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REVIEW

Protein quality control: the who's who, the where's and therapeutic escapes

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Abstract In cells the quality of newly synthesized proteins is monitored in regard to proper folding and correct assembly in the early secretory pathway, the cytosol and the nucleoplasm. Proteins recognized as non-native in the ER will be removed and degraded by a process termed ERAD. ERAD of aberrant proteins is accompanied by various changes of cellular organelles and results in protein folding diseases. This review focuses on how the immunocytochemical labeling and electron microscopic analyses have helped to disclose the in situ subcellular distribution pattern of some of the key machinery proteins of the cellular protein quality control, the organelle changes due to the presence of misfolded proteins, and the efficiency of synthetic chaperones to rescue disease-causing trafficking defects of aberrant proteins.

Keywords ERAD · Protein folding disease · Glucosidase II · Glucosyltransferase · EDEM1 · Endomannosidase · Chemical chaperones

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Introduction

Folding and assembly of proteins and their function depend on each other. Like in industrial production lines, in cells the quality of newly synthesized proteins is monitored in regard to proper folding and correct assembly in the early secretory pathway, the cytosol and the nucleoplasm (Bennett et al. 2005; Bukau et al. 2006; Dobson 2003; Ellgaard and Helenius 2003; Park et al. 2007; Ravid et al. 2006; Roth 2002; Sitia and Braakman 2003; Zhang and Kaufman 2006). Protein quality control is a basic cellular phenomenon through which aberrant proteins become eliminated. Aberrant proteins can occur as waste products at a certain rate during de novo synthesis, or are caused by cellular stress, or due to disease-causing mutations (Aridor and Hannan 2002; Gregersen et al. 2006; Kim and Arvan 1998; Kopito 2000; Lukacs et al. 1994; Petäjä-Repo et al. 2000; Schubert et al. 2000; Turner and Varshavsky 2000; Ward and Kopito 1994). Once recognized as non-native or incompletely assembled, those proteins will be removed and degraded by a process generally termed ERAD, for ER-associated degradation (Hirsch et al. 2004; Meusser et al. 2005).

For secretory and membrane proteins, the molecular machinery involved in the recognition, retention and dislocation of aberrant proteins has been identified to a certain

detail (Carvalho et al. 2006; Denic et al. 2006; Hirsch et al. 2003; Ismail and Ng 2006; Katiyar et al. 2005; Li et al. 2006; Lilley and Ploegh 2004; Schuberth and Buchberger 2005; Tsai et al. 2002; Ye et al. 2003, 2004). The ensuing final step consists of polyubiquitination of aberrant proteins condemning them for degradation by proteasomes (Eisele et al. 2006; Hochstrasser 1996; Jarosch et al. 2002; McCracken and Brodsky 2005; Wolf and Hilt 2004; Zwickl et al. 2002). In addition to various chaperones aiding proteins to achieve their proper conformation, various machinery proteins are involved in the recognition and retention of aberrant proteins. For glycoproteins, the importance of specific oligosaccharidic structures generated initially by trimming glucosidase II and UDP-glucose:glycoprotein glucosyltransferase and later on by ER-mannosidase I has been recognized (Helenius and Aebi 2004; Parodi 2000; Roth 2002; Roth et al. 2002). Glycoproteins bearing monoglucosylated oligosaccharides will be bound by calnexin or calreticulin. If the aberrant glycoproteins are considered, binding to calnexin or calreticulin will protect them temporarily from degradation. The complete deglucosylation by glucosidase II will result in their exit from the calnexin/calreticulin cycle. Subsequent trimming of mannose residue(s) of the oligosaccharide B branch by ER-mannosidase I opens the gate to dislocation and degradation of aberrant proteins. The link between the calnexin/calreticulin cycle and the dislocation process is apparently provided by two lectin-like proteins: EDEM1 (yeast ortholog Htm1p/Mnl1p) (Hosokawa et al. 2001; Jakob et al. 2001; Kanehara et al. 2007; Nakatsukasa et al. 2001; Oda et al. 2003) and Yos9p (mammalian orthologues OS-9 and XTP3-B) (Bhamidipati et al. 2005; Buschhorn et al. 2004; Gauss et al. 2006; Kanehara et al. 2007; Kostova and Wolf 2005; Szathmary et al. 2005).

Depending on the type of protein and the location of the lesion, different ERAD dislocation pathways have been identified (Carvalho et al. 2006; Denic et al. 2006; Ismail and Ng 2006; Schuberth and Buchberger 2005). Aberrant luminal proteins and membrane proteins with a defect in their luminal domain undergo the ERAD-L pathway, which is defined by the E3 ubiquitin ligase Hrd1p complex. The Hrd1p complex consists of several proteins including Hrd3p, an E2 complex (Ubc7p and its membrane-anchoring factor Cue1p), the Cdc48p complex (AAA-ATPase Cdc48p or p97, the Ufd1 and Npl4 cofactors, and the Ubx2p membrane anchor), Der1p, Yos9p, Kar2p (BiP) and Usa1p. It should be noted that the actual function of some of those proteins in the complex remains to be established. Aberrant membrane proteins with lesions in their cytosolic domain enter the ERAD-C pathway organized by the E3 ubiquitin ligase Doa10p complex. This complex is comparably simple and consists in addition to Doa10p only of the E2 complex and the Cdc48p complex. The ERAD-M pathway is followed by membrane proteins with a lesion in their transmembrane domain and involves only Hrd1p and Hrd3p. These dislocation pathways were established for yeast cells but most probably will apply to higher eukaryotes as well because of the evolutionary conservation of the ERAD pathways. The Doa10p complex also operates in the polyubiquitination of aberrant cytosolic and nuclear proteins, in addition to the ERAD-C pathway (Neuber et al. 2005; Ravid et al. 2006; Swanson et al. 2001).

This review will focus on how immunocytochemical labeling and electron microscopic analysis have helped to disclose the in situ subcellular distribution pattern of some of the key machinery proteins of the protein quality control, the organelle changes due to the presence of misfolded proteins, and the efficiency of synthetic chaperones to rescue disease-causing trafficking defects of aberrant proteins.

Machinery proteins of the protein quality control reside beyond the ER

For the quality control of glycoprotein folding, glucosidase II (Gls II) and UDP-glucose:glycoprotein glucosyltransferase (GT) in connection with the calnexin/calreticulin cycle are of eminent importance (Helenius and Aebi 2004; Parodi 2000; Roth 2002). Gls II is a luminal glycoprotein, which exists in two isoforms (Pelletier et al. 2000; Ziak et al. 2001) and does not contain known ER retention signals of the C-terminal KDEL type, nor any hydrophobic region characteristic of transmembrane proteins (Flura et al. 1997; Trombetta et al. 1996). As depicted in Fig. 1a, Gls II acts second to glucosidase I by removing the two inner α1,3-linked glucose residues (Brada and Dubach 1984; Burns and Touster 1982). The presence of three or two glucose residues on oligosaccharides can be considered to represent a trimming glyco-code whereas one glucose residue represents a trimming as well as folding glyco-code (Fig. 1b) (Jakob et al. 1998b). The involvement of Gls II and of mono-glucosylated oligosaccharides generated by the enzyme in the protein quality control is well documented (Hammond et al. 1994; Hebert et al. 1995; Jakob et al. 1998a, b). By confocal immunofluorescence, Gls II not unexpectedly exhibited a pattern typically observed for the ER as shown in Fig. 2b (Roth et al. 2003; Zuber et al. 2001). By high-resolution immunoelectron microscopy, ER localization of Gls II could be definitely established (Lucocq et al. 1986; Zuber et al. 2000, 2001). In addition to the rough ER including the nuclear envelope and the transitional ER, the smooth ER was also positive for Gls II. However, with the superior resolution of electron microscopic immunogold labeling, Gls II was additionally found in tubulovesicular clusters between transitional ER and the cis Golgi apparatus. They represent pre-Golgi intermediates

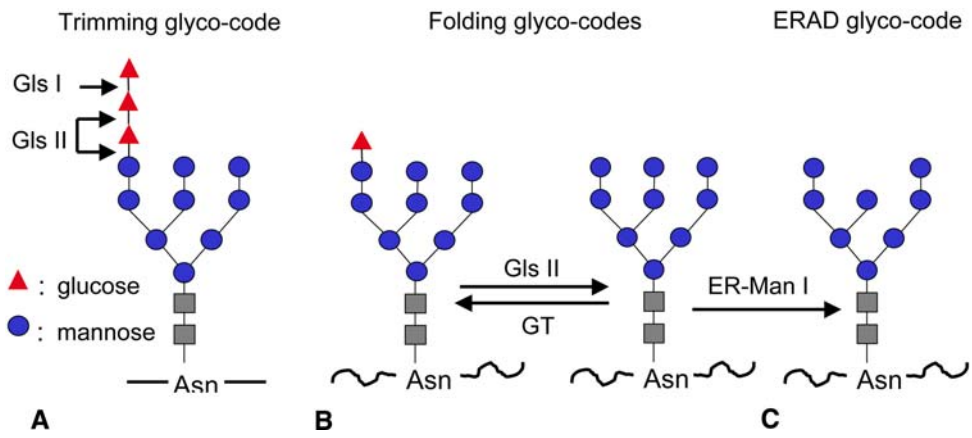

Fig. 1 Schematic presentation of the oligosaccharide trimming pathway by glucosidase I (*Gls I*), glucosidase II (*Gls II*), UDP-glucose:glycoprotein glucosyltransferase (GT) and ER-mannosidase I (ER-Man I)

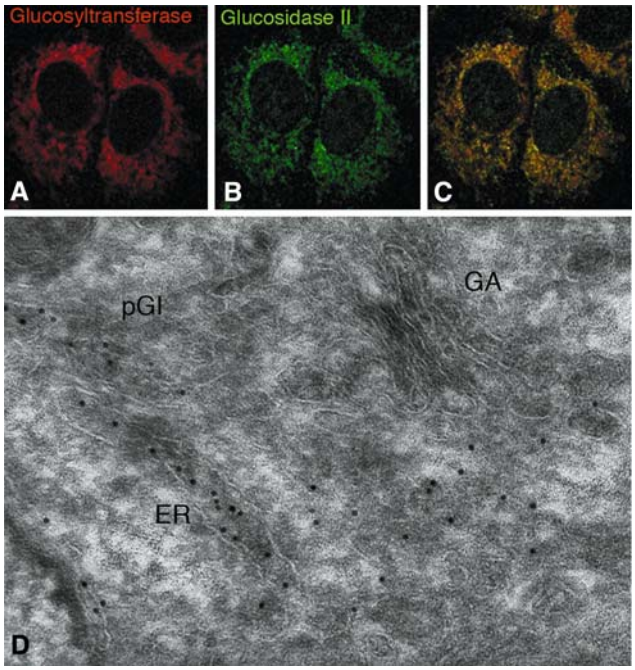

Fig. 2 Double confocal immunofluorescence for UDP-glucose:glycoprotein glucosyltransferase (**a**) and glucosidase II (**b**) demonstrates co-distribution (**c**) of the two protein quality control machinery proteins in cultured clone 9 hepatocytes. Immunogold localization of UDP-glucose:glycoprotein glucosyltransferase in an ultrathin frozen section of clone 9 hepatocytes reveals immunoreactivity in the rough ER (*ER*) including nuclear envelope and a pre-Golgi intermediate (*pGI*). The cisternal stack of the Golgi apparatus (*GA*) is not labeled

involved in antero- and retrograde transport of cargo (Appenzeller-Herzog and Hauri 2006; Bannykh and Balch 1997, 1998; Hammond and Glick 2000; Palade 1975; Saraste et al. 1987; Schweizer et al. 1988).

Mono-glucosylated oligosaccharides of glycoproteins are targeted by the calnexin/calreticulin cycle and after being deglucosylated by Gls II, will be targeted by GT if not correctly folded (Fig. 1b). GT apparently senses exposed patches of charged amino acids and reglucosylates the aberrant glycoproteins, which is followed by their re-entry in the calnexin/calreticulin cycle (Parodi et al. 1983; Sousa and Parodi 1995; Trombetta and Parodi 2003). When the subcellular distribution of GT was studied by confocal immunofluorescence, its labeling pattern (Fig. 2a) was alike that observed for Gls II (Fig. 2b; Zuber et al. 2001). By immunoelectron microscopy, GT was detectable in the rough ER including the nuclear envelope and the transitional ER as well as the smooth ER (Fig. 2d). Unlike Gls II, for which the labeling intensity over rough and smooth ER was equal, labeling intensity for GT over smooth ER was only 11% that of the rough ER. GT immunolabeling was also discovered in the pre-Golgi intermediates (Fig. 2d). Notably, the pre-Golgi intermediate immunolabeling for GT was approximately twice that of rough ER (Zuber et al. 2001). Double immunogold labeling for GT combined with the pre-Golgi intermediate marker ERGIC-53 and the COPII component sec23p (Hughes and Stephens 2008) proved the identity of the GT-labeled structures. Interestingly, specific immunogold labeling for calreticulin was also observed in the pre-Golgi intermediates (Zuber et al. 2000, 2001).

Together, these results provided new insight into the in situ subcellular organization of some key elements of the protein quality control machinery. Gls II, GT and calreticulin were not only present in the rough ER, as expected, but also in the smooth ER and unequivocally present in pre-Golgi intermediates. This pattern was found in different rat cell lines and tissues as well as *Drosophila* tissue and cell lines. The presence of three functionally closely associated proteins is a strong evidence for the involvement of pre-Golgi intermediates in protein quality control. Of course, immunolocalization provides no direct evidence for the functionality of the detected protein at a certain location. However, there is no reason to assume that Gls II, GT and calreticulin would be only functional in the ER. Studies in yeast have provided strong evidence that multiple, sequentially acting quality control checkpoints exist along the secretory pathway extending as far as to the Golgi apparatus (Arvan et al. 2002; Caldwell et al. 2001; Sayeed and Ng 2005; Taxis et al. 2002; Vashist et al. 2001; Vashist and Ng

2004; Younger and Chen 2006). In mammalian and insect cells, protein quality control is apparently not restricted to the ER, and the pre-Golgi intermediates appear to be involved in this fundamental cellular process as well. As will be discussed later, pre-Golgi intermediates represent not only a quality control checkpoint, but are also sites of accumulation of aberrant proteins.

The ERAD factor EDEM1 defines a novel vesicular ER exit pathway

As mentioned in the "Introduction", an impressive body of molecular and functional data exists concerning the macromolecular assemblies involved in the various ERAD pathways. The current well-founded basic conception of ERAD in yeast and higher eukaryotes is that the aberrant proteins after being removed from folding cycles are dislocated to the cytosol and eventually degraded by the 26S proteasome, and that this occurs in the ER. It is not clear whether this is a randomly occurring event or a more structured affair. Recent studies on EDEM1 in mammalian cells have provided preliminary evidence for a high level of subcellular organization.

The discovery that the Man_8 B isomer oligosaccharide was actively involved in ERAD-L (Fig. 1c) in yeast (Jakob et al. 1998a) and mammalian cells (Liu et al. 1999) paved the way to the identification of a lectin-like protein with sequence similarity to class I α1,2-mannosidases in yeast -Htm1p/Mnl1p- and mammalian cells -EDEM1- (Hosokawa et al. 2001; Jakob et al. 2001; Nakatsukasa et al. 2001). EDEM1 of mammalian cells is a soluble glycoprotein (Olivari et al. 2005; Zuber et al. 2007), which is regulated by the unfolded protein response (Hosokawa et al. 2001) and seems to connect the calnexin/calreticulin cycle to the dislocation process (Molinari et al. 2003; Oda et al. 2003). EDEM1 appears to exist in complex with the dislocation proteins Derlin-2 and -3, and the AAA ATPase p97 (Oda et al. 2006). It is not fully understood how EDEM1 interacts with aberrant proteins. However, there is evidence for interaction with ER-mannosidase I-trimmed oligosaccharides such as depicted in Fig. 1c (Hosokawa et al. 2003). Overexpression of EDEM1 has been shown to prevent formation of dimers of misfolded Null Hong Kong variant of alpha1-antitrypsin (Hosokawa et al. 2006). Notably, EDEM1 and ER-mannosidase I do not exist in complexes, which can be immunoprecipitated (Hosokawa et al. 2003).

Recently, the subcellular distribution of endogenous EDEM1 in various mammalian cell types was established with a specific anti-peptide antibody (Zuber et al. 2007). Unexpectedly, its immunofluorescence pattern did not correlate with that of calnexin and other ER marker proteins. Rather, an unusual pattern of well distributed punctate structures along with some localized finger-like structures was revealed (Fig. 3a–c). The distribution patterns of endogenous EDEM1 and that of overexpressed tagged EDEM1 were dramatically different: instead of a punctate, non-ER pattern, a typical reticular ER pattern plus punctate staining was observed (Zuber et al. 2007). This striking difference in subcellular distribution between endogenous EDEM1 and overexpressed tagged EDEM1 was confirmed by Optiprep density gradients. Endogenous EDEM1 was restricted to the densest fractions, whereas tagged EDEM1 showed the same broad distribution as observed for calnexin, sec61β, and Derlin-1 and -2 (Zuber et al. 2007). In this context, it needs to be emphasized that previous biochemical analyses of EDEM1 interaction with quality control machinery proteins and ERAD substrates were performed with cells transiently overexpressing tagged EDEM1 (Hosokawa et al. 2003; Molinari et al. 2003; Oda et al. 2003). The nature of the EDEM1 immunofluorescence pattern was clarified by immunogold labeling and serial section analysis (Fig. 3d–h). It revealed the presence of EDEM1-reactive buds along rough ER cisternae which apparently gave raise to ~150 nm vesicles. These buds and vesicles were devoid of a COPII coat, formed outside the canonical ER exit sites of the transitional ER and were not found in the tubulovesicular clusters of pre-Golgi intermediates (Fig. 3i). Occasionally, EDEM1 luminal immunolabeling in limited parts of distended ER cisternae was observed, which accounted for approximately 11% of the immunogold labeling for GT. Double confocal immunofluorescence for endogenous EDEM1 in rat hepatoma clone 9 cells stably expressing the Null Hong Kong variant alpha 1-antitrypsin showed co-distribution of the two proteins (Zuber et al. 2007). Together, these data revealed the existence of a vesicular transport pathway out of the rough ER through which the ERAD factor EDEM1 and an ERAD substrate became sequestered from the early secretory pathway. Through this pathway potentially harmful aberrant luminal proteins can be removed. These findings also indicate that the Gls II and GT containing pre-Golgi intermediates appear to be not involved in the dislocation of an ERAD-L substrate.

Endomannosidase assigns glucose trimming function to the Golgi apparatus

It is generally assumed the glucose trimming occurs exclusively by Gls I and II and, therefore, is limited to the ER and pre-Golgi intermediates. However, under conditions of inhibition of trimming glucosidases, formation of mature oligosaccharides has been observed. This apparent paradox could be explained by the existence of an alternate glucose-trimming pathway by neutral endo-alpha-mannosidase (Lubas and Spiro 1987, 1988; Moore and Spiro 1990, 1992; Spiro

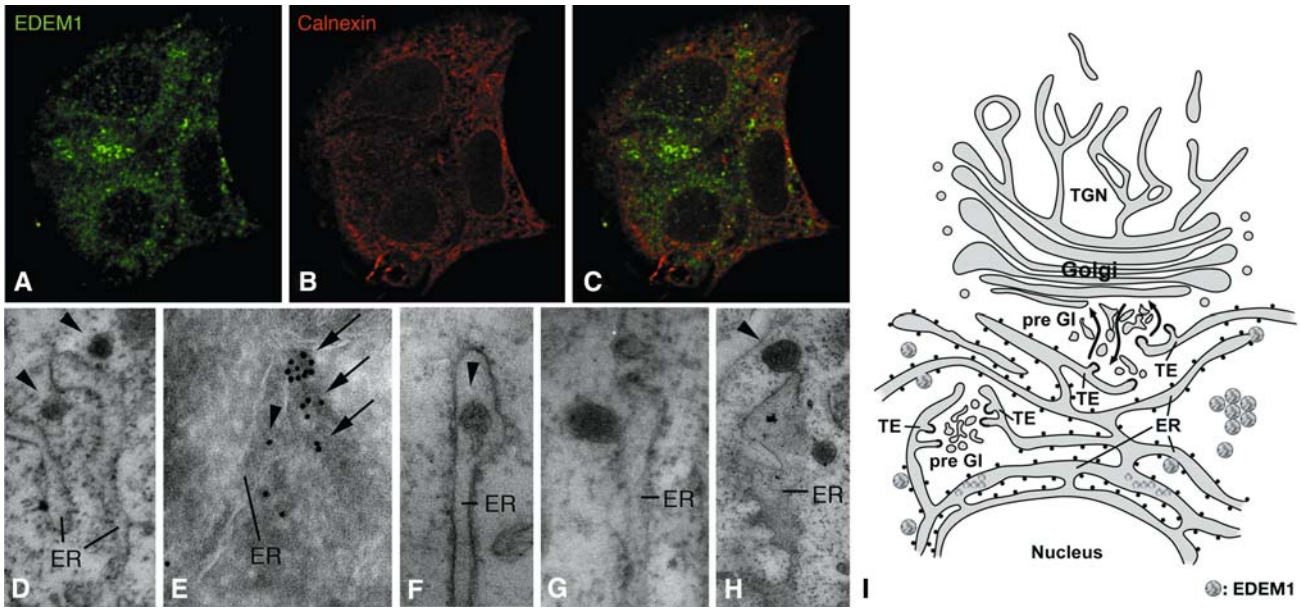

Fig. 3 Double confocal immunofluorescence for endogenous EDEM1 (**a**) and calnexin (**b**) reveals different distribution patterns for the two proteins (**c**) in human HepG2 cells. Detection of endogenous EDEM1 by immunogold labeling of ultrathin frozen sections (**e**) or pre-embedding immunoperoxidase labeling (**d**, **f**–**h**) reveals sparse labeling in the lumen of ER cisternae (*arrowheads* in **e** and **f**) and intense labeling over ER buds and vesicles pinching-off the ER (from Zuber et al. 2007). The subcellular distribution pattern of endogenous EDEM1 is schematically depicted in (**i**). In contrast to COPII-coated buds, which are formed at the transitional ER (TE) and give raise to COPII-coated vesicles present in pre-Goli intermediates (pre GI), EDEM1-positive buds occur outside the transitional ER and EDEM1-positive COPII-unreactive vesicles form clusters in the cytoplasm

2000). Endomannosidase is currently the only known endoglycosidase. In contrast to the trimming Gls I and II, it cleaves internally between the glucose-substituted mannose and the remaining oligosaccharide (Fig. 4a). Its substrate specificity (Fig. 4a) is basically that of Gls I and II (Moore and Spiro 1990, 1992; Rabouille and Spiro 1992). However, unlike Gls I and II, ER-mannosidase I trimmed mono-glucosylated oligosaccharides are a substrate of endomannosidase. The resulting Man_{8-5} GlcNAc isomer A is the specific product of endomannosidase. It should be noted that this oligosaccharide is no more a substrate for reglucosylation by GT. Biochemically, activity for endomannosidase was found to be enriched in Golgi membranes (Lubas and Spiro 1987). By immunofluorescence (Dong et al. 2000; Zuber et al. 2000), endomannosidase exhibited a crescent-shaped perinuclear staining and fine punctate staining throughout the cytoplasm which partially overlapped with immunofluorescence for Gls Golgi mannosidase II (Fig. 4b–d). High-resolution immunoelectron microscopy demonstrated endomannosidase in the peripheral and Golgi-associated pre-Golgi intermediates as well as cis and medial cisternae of the Golgi apparatus (Fig. 4e) (Zuber et al. 2000). Trans cisternae of the Golgi apparatus and the trans Golgi network were unreactive. Quantification revealed ~85% of the immunogold labeling for endomannosidase in the Golgi apparatus and ~15% in pre-Golgi intermediates. Although, both endomannosidase and Gls II could be detected in pre-Golgi intermediates by double immunogold labeling, they labeled different elements of the vesiculotubular clusters (Fig. 4e). Thus, endomannosidase and Gls I and II exhibited non-overlapping subcellular distributions (Roth et al. 2003; Zuber et al. 2000). Functionally, the presence of endomannosidase in the ER would interfere with the action of glucosyltransferase by preventing the reglucosylation of misfolded glycoproteins. Together, these findings demonstrating a predominantly Golgi apparatus localization of endomannosidase strongly indicated that glucose trimming of N-linked oligosaccharides is not limited to the ER.

Since glucose trimming is indispensable for the synthesis of mature oligosaccharide side chains, deglucosylation by endomannosidase in the Golgi apparatus ensures that this important process is not blocked. Further biochemical and morphological analyses demonstrated that Golgi apparatus localized endomannosidase-processed oligosaccharides of alpha 1-antitrypsin irrespective of their folding state (Torossi et al. 2006). From the literature, it is well known that disease-causing misfolded glycoproteins to a certain extent might escape the protein quality control and become secreted (Cox 2001; Desnick et al. 2001). As a case in point, in humans suffering from alpha1-antitrypsin deficiency, the Z-variant of alpha1-antitrypsin not only becomes partially secreted, but also is active as serine protease inhibitor (Cabral et al. 2002; Teckman and Perlmutter 1996). As experimentally shown for the Z-variant of alpha1-antitrypsin (Torossi et al. 2006),

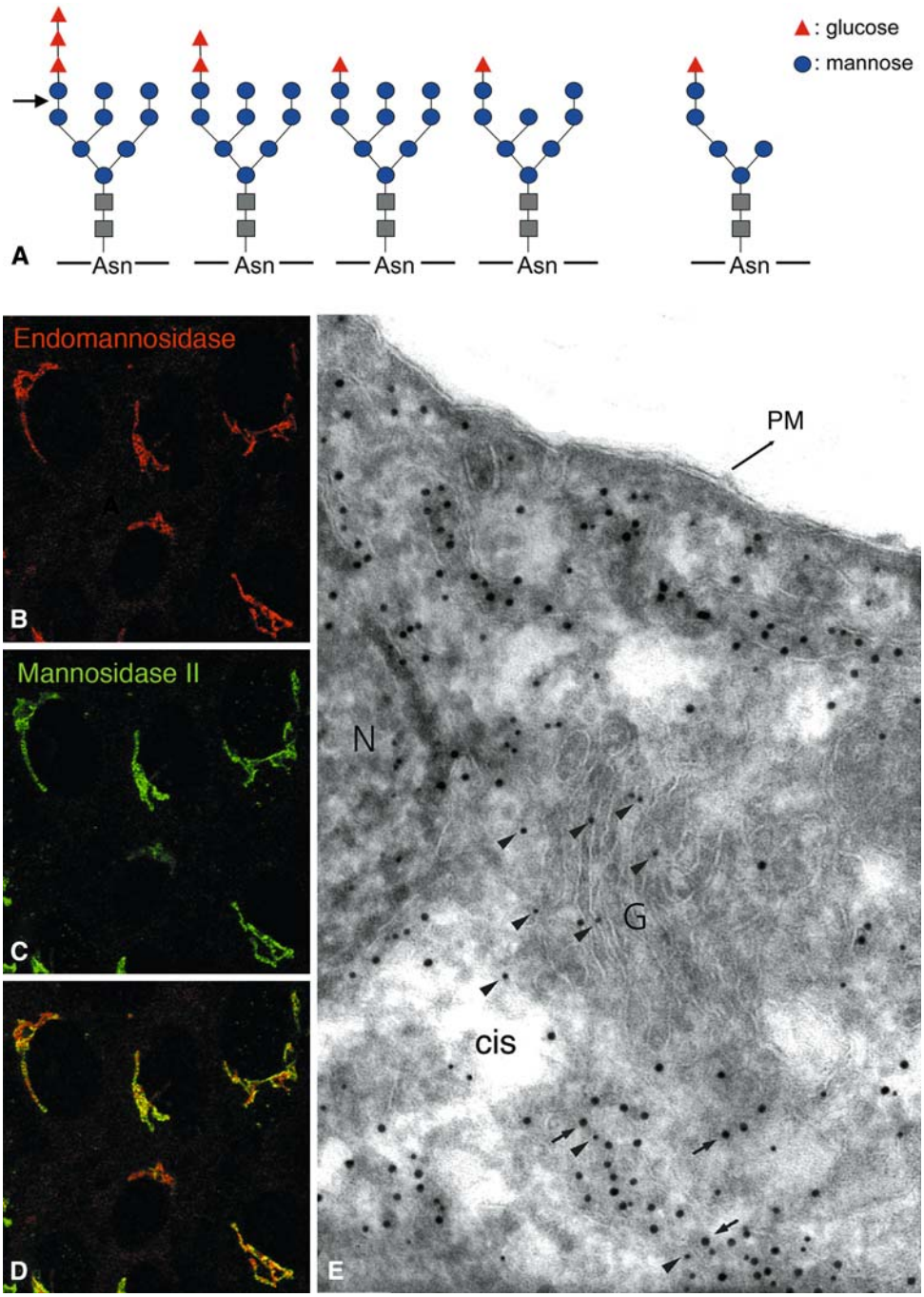

Fig. 4 The various oligosaccharidic substrates of endomannosidase are depicted in (**a**). Like trimming glucosidases, endomannosidase trimms $Gls_{1-3}Man_9GlcNAc_2$, and unlike trimming glucosidase II, monoglucosylated oligosaccharides with mannose-trimmed B and C branches. Double confocal immunofluorescence for endomannosidase (**b**) and Golgi mannosidase II (**c**) reveals co-distribution of the two enzymes (**d**) in clone 9 hepatocytes. Double immunogold labeling for endomannosidase (small gold particles, *arrowheads*) and glucosidase II (large gold particles, *arrows*) reveals endomannosidase localization in cis and middle Golgi apparatus cisternae (**g**), whereas glucosidase II is observd in rough ER including nuclear envelope. Non-overlapping immunogold labeling for both enzymes exists in pre-Golgi intermediates. *N*: nucleus, *PM*: plasma membrane. Micrographs **b**–**e** are from Zuber et al. (2000)

endomannosidase provided a back-up mechanism for its deglucosylation en route through the Golgi apparatus. Processing of its oligosaccharides to mature ones is apparently important for their proper trafficking and correct functioning.

Organelle changes due to intracellular accumulation of misfolded proteins

As a general rule, misfolded proteins become targeted by the protein quality control and following polyubiquitination will be degraded by proteasomes (Eisele et al. 2006; Hochstrasser 1996; Jarosch et al. 2002; McCracken and Brodsky 2005; Wolf and Hilt 2004; Zwickl et al. 2002). Depending on various factors such as the efficiency of the ubiquitin–proteasome system, the intracellular amounts of misfolded glycoproteins and their biophysical properties as well as interactions with other proteins, a whole spectrum of organelle changes can be observed in protein folding diseases.

For some protein folding diseases, no significant structural aberrations of the early secretory pathways could be

observed. An example is Fabry's disease, an inherited deficiency of lysosomal alpha-galactosidase A (alpha-Gal A), which causes progressive lysosomal glycosphingolipid accumulation (mainly globotriosylceramide Gb3) (Desnick et al. 2001). Disease-causing mutant alpha-Gal A could be shown by immunofluorescence to be retained in the ER where it existed in complexes with the chaperone BiP (Yam et al. 2005, 2006). From this, we concluded that recognition and ER-retention of the mutant alpha-Gal A by the protein quality control machinery constituted the mechanism leading to lysosomal deficiency in alpha-Gal A. Electron microscopic analysis of cultured fibroblast from Fabry patients harboring different mutations did not reveal significant changes of the morphology of the ER and the pre-Golgi intermediates. As expected, the fibroblasts contained numerous large lysosomes with characteristic multilamellar inclusions. Thus, the intracellularly retained mutant alpha-Gal A apparently became dislocated and was efficiently degraded by the ubiquitin–proteasome system. A similar situation was observed for a polytope membrane protein, aquaporin-2, whose folding mutants can cause renal diabetes insipidus (Canfield et al. 1997; Morello and Bichet 2001). The T126M mutant aquaporin-2 was found to be retained in the ER and efficiently degraded by proteasomes without causing ER dilatation (Hirano et al. 2003). ER retention and rapid proteasomal degradation are also hallmarks of the pulmonary form of alpha 1-antitrypsin deficiency (Lomas and Parfrey 2004; Sifers et al. 1988). However, other types of protein folding diseases have been shown to result in the distention of the ER cisternae. One example is the congenital hypothyroid goiter in which the mutant thyroglobulin is misfolded (Kim et al. 1996; Kim and Arvan 1998; Kim et al. 1998, 2000; Medeiros Neto et al. 1996). In disorders of procollagen biosynthesis, distended ER cisternae were also observed (Bogaert et al. 1992). Other examples are represented by LDL receptor class 2 mutants (Lehrman et al. 1987; Pathak et al. 1988).

There are protein folding diseases associated with both distended ER cisternae and enlarged pre-Golgi intermediates. A misssense mutation of the insulin 2 gene (*Cys96Tyr*) in Akita mice disrupting one of the two interchain disulfide bonds is associated with intracellular accumulation of misfolded proinsulin (Wang et al. 1999). This resulted in a significant increase of the volume density of dilated ER profiles and of the pre-Golgi intermediates (Fig. 5a, b) (Fan et al. 2007; Zuber et al. 2004). For the latter, a significant increase of the tubular elements was observed. Although the mutant proinsulin was degraded through proteasomes (Wang et al. 1999), its accumulation in the early secretory pathway caused an activation of the unfolded protein response and induced apoptosis (Oyadomari et al. 2002a; Oyadomari et al. 2002b). Other mutant proteins such as the cystic fibrosis (Kopito 1999; Riordan 1999) causing delta F508 variant of the chloride channel (Gilbert et al. 1998) and misfolded major histocompatibility complex class I protein (Hsu et al. 1991; Raposo et al. 1995), have been shown to accumulate in the expanded pre-Golgi intermediates.

Certain other misfolded proteins are accompanied by the formation of insoluble aggregates in the lumen of the ER, which physically precludes dislocation to the cytosol and exposure to proteasomes. The stress-induced so-called intracisternal granules in the pancreas of starved guinea pigs (Palade 1956), which are composed of aggregated proenzymes (Fig. 6a) (Geuze and Slot 1980; Pavelka and Roth 2005), form a classical example. For the liver-disease-causing alpha 1-antitrypsin Z variant, about 15% of the non-secreted mutant protein is polymerogenic and thus forms insoluble aggregates in the ER lumen, which cannot be degraded (Lomas et al. 1992, 2004) The Glu342Lys substi-

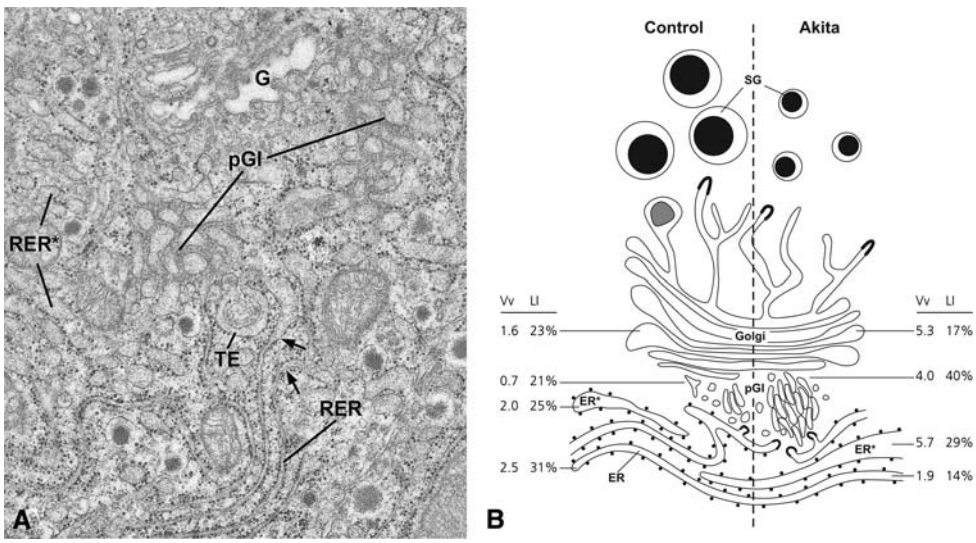

Fig. 5 Details of an insulin-producing pancreatic beta cell from Akita mice. The presence of misfolded proinsulin results in the local distention of rough ER cisternae (*RER**). *Arrows* point to regions of transition of non-distended ER (*RER*) to distended ER (*RER**). In addition, the pre-Golgi intermediates (*pGI*) are greatly enlarged. G: Golgi apparatus, TE: transitional ER. In (B), the organelle changes in terms of differences of their volume density (Vv) and differences in proinsulin distribution pattern (LI) are schematically shown (from (Zuber et al. (2004)

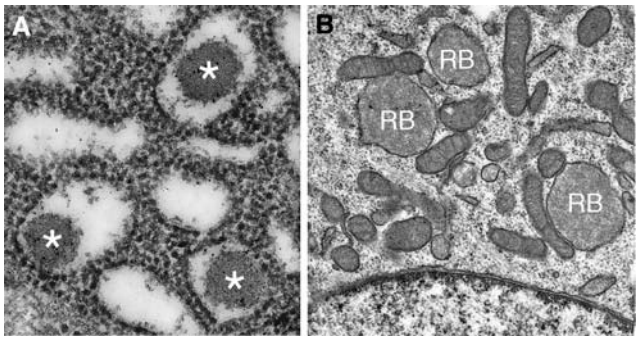

Fig. 6 **a** Intracisternal granules (*asterisks*) in the rough ER of exocrine rat pancreatic cells induced by puromycin treatment. These granules correspond to mini Russell bodies and are composed of aggregated proenzymes. **b** Russell bodies (*RB*) induced by heat shock in CHO cells. They represent distended parts of rough ER cisternae filled with protein aggregates. Note the structurally normal appearing rough ER cisternae in their neighborhood

tution of the Z-variant results in a spontaneous loop-sheet polymerization of the protein. In contrast to the above-mentioned protein folding diseases, which all have in common a loss-of-function pathogenesis, the Z-variant-caused alpha 1-antitrypsin deficiency seems to involve a pathologic gain-of-function pathogenesis (Hidvegi et al. 2005). The ER inclusions described above belong to the category of Russell bodies. Russell bodies represent subregions of the rough ER in which insoluble proteins accumulate (Fig. 6b) (Alanen et al. 1985; Kopito and Sitia 2000; Valetti et al. 1991). They are typically found in cells synthesizing mutant immunoglobulins (Alanen et al. 1985; Kopito and Sitia 2000; Mattioli et al. 2006; Valetti et al. 1991) but also in cells synthesizing, for instance, mutant myocilin. Mutations of the myocilin gene are associated with primary open-angle glaucoma (Tamm 2002). Mutant myocilins are secretion-incompetent and have been shown biochemically to form intracellular detergent-insoluble complexes (Gobeil et al. 2004; Jacobson et al. 2001; Sohn et al. 2002). In cultured cells transfected to express both mutant and wild-type myocilin, heteromeric, detergent–insoluble protein complexes were formed which were segregated into typical Russell bodies (Yam et al. 2007c). Thus, myocilin-caused open-angle glaucoma represents a protein folding disease. Its pathogenesis involves a pathological gain-of-function mechanism because of the interaction and complex formation of mutant with wild-type myocilin (Gobeil et al. 2004; Joe et al. 2003; Sohn et al. 2002; Yam et al. 2007c). As a consequence, unfolded protein response factors and pro-apoptotic factors were up-regulated and cells underwent apoptosis (Yam et al. 2007c) as detected by the appearance of lobulated nuclei and the TUNEL assay (Taatjes et al. 2008).

All the mutant proteins discussed above are luminal or membrane proteins. What happens to aberrant cytosolic and nuclear proteins? Same like ER proteins, one extreme situation is that they become efficiently degraded by cytosolic and nuclear proteasomes subsequent to polyubiquitination (Schubert et al. 2000; Turner and Varshavsky 2000). Thus, cytosolic and nuclear quality control in normal cells suppresses the formation of aggregates of aberrant proteins by degrading them. The other extreme is represented by the formation of cytosolic and nuclear inclusion bodies due to inefficient degradation of aberrant proteins by the ubiquitin–proteasome system. Cytosolic, non-membrane bounded inclusion bodies are generally called aggresomes (Corboy et al. 2005; Kopito and Sitia 2000). They consist of pericentriolar protein aggregates surrounded by a cage of intermediate (vimentin) filaments that are the most consistent component of aggresomes in addition to ubiquitin, proteasomes and molecular chaperones. Aggresomes can be induced experimentally by forced overexpression of aggregation-prone mutant proteins or by experimentally inhibiting proteasomes (Fig. 7) (Anton et al. 1999; Fan et al. 2007; Johnston et al. 1998; Wigley et al. 1999). On the other hand, it has been shown that protein aggregates can directly impair the function of the ubiquitin–proteasome system (Bence et al. 2001). The formation of aggresomes is a multi-step process, which depends on the intact microtubules. Aggresomes are formed by the coalescence of small protein aggregates transported from the cell's periphery along microtubules to centrioles (Garcia-Mata et al. 1999; Johnston et al. 1998; Kawaguchi et al. 2003; Vidair et al. 1966; Wigley et al. 1999; Wojcik et al. 1996). In the nucleus, the inclusion bodies can be found in association with the promyelocytic leukemia oncogenic domains (Anton et al. 1999).

Inclusion bodies have been observed in association with a number of chronic neurodegenerative diseases such as Parkinson's disease, Huntington's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Johnston et al. 2000; Kabashi and Durham 2006; Rubinsztein 2006; Selkoe 2003; Shults 2006; Soto 2003). Inclusion bodies named Lewy bodies are a morphological hallmark of Parkinson's disease and other neurodegenerative disorders (McNaught et al. 2002b; Olanow et al. 2004; Shults 2006). Lewy bodies in the dopaminergic neurons resemble aggresomes and represent spherical bodies commonly composed of a core of granular material and peripheral radiating filaments. They contain a variety of proteins such as alpha-synuclein, the alpha-synuclein-binding protein synphilin-1 torsin A, neurofilaments, ubiquitin, proteasomal subunits and various heat shock proteins as well as ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, ubiquitin ligase enzymes and proteasome activators. Furthermore, they contain centrosome-related gamma-tubulin and pericentrin. Thus, it has been proposed that the formation of Lewy bodies represents an aggresome-like response in dopaminergic neurons (McNaught et al. 2002c). Considering the observed impair-

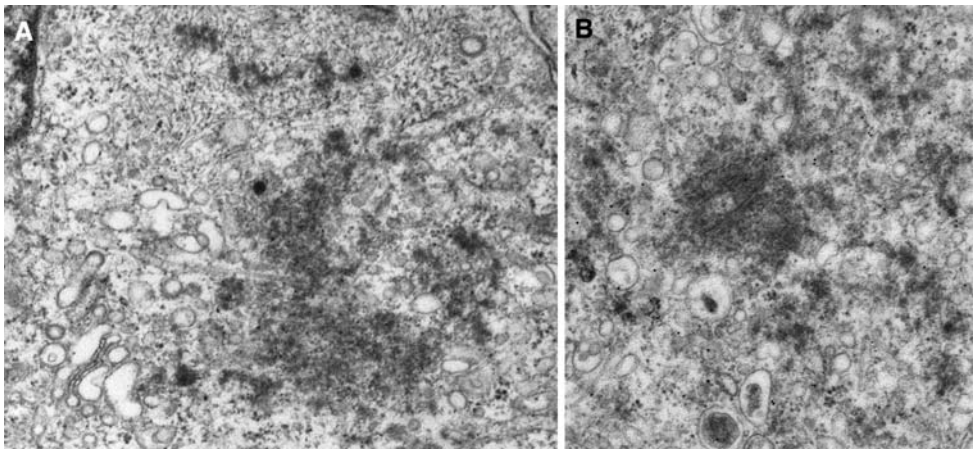

Fig. 7 Formation of pericentriolar aggresomes following proteasome inhibition by lactacystin in CHO cells stably expressing misfolded proinsulin. Irregularly shaped, electron dense flocculent material is present in the cytoplasm and surrounded by intermediate filaments (**a**). At higher magnification, the spatial relationship between the protein aggregates and a centriole can be seen (**b**). Micrographs from Fan et al. (2007)

ment of the ubiquitin–proteasome system in patients with Parkinson's disease (McNaught et al., 2001, 2002a, 2006), it is assumed that Lewy bodies, by segregating increasing levels of aberrant and potentially cytotoxic proteins, might protect the neurons (Olanow et al. 2004). It should be stressed, however, that the mechanism leading to selective neuronal death in Parkinson's disease is not fully understood and the role Lewy bodies might be playing needs to be studied further studies (Lindholm et al. 2006).

Synthetic chaperones for treatment of protein folding disease

The various protein folding diseases mentioned above can be classified based on the pathogenetic mechanism. Efficient proteasomal degradation of the misfolded protein is characteristic of the loss-of-function pathogenesis. This is the case in protein folding diseases such as cystic fibrosis, the lung form of alpha 1-antitrypsin deficiency, aquaporin 2-caused renal diabetes insipidus, Gaucher's disease and Fabry's disease. Here, the missing function of the degraded protein alone can be the cause of the clinical symptoms, or secondary effects due to substrate accumulation like in lysosomal storage diseases. Intracellular accumulation due to inefficient proteasomal degradation of misfolded proteins is representative of a pathological gain-of-function mechanism, which is combined with a loss of function. Intracellular accumulation of misfolded proteins associated or not with protein aggregation can result in the activation of the unfolded protein response leading to ER stress and apoptosis. A pathological gain-of-function mechanism can be also the cause of a dominant clinical course when the wild-type protein in complexes with the mutant protein is retained inside the cells. Examples for pathological gain-of-function pathogenesis-associated protein folding diseases are myocilin-caused open-angle glaucoma, familial hypophyseal diabetes insipidus, Parkinson's disease and Huntington's disease.

Many attempts have been made to at least partially correct the protein misfolding in order to overcome their trafficking defect and to alleviate ER stress. Among other approaches, small molecule synthetic chaperones have been used in order to shift the folding equilibrium of mutant proteins towards a more native state (Arakawa et al. 2006; Chaudhuri and Paul 2006; Cohen and Kelly 2003; Papp and Csermely 2006; Perlmutter 2002). Chemical chaperones include osmotically active substances such as DMSO, glycerol, polyols or deuterated water, and other compounds such as 4-phenylbutyric acid (Burrows et al. 2000; Lim et al. 2004; Liu et al. 2004; Pedemonte et al. 2005; Rubenstein and Zeitlin 2000; Tamarappoo and Verkman 1998; Tveten et al. 2007; Welch and Brown 1996). Other substances such as enzyme inhibitors (Fan et al. 1999; Matsuda et al. 2003; Sawkar et al. 2002) and receptor ligands or antagonists {Petäjä-Repo, 2002 #16211;Egan, 2002 #12283} have been shown to function as pharmacological chaperones.

Here, we have chosen two examples from our work to demonstrate how immunocytochemistry and microscopy in combination with biochemical analyses can be applied to demonstrate the functionality of a chemical and a pharmacological chaperone in rescuing the consequence of disease-causing protein misfolding. It has been mentioned above that open-angle glaucoma-causing mutant myocilin forms insoluble protein aggregates in the ER lumen (Russel bodies), which result in ER stress and apoptotic cell death (Yam et al. 2007b). Among the other tested chemical chaperones, treatment with sodium 4-phenylbutyrate significantly reduced the amount of intracellular detergent–insoluble myocilin aggregates and thereby the number of Russel bodies in the cells (Fig. 8a–c), diminished mutant myocilin interaction with calreticulin and restored the secretion of mutant myocilin. As a consequence, the ER stress was released and most interesting, the apoptosis rate was reduced close to levels observed in control cells expressing wild-type myocilin (Fig. 8d). Thus, sodium

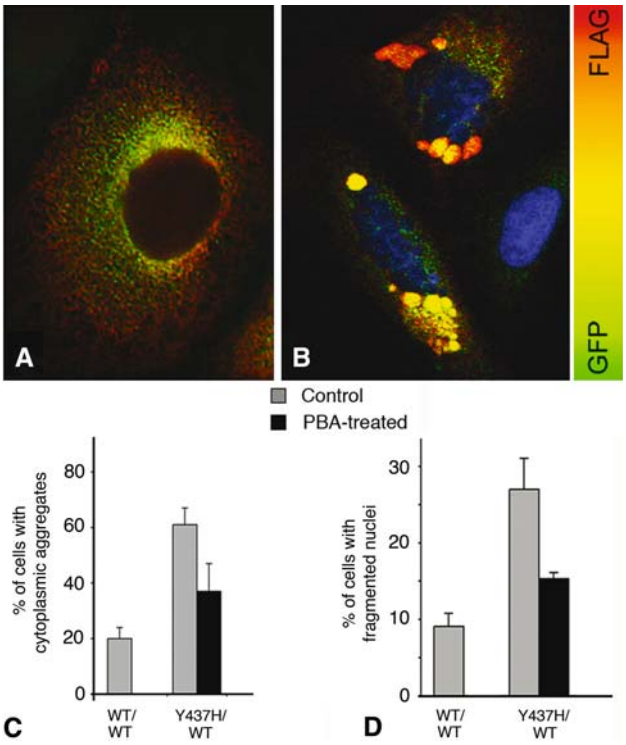

Fig. 8 Confocal double fluorescence of HEK 293 cells stably expressing GFP-wt and FLAG-wt myocilin reveals an ER and fine punctate pattern (**a**). HEK 293 cells coexpressing GFP-mutant myocilin and FLAG-wt myocilin exhibit distinct cytoplasmic aggregates, which correspond to Russell bodies (**b**). Treatment with the chemical chaperone sodium 4-phenylbutyrate results in reduction of the percentage of cells with myocilin-containing Russell bodies (**c**) and a drastic reduction of the apoptosis rate (**d**). *Empty columns* in **d** show values for untreated cells and filled columns for sodium 4-phenylbutyrate treated cells. From Yam et al. (2007a)

4-phenylbutyrate exerts a beneficial effect by protecting the cells from the deleterious effects of mutant myocilin. Since sodium 4-phenylbutyrate is a tissue and cell-permeable molecule, it holds the potential for topical administration in the treatment of myocilin-caused primary open-angle glaucoma.

The second example concerns Fabry's disease, a lysosomal storage disorder caused by a deficiency of alpha-Gal A activity in lysosomes that results in the accumulation of glycosphingolipid globotriosylceramide (Gb3). The lysosomal trafficking of mutant alpha Gal A is impaired because the enzyme is retained in the ER by the protein quality control (Yam et al. 2005). Others had demonstrated that the activity of mutant alpha-Gal A in vitro at neutral pH could be stabilized with the competitive enzyme inhibitor 1-deoxygalactonorijimycin (DGJ) (Fan et al. 1999). Treatment of cells expressing mutant alpha-Gal A with a non-inhibitory dose of DGJ enhanced the intracellular enzyme activity (Yam et al. 2005, 2006). In addition, we could demonstrate by immunofluorescence and quantitative immunogold labeling that the mutant enzyme was redistributed from the ER to lysosomes and that this trafficking was mannose 6-phosphate-dependent. The DGJ treatment resulted in release of mutant alpha-Gal A from the chaperone BiP and in its conversion in the mature lysosomal form. Double confocal immunofluorescence and immunogold labeling demonstrated that the lysosomal Gb3 storage was cleared and that the size of the lysosomes became normalized (Yam et al. 2005, 2006). Together, this demonstrated that DGJ exhibited a chaperone-like effect and induced the trafficking of ER-retained mutant alpha Gal A to lysosomes where the enzyme was catalytically active. Therefore, the pharmacological chaperone DGJ potentially offers a convenient and cost-efficient therapeutic alternative to enzyme replacement therapy.

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