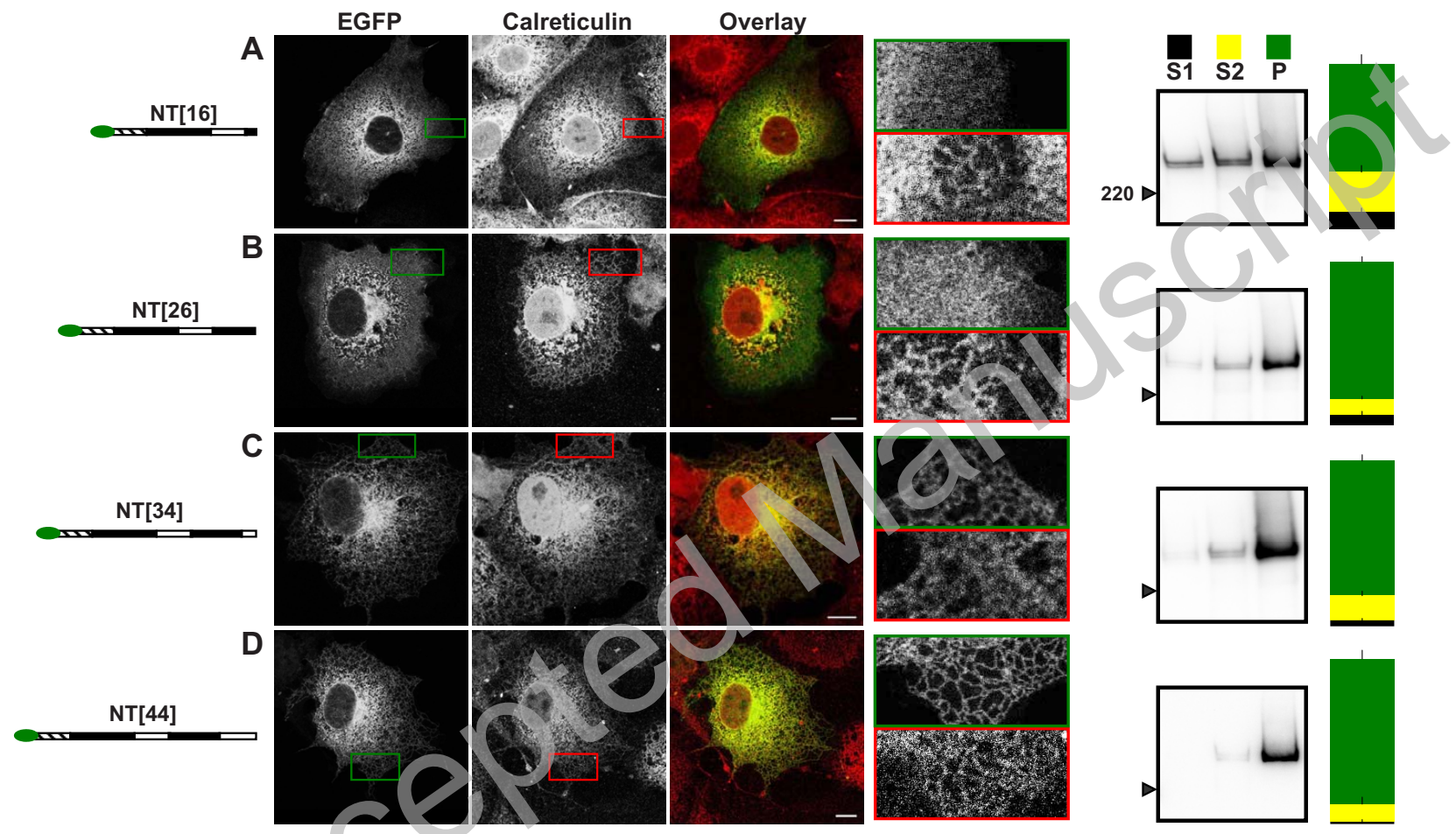
Pantazaka and Taylor, Fig. 1



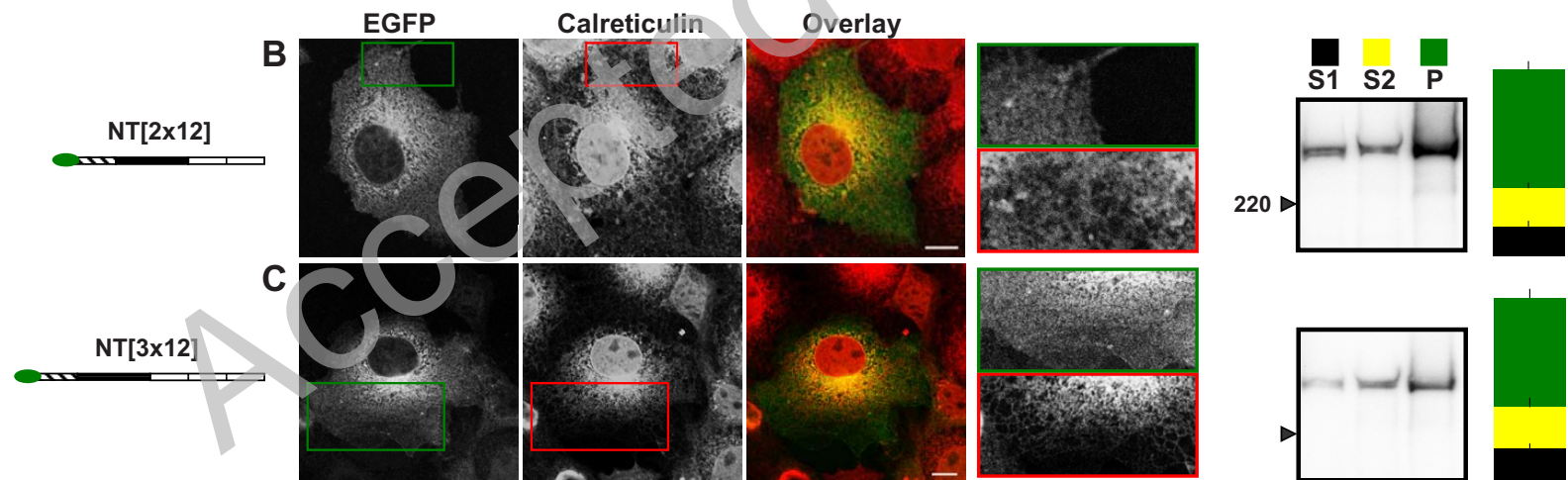
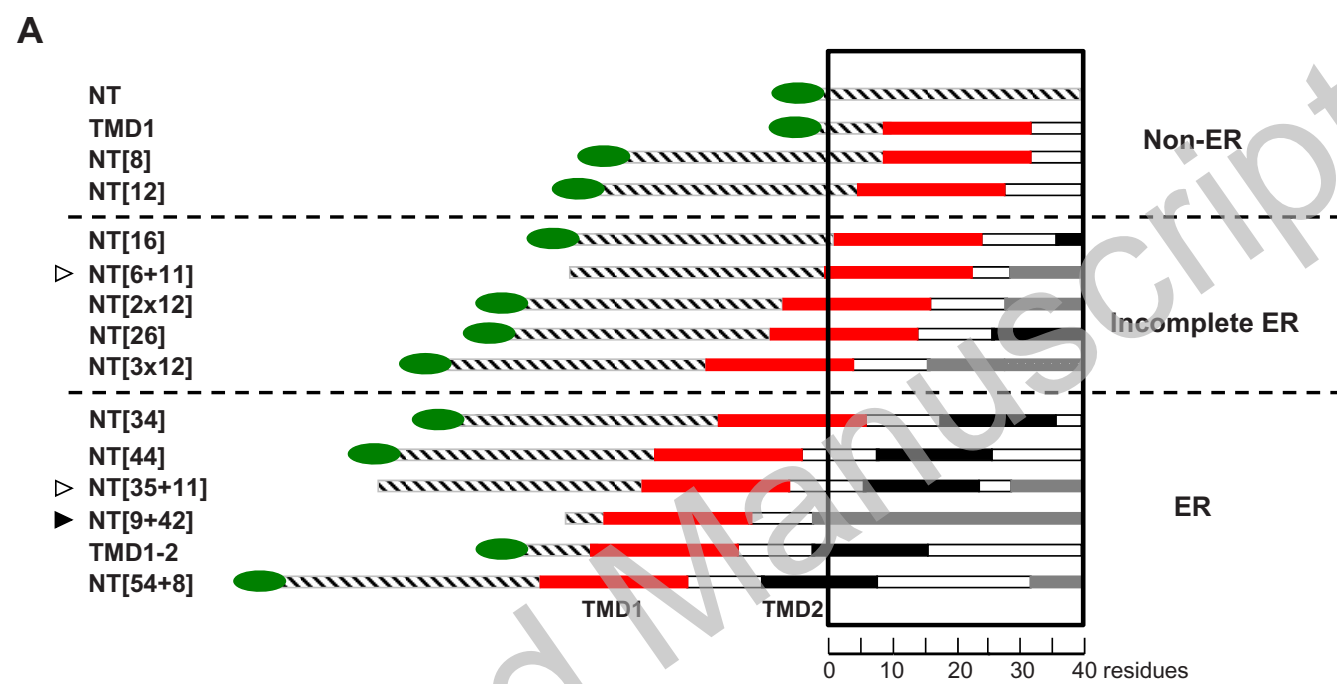
Pantazaka and Taylor, Fig. 2



Pantazaka and Taylor, Fig. 3



Pantazaka and Taylor, Fig. 4



Pantazaka and Taylor, Fig. 5