

# ER Reorganization is Remarkably Induced in COS-7 Cells Accumulating Transmembrane Protein Receptors Not Competent for Export from the Endoplasmic Reticulum

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**Abstract** The newly synthesized mutant L501fsX533 Frizzled-4 form and the alpha3beta4 nicotinic acetylcholine receptor expressed in the absence of nicotine accumulate in the endoplasmic reticulum of COS-7 cells and induce the formation of large areas of smooth and highly convoluted cisternae. This results in a generalized block of the transport to the Golgi complex of newly synthesized proteins. Intriguingly, both effects happen peculiarly in COS-7 cells; HeLa, Huh-7, and HEK293 cells expressing

the two receptors at similar level than COS-7 cells show normal ER and normal transport toward the plasma membrane. These results question the conclusion that a dominant-negative mechanism would explain the dominance of the mutant L501fsX533 Fz4 allele in the transmission of a form of Familial exudative vitreoretinopathy. Moreover, they indicate that the coordination of endoplasmic reticulum homeostasis in COS-7 cells is particularly error prone. This finding suggests that COS-7 cells may be extremely useful to study the molecular mechanisms regulating endoplasmic reticulum size and architecture.

**Keywords** Frizzled-4 L501fsX533 · Alpha3beta4 nicotinic acetylcholine receptor · COS-7 cells · ER reorganization

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## Introduction

Mutations frequently cause misfolding and retention in the endoplasmic reticulum (ER) of transmembrane (TM) receptor, channel, pump, or transporter proteins destined to the plasma membrane (Frump et al. 2013; Iram & Cole 2012; Ishii et al. 2014; Kleizen & Braakman 2004; Matsuda & Yuzaki 2002; Nechamen & Dias 2003; Ohgane et al. 2010; Saliba et al. 2002; Tjeldhorn et al. 2011; Wickert et al. 2013). These mutant TM proteins may exert a dominant-negative effect, blocking by oligomerization/aggregation the intracellular trafficking to the cell surface of the wild-type (wt) chains, thus determining a loss of function (Beaumont et al. 2007; Brothers et al. 2004; Bulenger et al. 2005; Calebiro et al. 2005; Guan et al. 2009; Lee et al., 2000; McElvaine & Mayo 2006; Sairam et al. 1996; Zhu & Wess 1998) that would explain the autosomal dominant (AD) inheritance of a number of

genetic disorders (Brothers et al. 2004; Kaykas et al. 2004; Ulloa-Aguirre et al. 2004). Among such AD disorders is a rare form of Familial exudative vitreoretinopathy (FEVR) due to the Frizzled-4 (Fz4) receptor mutant L501fsX533 (henceforth referred to as Fz4-FEVR). FEVR is due to the loss of Fz4 signaling from the cell surface during retinal development (Gariano & Gardner 2005; Ye et al. 2010). The mutant Fz4-FEVR receptor has a different C-terminal cytosolic tail, accumulates in the ER of transfected cells (Robitaille et al. 2002), and it is thought to trap its wt counterpart in the ER of transfected COS-7 cells by improper heteroligomerization (Kaykas et al. 2004). We have recently shown that the mutated tail of Fz4-FEVR has acquired a helix-loop-helix amphipathic structure that has affinity for the membrane and induce aggregation by affecting the folding of the luminal portion of the protein (Lemma et al. 2013). Carrying on these results, here we report the surprising finding that the accumulation of Fz4-FEVR in the ER of COS-7 cells has a dramatic ER reorganization effect. This effect is not observed in other cell lines and results in a generalized impairment of the intracellular transport to the Golgi complex of co-transfected newly synthesized TM proteins.

Intriguingly, such COS-7 cell-dependent ER reorganization is not a specific feature of Fz4-FEVR; we found a very similar effect with a different receptor accumulating in the ER, the wt pentameric  $\alpha 3\beta 4$  neuronal acetylcholine receptor (nAChR). Indeed, in non neuronal cells,  $\alpha 3\beta 4$  nAChR is mainly retained at the level of the ER even if it has been demonstrated that chronic nicotine treatment promotes its exit from the ER and its arrival at the plasma membrane (Mazzo et al. 2013). That the findings obtained with the two reporters highlight a peculiar sensitivity of COS-7 cells to the accumulation of TM proteins in the ER, and it is strongly suggested by the ultrastructural analysis: this shows very similar morphological features of the reorganized ER in either Fz4-FEVR or  $\alpha 3\beta 4$  expressing cells. The outcomes of these findings on the molecular mechanism underlying AD-FEVR, and of future studies on the regulation of ER structural rearrangements, are discussed.

## Materials and Methods

### Reagents

All of the culture reagents were obtained from Sigma-Aldrich (Milan, Italy). The solid chemical and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milan, Italy), Serva Feinbiochemica (Heidelberg, Germany), Delchimica (Naples, Italy), and BDH (Poole, United Kingdom). Protein

A-Sepharose CL-4B and the enhanced chemiluminescence reagents were obtained from Roche (Milan, Italy).

### Antibodies

The following primary antibodies were used: rabbit polyclonal anti-HA antibody and mouse monoclonal anti-FLAG antibody (Sigma-Aldrich, Milan, Italy); mouse monoclonal anti-HA antibody (Sigma-Aldrich, Milan, Italy and Santa Cruz Biotechnology Inc., Heidelberg, Germany); mouse monoclonal anti-CD8a glycoprotein (Ortho, Raritan, NJ); mouse monoclonal anti-c-Myc (Santa Cruz Biotechnology Inc., Heidelberg, Germany); rabbit polyclonal anti-Calnexin and mouse monoclonal anti-PDI clone 1D3 (SPA-891) (Stressgen, Ann Arbor, MI); and monoclonal anti-VSVG (IE9F9) (KeraFAST, Boston, MA). Rabbit polyclonal anti- $\alpha 3$  and anti- $\beta 4$  nAChR subunits were previously described (Mazzo et al. 2013).

The secondary antibodies used for western blot analysis were peroxidase conjugated anti-mouse and anti-rabbit IgG (Sigma-Aldrich, Milan Italy). The secondary antibodies used for immunofluorescence analysis were Texas-Red-conjugated anti-mouse and anti-rabbit IgG, FITC-conjugated goat anti-mouse and anti-rabbit IgG, Cy5-conjugated goat anti-mouse and anti-rabbit IgG, anti-rabbit and anti-mouse DyLight 549-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and anti-rabbit and anti-mouse Alexa 488 secondary antibodies (Life Technologies (Carlsbad, CA).

### cDNA Cloning and Plasmid Construction

The expression vectors pCDNA5/TO for the mutant Fz4-L501fsX533 (Fz4-FEVR) and pFLTRbT8 for human CD8 have been described elsewhere (D'Angelo et al. 2009; Iodice et al. 2001); the plasmid to express the VSVG protein with GFP-tag at its C-terminus coded by the ts O45 strain was provided by Jennifer Lippincott-Schwartz (NICHD, NIH, Bethesda, MD). Human  $\alpha 3$ -pcDNA3 and  $\beta 4$ -pcDNA3 were kind gifts of Dr. Sergio Fucile (University of Rome, Italy).

### Cell Culture, Transfection, and Immunofluorescence

Cells were routinely grown at 37 °C in Dulbecco's modified eagle's medium (DMEM), containing 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin and transfected using FuGene 6.0 (Roche, Milan, Italy) or using the Jet-PEI reagent (Polyplus transfection), according to the manufacturer's instructions, using a ratio of 1  $\mu$ g plasmid DNA/2  $\times 10^5$  cells (ratio DNA:Jet-PEI, 1  $\mu$ g/3  $\mu$ l). COS-7 cells transfected to

express VSVG-GFP were shifted 1 h post-transfection at 32 °C for 48 h (D'Agostino et al. 2011). After transfection, cells were fixed in 4 % paraformaldehyde, permeabilized in 0.3 % triton X-100, and immunostained with the antibodies listed above. Single confocal images were acquired at 63X magnification (NA 1.4) on a LSM510 Meta or LSM710 confocal microscope (Carl Zeiss, Jena, Germany) Lasers: Argon (488) and HeNe 543 and emission filters: BP 500–530 and 560–590. z-stack and confocal images were taken with Axiovert 200 M Microscope (Carl Zeiss) equipped with Spinning. Disk confocal system LCI MLS (PerkinElmer) using the 63X PlanApo lens (NA 1.4) and the 50 mW solid state diode laser 488/561 (emission filters: 510–550, 580–620). To quantify by immunofluorescence the expression level of the relevant proteins made in Huh-7, HeLa, and COS-7 cells, z-stacks images from unbiased fields were taken by keeping constant (between HeLa and COS-7 expressing nAChRs and Huh-7 and COS-7 expressing Fz4-FEVR, respectively) all the parameters of the acquisition (laser intensity, gain, and background). Cells showing a saturated signal were excluded, and the sum of the integrated density of the signal of each plane of the z-stack for each cell was calculated using NIH ImageJ. For each case, at least 15 cells were considered.

### Electron Microscopy

For routine electron microscopy, Cos-7 cells were fixed with 1 % glutaraldehyde prepared in 0.2 M HEPES buffer, pH 7.3. Cells for pre-embedding immunoelectron microscopy were fixed, permeabilized, and labeled as described previously (Polishchuk et al. 2012). From each sample, thin 65 nm sections were cut using a Leica EM UC7 ultramicrotome. EM images were acquired from thin sections using a FEI Tecnai-12 electron microscope (FEI, Eindhoven, the Netherlands) equipped with a VELETTA CCD digital camera (Soft Imaging Systems GmbH, Munster, Germany).

## Results

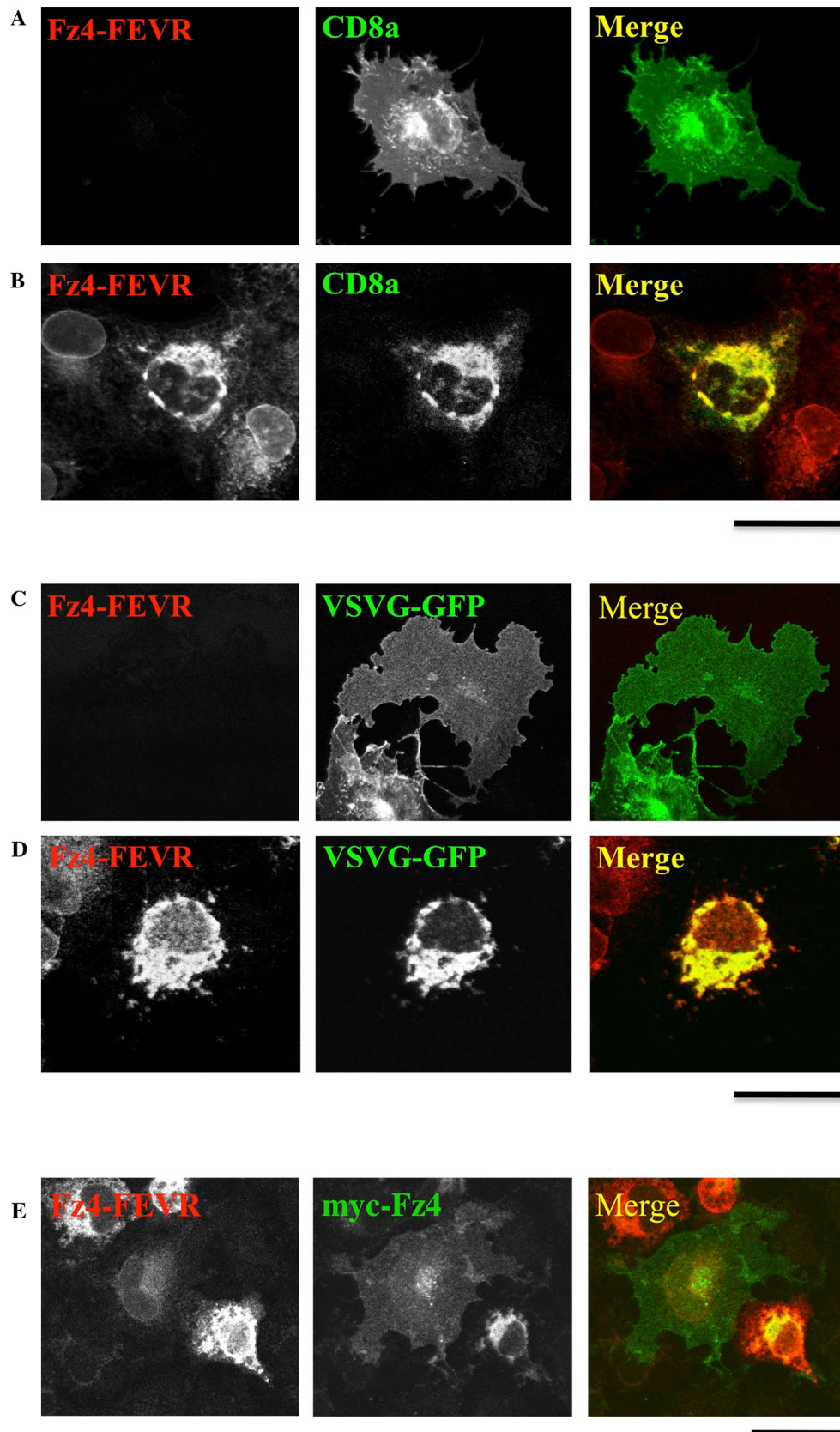
### Accumulation of Fz4-FEVR in the ER Prevents Transport to the Golgi Complex of Different TM Proteins

In order to study the dominant effect of Fz4-FEVR on wt Fz4, we transiently co-transfected COS-7 cells with HA-tagged Fz4-FEVR and myc-tagged Fz4 in the plasmid DNA in the ratio of 5:1, respectively, as previously reported (Kaykas et al. 2004). The immunofluorescence analysis showed that Fz4 and Fz4-FEVR when expressed

alone were mainly located on the cell surface and in the ER, respectively (Fig. S1a, b); whereas, both proteins were clearly localized in the ER when co-expressed (Fig. S1c), fully confirming the previously reported results (Kaykas et al. 2004). However, the ER visualized by Fz4-FEVR in these cells did not appear normal. Thus, we substituted Fz4 with two different TM reporter proteins, i.e., the human co-receptor CD8a and the C-terminally GFP-tagged form of the mutant G glycoprotein were coded by the ts-045 strain of Vesicular Stomatitis virus (VSVG-GFP). Surprisingly, the same dominant-negative effect of Fz4-FEVR was observed. As shown in Fig. 1a, c, both CD8a and VSVG-GFP were detected on the PM of COS-7 cells when expressed alone, while in the presence of Fz4-FEVR both reporters co-localized with the mutant receptor in the ER (Fig. 1b, d, respectively). The relevance of this result is further underlined by the notion that both reporters are among the fastest proteins to move from the ER to the Golgi complex and the PM (Lotti et al. 1992; Pascale et al. 1992). Interestingly, COS-7 cells expressing lower level of HA-Fz4-FEVR normally located wt myc-Fz4 on the PM, but never HA-Fz4-FEVR (Fig. 1e). In addition, when Fz4 wt substituted Fz4-FEVR in the co-transfections with both reporters, either Fz4 and VSVG or Fz4 and CD8a were expressed on the cell surface (data not shown). Thus, we concluded that the accumulation of Fz4-FEVR in the ER severely impairs the transport to the Golgi complex.

### The Dominant-Negative Effect of Fz4-FEVR is Cell-Dependent and Correlates with Alteration of the ER

To further investigate the dominant-negative effect of Fz4-FEVR, we co-transfected HA-Fz4-FEVR and myc-Fz4 in HEK293 and Huh-7 cells with the same 5:1 plasmid DNA ratio used in COS-7 cells. As shown in Fig. 2a, while in COS-7 cells, the dominant-negative effect of Fz4-FEVR on Fz4 wild type was clearly detectable, in both HEK293 and Huh-7 cells, the over-expression of Fz4-FEVR apparently did not affect the capacity of Fz4 wild type to reach the plasma membrane. This phenomenon was particularly evident in Huh-7 cells that are quite large and flat and exhibit a well-defined and extended ER when observed with light microscopy (D'Agostino et al. 2011; Stornaiuolo et al. 2003). Clearly, only in COS-7 cells, the accumulation in the ER of Fz4-FEVR affected the physiological organization of the ER, as shown by the altered localization of the ER marker Calnexin (Fig. 2b, compare high with low). In addition, we found that COS-7 cells transiently transfected with HA-Fz4-FEVR alone showed this altered ER morphology in a time-dependent manner (Fig. 2c).



◀ **Fig. 1** Fz4-FEVR traps other TM receptors in the ER. COS-7 cells grown on coverslips were transiently transfected to express the indicated proteins alone or together with Fz4-FEVR as in Fig. S1C. Forty-eight-hour post-transfection, the cells were subjected to confocal immunofluorescence microscopy analysis. Fz4 and Fz4-FEVR were revealed as in Fig. S1; CD8a and VSVG proteins by mouse monoclonal anti-CD8a antibody and GFP autofluorescence, respectively. Scale bar 10  $\mu\text{m}$

### ER Reorganization and Trafficking Impairment in COS-7 Cells are Induced by Accumulation of nAChR

It has been recently shown that  $\alpha 3\beta 4$  nAChR accumulates in the ER of transfected HeLa cells although correctly assembled in pentamers (Mazzo et al. 2013). Transport to the PM was achieved in HeLa cells only in the presence of nicotine, most relevant to the present work, and a normal ER organization was detected in the transfected cells accumulating  $\alpha 3\beta 4$  in the ER in the absence of nicotine (Mazzo et al. 2013). In contrast, when we transfected in parallel COS-7 and HeLa cells, a striking difference was observed in the distribution of the  $\alpha 3\beta 4$  and the ER marker PDI (Fig. 3a, b). nAChR was accumulated in the ER in both instances, but while in HeLa cells, an altered ER was detected only in a very minor percent of transfected cells, more than 80 % of transfected COS-7 cells showed a drastic reorganization of the ER. Furthermore, COS-7 cells were co-transfected as shown in Fig. 1d, but substituting Fz4-FEVR with  $\alpha 3\beta 4$  nAChR. Strikingly, all cells showing a reorganized ER for the accumulation of  $\alpha 3\beta 4$  had the VSVG-GFP reporter accumulated in the ER (Fig. 3c, central row), while the cells expressing only VSVG-GFP or low level of the co-transfected  $\alpha 3\beta 4$  showed a strong labeling of the viral reporter on the plasma membrane (Fig. 3c, upper and lower rows, respectively).

### Fz4-FEVR and $\alpha 3\beta 4$ are Expressed in similar Amount in the Different Cell Lines Analyzed

All our findings suggested that COS-7 cells are peculiarly sensitive to the accumulation in the ER of TM proteins. Alternatively, this phenomenon could be simply due to a largely higher expression of the transfected proteins in COS-7 cells. Western blot analysis showed that the relative amount of Fz4-FEVR synthesized in Huh-7 and COS-7 cells was very similar (data not shown). However, the immunofluorescence results indicated a significant variability in the expression level of both Fz4-FEVR and  $\alpha 3\beta 4$  in individual cells, thus a more rigorous analysis was needed to test the above hypothesis. The total amount of HA-Fz4-FEVR and  $\alpha 3\beta 4$  expressed in COS-7, Huh-7, and HeLa cells was evaluated by measuring the integrated

density of the immunofluorescence signal (see “Materials and Methods” section for details). As illustrated in Fig. 4, similar expression levels of the two receptors were detected in COS-7 and Huh-7 cells, and in COS-7 and HeLa cells, respectively. Therefore, the altered ER organization seen in COS-7 cells is not due to a higher expression level of HA-Fz4-FEVR and  $\alpha 3\beta 4$  proteins in these cells.

### Reorganization of the ER in Smooth and Highly Convoluted Tubular Structures in COS-7 Cells Accumulating Fz4-FEVR or $\alpha 3\beta 4$

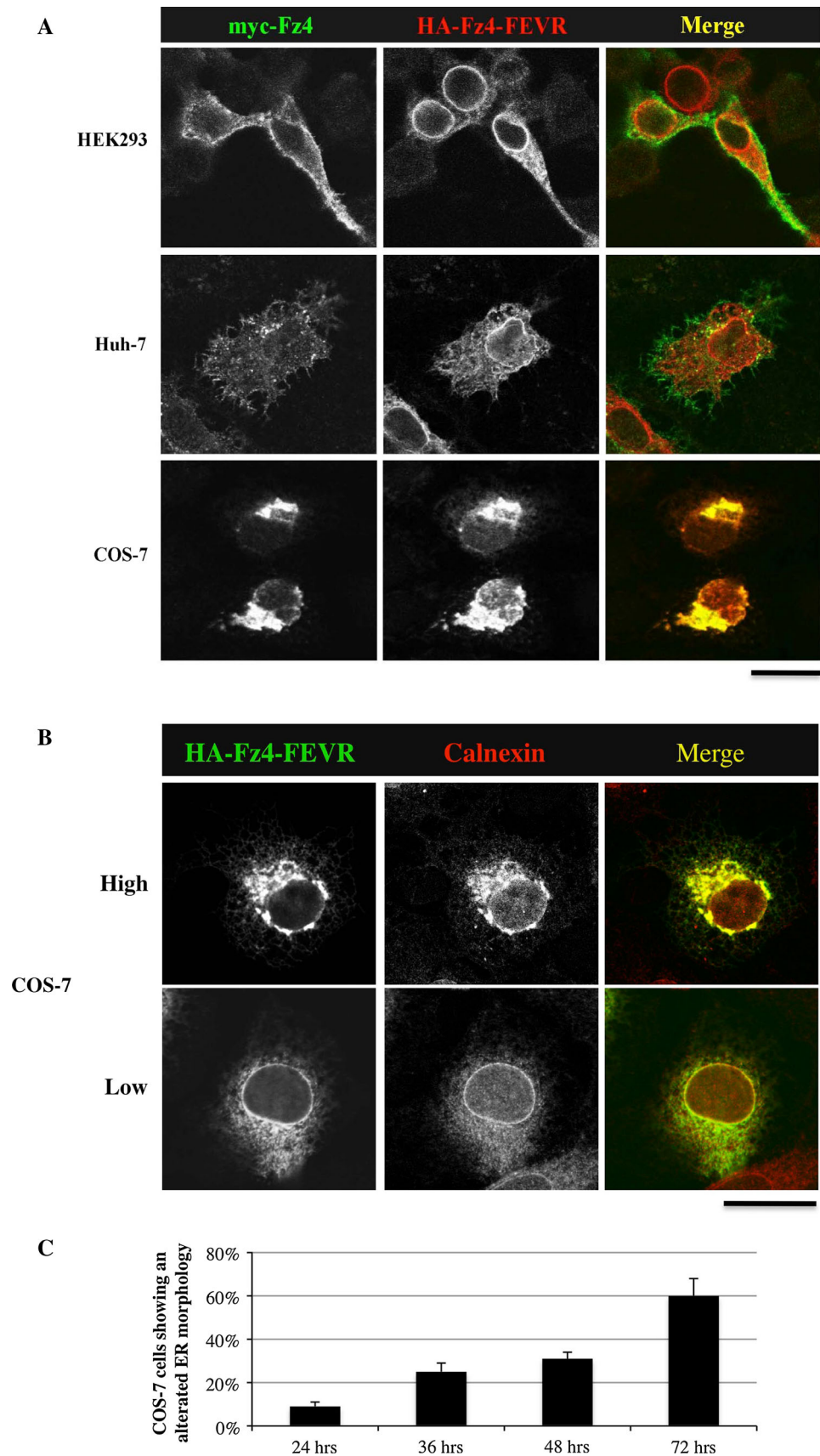
To better visualize the effect on ER morphology caused by accumulation Fz4-FEVR and  $\alpha 3\beta 4$  nAChR, transiently transfected COS-7 cells were analyzed by conventional transmission electron microscopy 48-h post-transfection. As shown in Fig. 5a and b, no transfected cells showed the canonical organelles' morphology, typical of healthy cells, with long and regular ER cisternae. In contrast, in specimens of COS-7 cells transiently transfected with the mutant Fz4-FEVR (Fig. 5c, d) or with  $\alpha 3\beta 4$  (Fig. 5e, f), the expected percentage of cells showed a dramatic reorganization of large area of the ER in highly convoluted smooth cisternae. Remarkably, the morphological features of these areas were very similar in COS-7 cells accumulating either Fz4-FEVR or  $\alpha 3\beta 4$  proteins (Fig. 5, compare panels c, d with e, f). The great majority of the cells with a reorganized ER had these areas of convoluted smooth cisternae: only few cells showed either swollen cisternae or onion-like structures (data not shown). An immunoelectron microscopy analysis was also performed in parallel and fully confirmed that the areas of convoluted smooth tubular structures were present only in transfected cells and accumulated the expressed protein (data not shown).

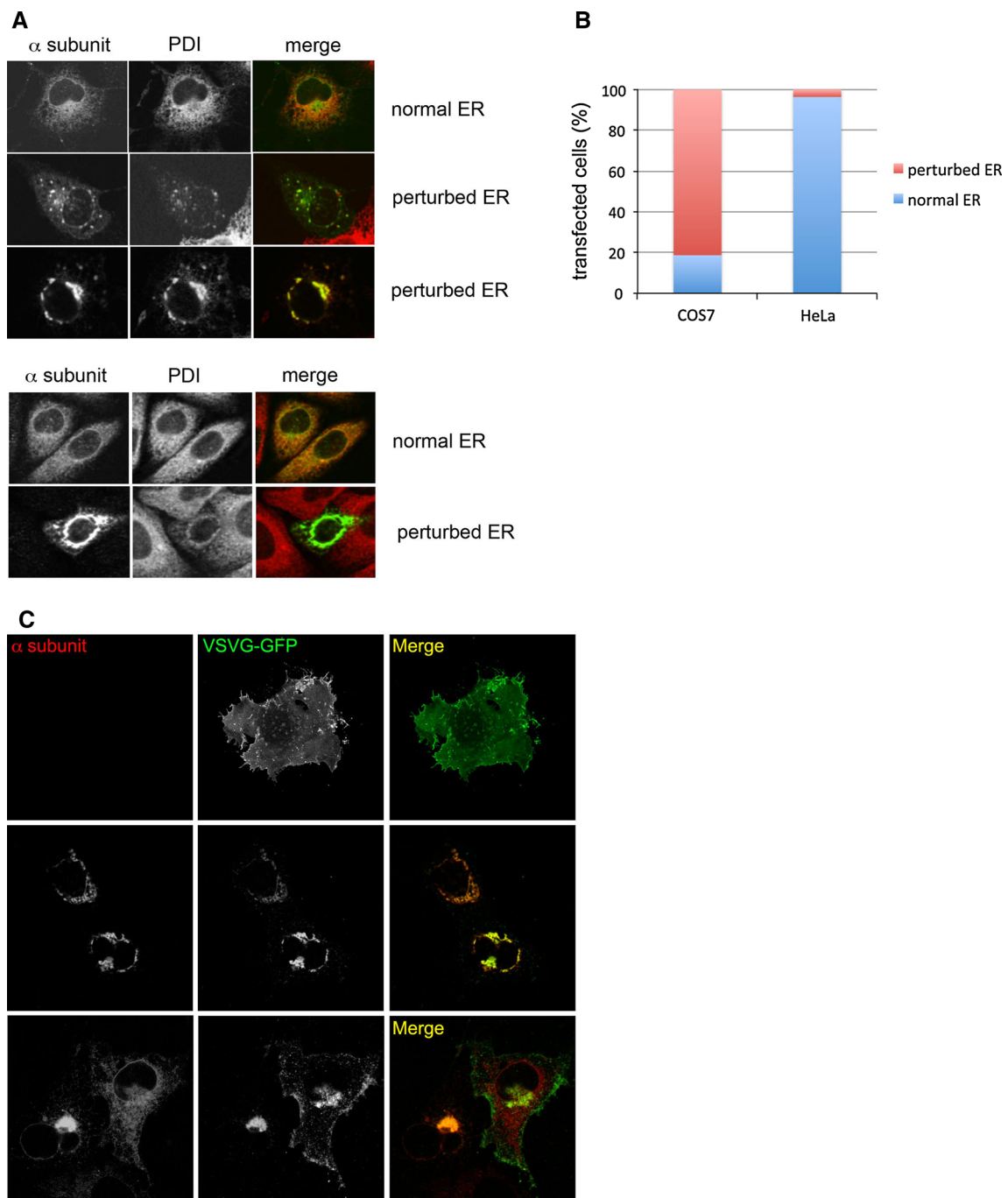
### Discussion

Three main results are presented in this work. First, the accumulation of two very different TM proteins not competent for ER export, the Fz4-FEVR mutant receptor belonging to the GPCR F family and the  $\alpha 3\beta 4$  nAChR (Lemma et al. 2013; Mazzo et al. 2013), induces a dramatic and almost undistinguishable effect on the ER in COS-7 cells: the generation of large areas of smooth and highly convoluted cisternae. This finding is supported by the immunofluorescence analysis of the distribution of the two receptors as well as of two main ER marker proteins as Calnexin and PDI (Figs. 1–4), and directly demonstrated by conventional transmission electron microscopy (Fig. 5), as well as by immunoelectron microscopy. Thus, most

**Fig. 2** Cell-dependent trapping of Fz4 by Fz4-FEVR.

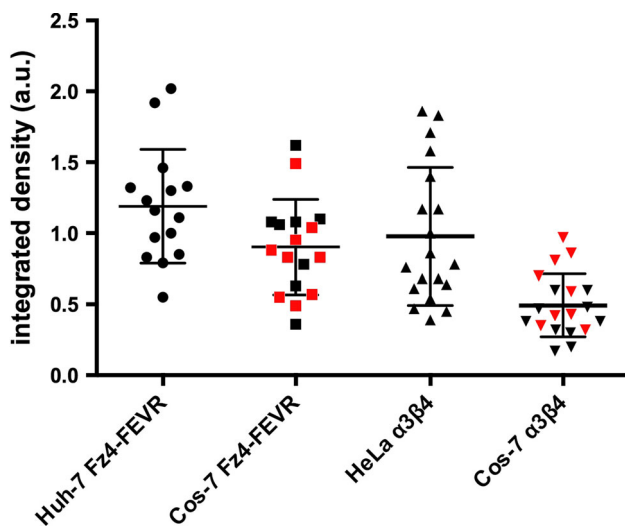
**a** HEK293, Huh-7, and COS-7 cells were transiently co-transfected to express HA-Fz4-FEVR and myc-Fz4 as in Figs. S1 and 1, and after 48 h, they were subjected to confocal immunofluorescence microscopy analysis. Note the surface expression of Fz4 in HEK293 and Huh-7 but not in COS-7 cells. **b** COS-7 cells, transiently transfected with HA-Fz4-FEVR alone for 48 h, were subjected to confocal immunofluorescence microscopy analysis to reveal the localization of HA-Fz4-FEVR and calnexin proteins, respectively. Note that altered ER morphology is detected only in cells expressing high levels of Fz4-FEVR. *Scale bar* 10  $\mu$ m. **c** Quantification of the time-dependent alteration of ER morphology in COS-7 cells transfected with HA-Fz4-FEVR alone. Cells were processed for confocal immunofluorescence microscopy analysis as in panel **b**;  $n = 50$  for each time point





**Fig. 3** Accumulation of nAChR induces ER reorganization and blocks transport of VSVG in COS-7 cells. **a** COS-7 and HeLa cells (*upper* and *lower panels*, respectively) were transfected to express both  $\alpha 3$  and  $\beta 4$  nicotinic receptor subunit, and after 24 h processed for confocal immunofluorescence microscopy analysis to reveal the localization of the receptor (with anti- $\alpha 3$  antibody) and of the ER marker PDI. Examples of cells showing normal or perturbed ER are shown. *Scale bar* 10  $\mu$ m. **b** quantitation of the number of transfected COS-7 and HeLa cells expressing  $\alpha 3\beta 4$  nicotinic receptor and showing altered ER morphology. Cells were processed for confocal

immunofluorescence microscopy analysis as in panel **a**, and the percentage of cells with a perturbed (*red*) or normal ER was counted; ( $n = 90$  and  $83$  for COS-7 and HeLa cells, respectively). **c** COS-7 cells were transfected to express VSVG-GFP alone or together with  $\alpha 3$  and  $\beta 4$  subunit as in Fig. 1 (using a plasmid molar ratio between nicotinic subunits and VSVG of 5:1). Twenty-four hours after transfection, cells were fixed and processed for confocal immunofluorescence microscopy analysis with anti- $\alpha 3$  antibody to reveal nAChR and anti-VSVG antibody to enhance the signal of VSVG-GFP. *Scale bar* 10  $\mu$ m (Color figure online)



**Fig. 4** Transfected Fz4-FEVR and  $\alpha 3\beta 4$  are expressed in very similar amount in the different cell lines analyzed. Quantitation of the level of Fz4-FEVR or  $\alpha 3\beta 4$  nAChR expressed in Huh-7 and COS-7 cells, and in HeLa and COS-7 cells, respectively. See “Materials and Methods” section for details. In red are indicated cells with perturbed ER. 47 % of the COS-7 cells expressing  $\alpha 3\beta 4$  were not saturated. The Prism scattered dot blot is shown. The mean value and the standard deviation are indicated. Note that the average expression levels of Fz4-FEVR and  $\alpha 3\beta 4$  are even higher in Huh-7 and HeLa cells, respectively, than in COS-7 cells (Color figure online)

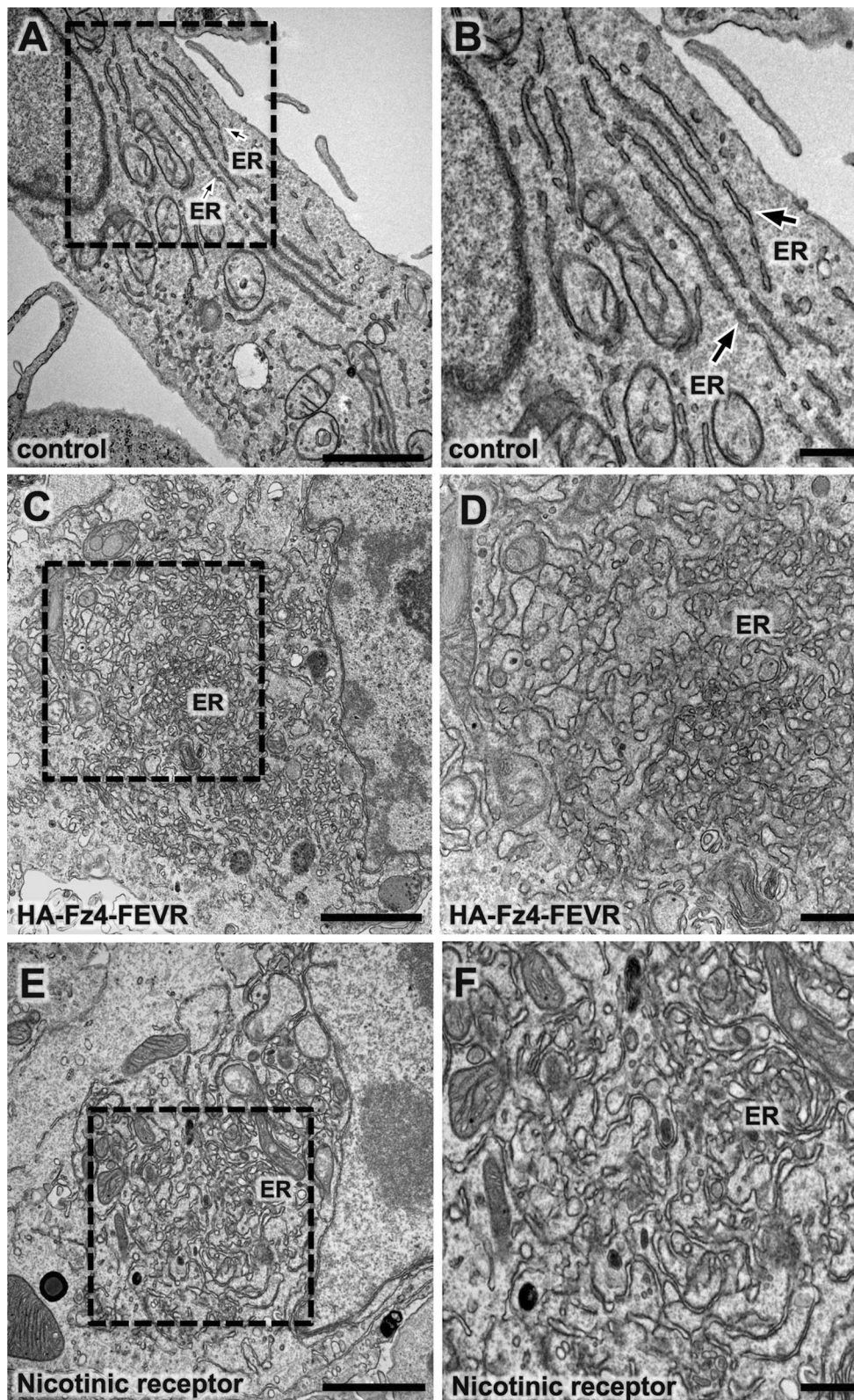
likely, COS-7 cells display a general response to the excessive presence of multispan TM proteins in the ER, not to specific features of Fz4-FEVR or  $\alpha 3\beta 4$  nAChR.

Second, in COS-7 cells with such reorganized ER, the export toward the Golgi complex is blocked. Fz4, CD8a, and VSVG glycoproteins are kept in the reorganized ER with Fz4-FEVR (Figs. S1, 1). Similarly, VSVG accumulates in the reorganized ER by the expression of  $\alpha 3\beta 4$  receptor (Fig. 3). In contrast, in cells expressing lower level of Fz4-FEVR or  $\alpha 3\beta 4$ , no ER reorganization is observed and normal transport to the plasma membrane of co-transfected Fz4 and VSVG takes place (Figs. 1, 3). Thus, these results question a previous suggestion (Kaykas et al. 2004) that the specific heterologomerization of Fz4 and Fz4-FEVR would be the key to explain the dominance of the Fz4-FEVR allele in heterozygous patients and the consequent dominant inheritance of this rare form of FEVR (Robitaille et al. 2002). Clearly, co-transfection experiments in heterologous cell lines represent a useful model system to study the molecular mechanism of the disease, but too far from the cells of the developing retina of human fetuses to allow solid conclusions. Our results do not solve the question of the dominant inheritance either, but they suggest that other causes such as haploinsufficiency may well explain the dominance of the Fz4-FEVR allele, as recently shown for several other FEVR-associated

missense mutations of Fz4 that result in ER retention of the mutant but not of wt Fz4 chains (Milhem et al. 2014).

Third, ER reorganization is not observed, or is extremely minor, in Huh-7, HEK293, and HeLa cells expressing either Fz4-FEVR or  $\alpha 3\beta 4$  nAChR (Figs. 2–4). This finding is intriguing and much work is needed to shed light on its direct cause. COS-7 cells in culture divide at similar rates and are transfected at similar efficiency to HeLa, Huh-7, and HEK293 cells. The average amount of expression of the transfected protein per cell results similar between the four cell lines (measured by either immunofluorescence or western blot analysis). This last finding makes it unlikely that in COS-7 cells episomal plasmid replication or ERAD rates may occur at higher and lower rates, respectively. The observed ER reorganization happens with some variability within transfected COS-7 cells, and the  $\alpha 3\beta 4$  nAChR appears to be somehow a more efficient trigger (80 % of cells at 48 h post-transfection); however, with time, the great majority of transfected COS-7 cells show the reorganized ER also when expressing Fz4-FEVR (30–60 %), while the amount of ER reorganization in the other cell lines examined is always negligible.  $\alpha 3\beta 4$  nAChR is slightly better expressed in COS-7 cells than in Fz4-FEVR (as judged by the higher number of cells with saturated fluorescence signal), but the crucial finding shown in Fig. 4 indicates that there is no correlation between level of expression and ER reorganization with both mutant receptors. Thus, the most likely explanation at the moment is that the molecular mechanisms that coordinate ER homeostasis in COS-7 cells are poorly prone to adapt to rapidly arising new needs. It is well known that the ER adjusts its size, thus modulating protein and lipid synthesis, to adapt to requirements of the lumen or to increased concentrations of ER resident membrane proteins (Orrenius et al. 1965; Szczesna-Skorupa et al. 2004; Vergeres et al. 1993). This ER proliferation/reorganization may have different morphological outcomes: stacked cisternae of the outer nuclear envelope or distributed in the cell, sinusoidal ER, concentric membrane whorls, or crystalloid ER (Borgese et al. 2006). ER reorganization is also determined by accumulation of proteins devoid of luminal domain, so the triggering factor can not depend only on the luminal folding machinery but may also be linked to events occurring within the lipid bilayer (Vergeres et al. 1993). Recent reports have indicated the possible involvement of the ATF6 branch of the unfolded protein response (Maiuolo et al. 2011), but the molecular mechanism and the signaling pathways that trigger ER proliferation are largely unknown yet. Our results clearly suggest to use COS-7 cells in a comparative fashion with other cell lines to decipher these fundamental mechanisms: being much more sensitive to the amount of membrane protein accumulated in the ER, COS-7 cells should facilitate the difficult search





**Fig. 5** Electron microscopy analysis of the ER reorganization induced by Fz4-FEVR and  $\alpha 3\beta 4$  nAChR. COS-7 cells were mock- or transiently transfected with the indicated constructs. Forty-eight-hour post-transfection, the cells were fixed and processed for

conventional transmission electron microscopy. Panels **b**, **d**, and **f** show larger magnification of the *boxed areas* in **a**, **c**, and **e**, respectively. *Scale bars a, c, e 2 μm; b, d, f 500 nm*

of the molecular components involved in regulating ER size and architecture.

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