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Epidermal Lamellar Granules Transport Different Cargoes as Distinct Aggregates

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Abbreviations used: CatD, cathepsin D; Cdsn, corneodesmosin; GlcCer, glucosylceramides; KLK, kallikrein; LGs, lamellar granules; PB, phosphate buffer, pH7.4; PBS, phosphate buffered saline; TGN, trans-Golgi network

Keywords: Keratinocytes; Electron microscopy; Immunoelectron microscopy; Trans-Golgi network

Summary

Lamellar granules (LGs) of the epidermis appear as discrete round or oblong shaped granules in classical transmission electron micrographs, but a recent cryo-transmission electron microscopy study has claimed that LGs are in fact branched tubular structures. LGs contain various cargoes including lipids, hydrolytic enzymes, and several other proteins. It is not known whether there are any differences in the timing of expression among them and whether they are sorted into the granules individually or collectively. In order to address these questions, we studied the expression of glucosylceramides (GlcCer), cathepsin D (CatD), corneodesmosin (Cdsn), kallikrein (KLK) 7, and KLK8 in normal human epidermis using confocal laser scanning microscopy and immunoelectron microscopy. The results were consistent with the model that LGs are parts of a branched tubular structure. In this structure, all the components were shown to be distributed as separate aggregates. In the trans-Golgi network (TGN), bulbous protrusions containing GlcCer, Cdsn, KLK7 and KLK8, and small CatD-positive vesicles were observed. The molecules were shown to be delivered to the apical region of granular keratinocytes. The present study provides strong evidence for the sequential synthesis and independent trafficking of various LG cargoes, including for the first time CatD and KLK8, from TGN.

Lamellar granules (LGs), also known as keratinosomes, lamellar bodies, membrane-coating granules, and Odland bodies, are specialized secretory granules of the keratinizing stratified squamous epithelia and are thought to be essential in barrier formation and desquamation (Odland and Hobrook, 1981; Hayward, 1979). LG components include glucosylceramides (GlcCer) and other lipids (Madison et al, 1998), various hydrolytic enzymes, such as proteases, acid phosphatases, glucosidases, and lipases (Grayson et al, 1985; Freinkel and Traczyk, 1985; Freinkel and Traczyk, 1983; Madison et al, 1998; Hayward, 1979; Wolff and Schreiner, 1970), and other proteins including corneodesmosin (Cdsn) (Serre et al, 1991). LGs have been observed by electron microscopy as round or oblong, membrane-delimitated, lamellate organelles (Hayward, 1979; Odland and Holbrook, 1981; Landmann, 1988). The classical view has been that LGs are produced as discrete granules in the stratum spinosum, probably from the Golgi apparatus, and then migrate to the cell surface, fuse with the plasma membrane, extruding their contents in the outer stratum granulosum. However, this view was recently challenged by Norlen, who proposed the 'membrane folding' model in which the trans Golgi network (TGN) and LGs as well as the intercellular space at the border zone between the stratum granulosum and stratum corneum are part of one and the same continuous membrane structure (Norlen, 2001).

Although epidermal granular keratinocytes are rich in LGs, they have not been fully appreciated as specialized secretory cells. Only recently, Elias et al. focused on this issue and characterized the most superficial granular keratinocytes as secretory granular cells (Elias et al, 1998). LGs are considered to be lysosome-related organelles (Madison et al, 1998), but it is not known whether these cells use the same sorting system for LGs as that used for classical lysosomes. Although a number of papers on lipid and lysosomal enzymes associated with LGs have been published, comparative analysis of the expression timing and localization of the various LG cargoes has yet to be performed. It is not known whether there is a single species of LG having mixed cargoes or distinct species of LGs transporting different cargoes, nor is it known whether different LG cargoes are synthesized simultaneously. In order to elucidate the overall structure of LGs as well as the synthetic and trafficking mechanisms of LG contents, we studied the localization of GlcCer, cathepsin D (CatD), kallikrein (KLK)7, KLK8 and Cdsn and found that each formed distinct aggregates. Ultrastructural images of LGs were consistent with parts of a branched tubular structure.

Materials and Methods

Antibodies

The following antibodies were used: two mouse anti-Cdsn antibodies (G36-19, F28-27) (Serre et al, 1991; Guerrin et al, 1998); rabbit anti-KLK 7 (Tanimoto et al, 1999); rabbit anti-KLK8 (Underwood et al, 1999); rabbit anti-GlcCer (Brade et al, 2000) (Glycobiotech, Kukels, Germany); rabbit anti-CatD (Oncogene, San Diego, CA); mouse anti-CatD (Oncogene); and sheep anti-TGN46 (Serotec, Oxford, UK). For immunofluorescence, the following secondary reagents were used: Alexa-Fluor 488 goat anti-rabbit IgG highly cross-absorbed (Molecular Probes, Eugene, OR); Cy3-labeled goat anti-mouse IgG (Amersham Bioscience, Buckinghamshire, UK); and Cy3-conjugated affinity-purified donkey anti-sheep IgG (Chemicon International, Temecula, CA). Secondary antibodies used for electron microscopy were 10-nm or 5-nm gold-conjugated goat anti-rabbit IgG (Amersham Bioscience), 10-nm or 5-nm gold-conjugated goat anti-mouse IgG (Amersham Bioscience), and 10-nm gold-conjugated donkey anti-sheep IgG (BBInternational, Cardiff, UK).

Immunofluorescence analysis

Biopsies of normal skin from healthy donors were immersed in 2% paraformaldehyde/ phosphate buffered saline (PBS) for 1 h at 4° C, rinsed with PBS and incubated with 100 mM glycine/PBS for 1 h at 4° C. After rinsing in PBS, samples were embedded in O.C.T. compound (Sakura Finetechnica, Tokyo, Japan). For immunofluorescence analysis, 6 micro meter-thick cryostat sections of the samples were cut and mounted on slides, and incubated with primary antibodies diluted in PBS for 1 h at 37° C. After washing in PBS, fluorescent secondary antibodies were applied for 30 min at 37° C. For double-labeling with antibodies raised in different animals, a mixture of primary antibodies was applied and this was followed by incubation with a mixture of secondary antibodies-conjugated with different fluorescent dyes. Nuclei were stained with propidium iodide (Sigma Chemical, St Louis, MO) or TO-PRO-3 (Molecular Probes). Slides were mounted using a ProLong Antifade Kit (Molecular Probes). Fluorescence images were observed using a Fluoview FV500 confocal laser scanning microscope equipped with an argon-ion laser (excitation lines at 488 nm), a Helium Neon Green laser (excitation

lines at 543 nm), and Helium Neon Red laser (excitation lines at 633 nm) and mounted on a Olympus BX61 microscope (Olympus). Imaging was performed using Fluoview version 2.0 software (Olympus America Inc, Melville, NY).

Immunoelectron microscopy

Post-embedding immunoelectron microscopy using Lowicryl K11M resin and Lowicryl HM20 resin was performed as described previously (Nakane et al, 2002; Allen et al, 2001). Immunoelectron microscopy on ultrathin cryosections was carried out according to the Tokuyasu method (Tokuyasu, 1989) with slight modifications. Briefly, normal human skin biopsies fixed with 2% paraformaldehyde/PBS and treated with 100 mM glycine/PBS, as described above, were cut into small pieces and incubated in 2.3 M sucrose/0.1 M phosphate buffer, pH 7.4 (PB) for 2 days at 4° C, mounted on aluminum pins and cryofixed by immersing into liquid propane cooled to -190° C (Reichert KF80 cryo-fixation apparatus; Leica, Wien, Austria). These were sectioned at -100° C using a Reichert Ultracut-N with an FC-4D cryoattachment (Leica) to an estimated thickness of 100 nm and the sections were picked up with 2.3 M sucrose/PB and mounted on nickel grids covered with Formvar support film. After washing with PB, sections were single or double labeled with primary antibodies and secondary antibodies conjugated with colloidal gold particles, as described previously (Ishida-Yamamoto et al, 1996). Sections were post-fixed in 1% glutaraldehyde/PB for 5 min at room temperature. This was followed by staining with 2% neutral uranyl acetate for 10 min at room temperature and embedding in a solution of 1.5% polyvinyl alcohol (Sigma Chemical) and 1.5% uranyl acetate. For all immunohistochemistry, negative controls included incubation in the presence of a secondary antibody alone, and incubation with unrelated primary antibodies.

Results

The two Cdsn antibodies gave essentially the same immunostaining patterns, as did the two CatD antibodies.

Immunofluorescence microscopy

In order to characterize the timing of expression and sub-cellular localization of LG-associated molecules

in normal human skin, immunofluorescence analysis was performed (Fig 1). KLK7 and KLK8 were expressed in the most superficial granular layer (Figs 1a, b). Cdsn expression appeared slightly earlier than KLK7 and KLK8 (Figs 1a, b), while GlcCer was expressed slightly earlier than Cdsn (Figs 1c-e). There were some overlaps between different molecules in these light microscopic images, but on the ultra structural level, each component formed distinct aggregates (see below). CatD was expressed in all living cell layers, with the greatest intensity in the stratum granulosum (Fig 1f). Localization of these molecules at the apical regions of granular layer keratinocytes was appreciated in some pictures (Figs 1a, b, d). At higher magnifications, the immunoreactivities appeared as small globules in the cytoplasm. In order to study the possible relationship with TGN, we performed double staining with TGN46, a glycoprotein localized primarily to TGN (Ponnambalam et al, 1996). The whole living epidermal cell layer displayed pronounced perinuclear TGN46 signals (Fig 2a), and staining for LG-associated molecules partially overlapped with that for TGN46 (Figs 2 b - d).

Immunoelectron microscopy

In order to more precisely define the cellular compartments containing LG-associated molecules, immunoelectron microscopy was performed. We used two different methods: post-embedding and cryo-ultramicrotomy methods. In the first method, LGs appeared as isolated granules as in conventional transmission electron microscopy (Figs 3a, b). However, the cryo-ultramicrotomy method demonstrated different images of LGs, namely branched tubules with a partial internal lamellar structure (Figs 3c-e). We used this method for the rest of the present study, because the membrane structure of keratinocytes was better preserved than with the post-embedding method. The LG-molecule positive structures were continuous with the TGN that was labeled with TGN46 (Figs 4, 5e). At the cell boundary between the stratum granulosum and stratum corneum, the membranes of these structures were continuous with the plasma membrane (Figs 6a, b).

Cdsn, KLK7, KLK8, CatD and GclCer were all localized within the tubular structures in a segregated fashion (Figs 3 - 9). Among them, the structures containing GlcCer are the most abundant (Fig 5) Nevertheless, some parts of the tubular structure were immuno-negative to GlcCer, which suggests the heterogeneous nature of LGs (Figs 5a - c). In TGN, bulbous protrusions were labeled for Cdsn, KLK7,

KLK8 and GlcCer as densely as LGs at the apical part of the cells (Figs 4b, c, 5d, e, 7a). CatD labeling in TGN was associated with small vesicles (Figs 8b, c). In an attempt to characterize the exit sites for LG-associated molecules, we tried to find morphologically recognizable clathrin-coated vesicles and buds at the TGN, but we could not find them. Lysosome-like structures were not found in the cells with LGs either. At the stratum granulosum-stratum corneum junction, Cdsn was specifically localized at the desmoglea of the desmosomes, but no other labels showed any particular association with the desmosomes (Figs 6a, 6b, 8a, 9b). (Mouse monoclonal antibodies to CatD did not provided high enough labeling densities for immunoelectron microscopy. Therefore, double labeling of LG-molecules was performed with Cdsn monoclonal antibodies and one of the other rabbit polyclonal antibodies against KLK7, KLK8, GclCer and CatD.)

Discussion

In this study, we analyzed the localization of several LG-associated molecules and for the first time present evidence for distinct timing of the synthesis and separate transport of these cargoes from the TGN.

Different LG cargoes are expressed at different times during keratinocyte differentiation and are transported separately

The molecules of interest for this study were Cdsn, KLK7, KLK8, GlcCer and CatD. Cdsn is an epidermal glycoprotein and defects in its gene are manifested as the inherited human disease hypotrichosis simplex of the scalp (Levy-Nissenbaum et al, 2003). It is stored within LGs, secreted and incorporated into the extracellular core of desmosomes (Serre et al, 1991; Allen et al, 2001; Simon et al, 2001b), and this was confirmed in the present study. KLK7 and KLK8 are relatively new members of the kallikrein family (Yousef et al, 2000). KLK7 is also known as stratum corneum chymotryptic enzyme and is thought to be involved in the degradation of intercellular cohesive structures of the stratum corneum and desquamation (Egelrud, 1993). Its localization within LGs has been shown before (Sondell et al, 1995), and was confirmed in the present study. KLK8, also known as neuropsin, tumor-associated differentiation expressed gene-14 and ovasin, has been detected in differentiated epidermal keratinocytes (Inoue et al, 1998; Kuwae et al, 2002), but its ultrastructural localization has not been studied before. In

the present study, we found that KLK8 is localized within the TGN, LGs and intercellular spaces between the stratum granulosum and stratum corneum. Whether KLK8 may also play a role in desquamation remains to be tested. GlcCer are widely distributed in biological systems and also represent the major precursors of the epidermal ceramides, which are essential components of the skin permeability barrier (Holleran et al, 1993). They were detected in LGs and TGN-like compartments by immunoelectron microscopy (Vielhaber et al, 2001), and this observation was confirmed in the present study. CatD is an aspartic protease expressed in a wide range of tissues including the epidermis (Horikoshi et al, 1998). Its ultrastructural localization in lysosomes and Golgi apparatus has been shown in various cell types (van Dongen et al, 1984), but its localization in the keratinocytes was not previously known. The present study indicated for the first time its presence in epidermal LGs, and its secretion at the apical surface of keratinocytes. CatD released from LGs may catalyze extracellular substrates and be involved in the desquamation process as suggested by previous biochemical studies (Horikoshi et al, 1998; 1999). The importance in desquamation of several other cathepsins, such as cathepsin L2, was also recently evidenced (Bernard, et al. 2003).

Two steps of corneodesmosome degradation have been described as necessary for desquamation to occur (Simon et al, 2001a). In the first one, at the stratum compactum to disjunctum transition, only non-peripheral corneodesmosomes of the surface of corneocyte discs are proteolysed, resulting in the persistence of the junctional structures at the periphery of the corneocytes. These are finally degraded in the upper stratum corneum. The reasons of the protection of peripheral corneodesmosomes are unknown. Our results showing LG exocytosis at the apical pole of keratinocytes may give an explanation; indeed proteases may be delivered in this way closed to the surface of the cells, and have an easier access to non-peripheral corneodesmosomes.

We then investigated whether LGs are single species containing a mixture of various molecules. The results of the immunofluorescent study demonstrate that the timing of the expression of each LG-associated molecule is unique. In immunoelectron microscopy, each cargo occupied a distinct domain within the TGN and LGs. This strongly suggests that the various LG cargoes are transported independently through the TGN and LGs. The mechanisms for the separated transport are not clear at this moment, but selective aggregation and condensation of each cargo may prevent mixing up different

contents within the continuous structures of LGs as suggested for many other secretory proteins (Dannies 2001). As to the biological significance of the separate transport of these compounds, it is interesting to note that Cdsn is a substrate of KLK7 (Simon et al, 2001b). While the environment within the LG is acidic, the activity of KLK7 is optimal at pH 7-8, but is still significant at pH 5.5 (Egelrud and Lundstršn, 1991). Therefore separate transport of KLK7 and Cdsn might be essential in order to prevent premature proteolysis of Cdsn.

LGs are the only known secretory granules present in epidermal keratinocytes, but little is known about the molecular machinery involved in protein and lipid sorting. One of the most popular models proposed for the biogenesis of secretory granules in conventional secretory cells, such as neuroendocrine and endocrine cells, is the 'sorting for entry' model (Tooze, 1998) prior to secretory granule formation. The process is initiated by selective aggregation, which may serve both a concentration and a sorting function, of the secretory proteins in the TGN. The images obtained in the present study clearly show aggregates of KLK7, KLK8, Cdsn and GlcCer in the TGN, thus suggesting that LGs may utilize a similar initiation mechanism for molecular sorting.

LGs are found in the upper spinous and granular cells and are considered to be lysosome-related organelles (Wolff and Holubar, 1967; Chapman and Walsh, 1989). Classical lysosomes in the normal epidermal keratinocytes are small in numbers and they are usually found in the basal and lower spinous cells (Wolff and Schreiner, 1970). Both biochemical and cytochemical data have confirmed the existence of acid hydrolases in LGs (Freinkel and Traczyk, 1985; Grayson et al, 1985; Madison et al, 1998; Hayward, 1979; Wolff and Schreiner, 1970). Their acidic internal pH, a hallmark of endosomes, lysosomes, and related organelles has also been reported (Chapman and Walsh, 1989). Based upon the combined secretion of lipids and lysosomal enzymes from LGs, Madison et al. placed LGs in the category of dual-function organelles or secretory lysosomes, examples of which are melanosomes and the lytic granules of natural killer cells (Madison et al, 1998). LGs may use a combination of lysosomal- and secretory granule-sorting mechanisms, or may have their own unique sorting systems considering the unique overall structures (see below).

One of the best-characterized systems of protein sorting is that of lysosomal hydrolases, which are separated from non-lysosomal proteins after transport through the Golgi complex (Kornfeld and

Mellman, 1989). This system typically involves mannose-6-phosphate groups attached to the carbohydrate moieties of the hydrolases, a transmembrane protein mannose-6-phosphate receptor, and clathrin/AP1 (adaptor protein 1)-coated vesicles (Hille-Rehfeld, 1995; Lewin and Mellman, 1998). However, a mannose-6-phosphate receptor-independent route also exists. CatD is a classic lysosome enzyme and is transported via clathrin-coated vesicles in other types of cells (Yokota et al, 1990). In the present study, we were unable to detect clathrin-coated vesicles. This may be due to either technical reasons and/or a poorly developed lysosome system involving coated-vesicles in the differentiated keratinocytes. Further investigation using double staining with clathrin or some adaptor complexes should be performed in order to determine how lysosomal proteins are sorted in LG-bearing keratinocytes, and to see whether we should consider LGs as specialized lysosomes after all.

LGs appear differently depending on the tissue processing methods

Traditionally, LGs are regarded as isolated granules. Norlen, however, proposed the 'membrane folding' model where the TGN, LGs, and the intercellular space at the border zone between the stratum granulosum and stratum corneum are parts of the same continuous membrane structure (Norlen, 2001). Norlen et al. used a cryotransmission electron microscopy technique and provided evidence to support this hypothesis (Norlen et al, 2003). They vitrified the human skin samples by high-pressure freezing, cryo-sectioned them and observed the sections under a cryoelectron microscope. In this method, chemical fixation, dehydration, and staining used in classical electron microscopy techniques were completely avoided, and structural images of biological specimens with minimal artifacts were obtained. They observed new branched tubular structures containing non-lamellar or partly lamellar material and considered these to correspond to LGs. This view is also consistent with the electron microscopic images obtained by Elias (Elias et al, 1998). His group showed an extensive intracellular tubulo-reticular cisternal membrane system within the apical cytosol of the outermost granular keratinocytes. This system was composed of a widely distributed TGN-like network associated with arrays of contiguous LGs and deep invaginations of the stratum granulosum/stratum corneum interface. Madison et al. stated that LGs showed shapes consistent with cross-sections of tubules or buds from tubules as well (Madison et al, 1998).

The present study indicates that the structure of LGs appear differently depending on the tissue processing methods. Post-embedding immunoelectron microscopy yielded views of LGs appearing as isolated granules, but cryo-ultramicrotomy showed continuous beaded structures consistent with parts of a branched tubular structure. Because the morphology of biological membranes is better preserved in the latter method, and considering the similarities between our results and those of Norlen et al., we propose the image shown schematically in Figure 10 to be representative of the in vivo structure of LGs. We tentatively draw anastomosing tubular image of LGs here, but simple blanching structure is also compatible with our observations. Further works have to be done to verify this model. Neither the present immunofluorescent staining of various LG-molecules nor 3D reconstruction of these images (data not shown) showed tubular structures, but only granular structures representing aggregations of these molecules. Perhaps in the future, techniques such as high-voltage electron microscopy-tomography of cryo-fixed samples may provide more accurate images of LGs as applied for another cell system (Ladinsky et al, 1999). There are still many more to be studied about the mechanisms responsible for the assembly of LGs. Only recently involvement of caveolin in the LG-assembly was proposed (Sando et al, 2003). We have to test if it is compatible with our model in the future studies.

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Figure legends

Fig 1. The timing of expression of individual LG cargoes is unique. Cdsn is expressed in the granular layer, slightly earlier than KLK7 and KLK8 in the epiderms (a, b), while GlcCer is expressed in the superficial epidermis (c, d), earlier than or simultaneously with Cdsn (e). CatD is expressed in the whole epidermis, with the strongest expression being in the stratum granulosum (f). Note apical localization of LG-associated molecules (arrows). DNA staining is colored blue in a and b, and red in c, d and f. Detected antigens are colored as indicated in each figure. Differential interference microscopy image is superimposed upon immunofluorescent images in figure d.

Fig 2. LG cargo staining is overlapped with that of the TGN. (a) A perinuclear pattern of TGN46 staining is noted among living epidermal cells. (b - d) Double staining of TGN46 and LG cargo. Double positive staining appears yellow. TGN46 staining is red, other antigens are colored green, and DNA staining is blue. Confocal laser scanning microscopy.

Fig 3. LGs appear differently depending on electron microscopic techniques. Cdsn immunoelectron microscopy. Isolated granules in post-embedding immunoelectron microscopy samples embedded in Lowicryl HM20 resin (a, b). Branched tubular structures with partial internal lamellar structure are seen in the cryo-ultramicrotomy sections (c-e). The area marked with a square in figure c is shown in figure d at a higher magnification. Cdsn is seen within these structures. Bar: 100 nm.

Fig 4. LG cargo aggregates in sack-like protrusion of the TGN. TGN46 is associated with the TGN at the trans face of the Golgi apparatus (Golgi) near the nucleus (Nu) (a). Cdsn labels are aggregated in the sack-like protrusions extending from the TGN46-positive tubules (b, c). Note the laminated internal structure (white arrow) in the protrusion. Sizes of the colloidal gold particles are indicated in each figure. Bar: 100 nm. 1μm in the inset in figures a and b.

Fig 5. GlcCer in LGs. (a - c) Abundant labels are associated with vesiculo-tubular structures that appear to represent cross sections of branched tubular structures. Note that there are still immuno-negative portions in these structures (black arrows) suggesting heterogeneity in the contents of the LGs. (d, e) Sack-like protrusions (white arrows) extending from the TGN (black arrows). The area shown in figure a corresponds to the area marked with a square in the inset. 10-nm gold single labeling of GlcCer (a - d) and double labeling of TGN46 with 10-nm gold and GlcCer with 5-nm gold (e). d, desmosome. SC,

stratum corneum. Bar: 1μm in the inset in figure a, and 100 nm in the others.

Fig 6. Secretion of LGs and separate transport of LG-contents. (a, b) Apical surface of a granular cell. GlcCer labels (10-nm gold) are seen in the inter-cellular spaces (ICS) above the granular cells. Some LGs are secreted into the ICS (white arrow) and others are about to be secreted (black arrows). (c, d) Localization of Cdsn and GlcCer. Note that Cdsn (5-nm gold labels) and GlcCer (10-nm gold labels) are localized separately in the beaded structure of LGs (d). The areas marked with squares in figures a and c are shown at higher magnifications in figures b and d, respectively. Bar: 1μm in a and c, 100 nm in b and d. SC, stratum corneum.

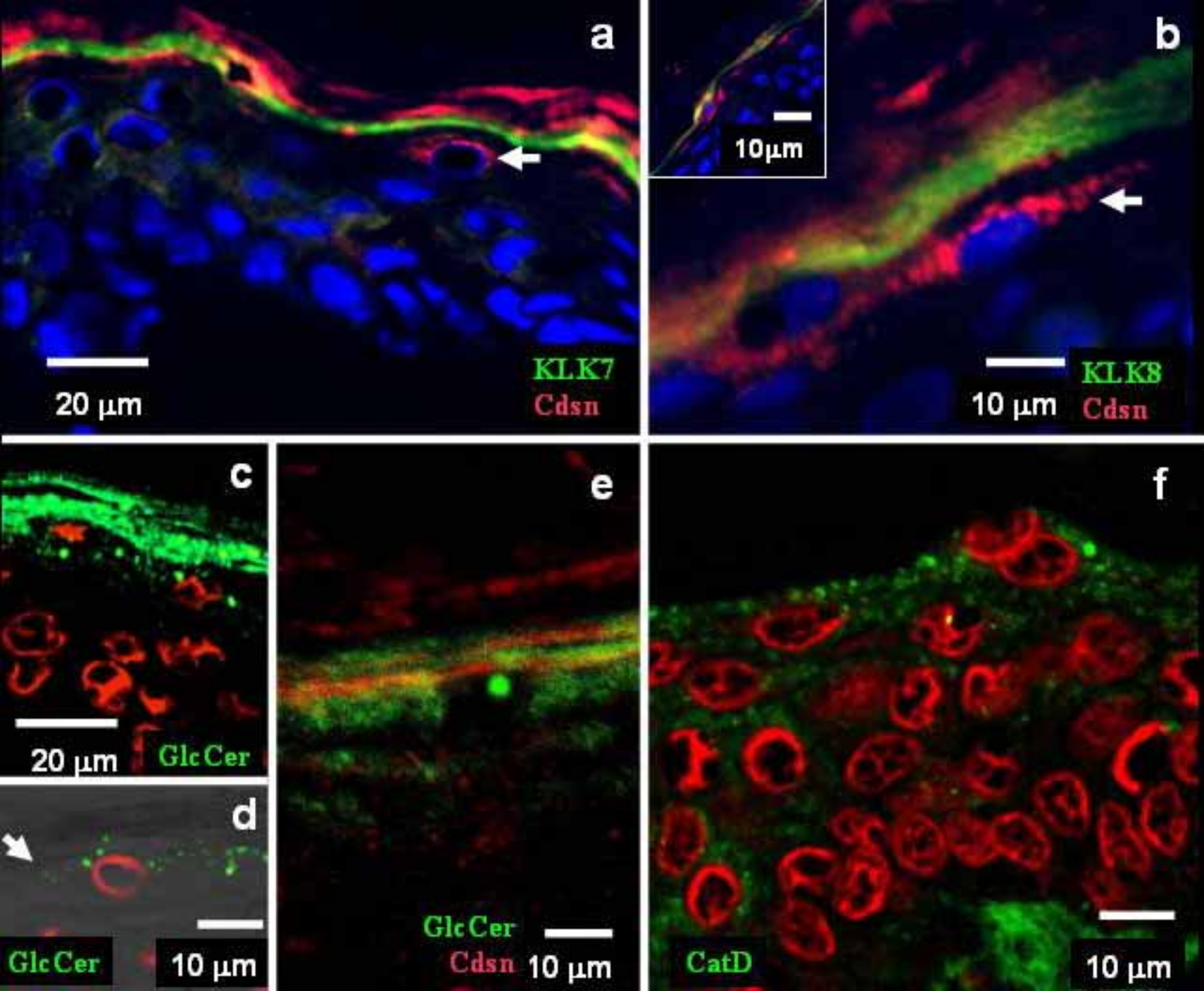
Fig 7. Aggregation of LG proteins. Cdsn and KLK8 labels are associated with separate aggregates in the sack-like protrusion of Golgi apparatus (Golgi) in a, and interconnected vesicles of different sizes in b and c. Figure c shows apical portion of the granular cell where LGs are very close to the cell surface. d, desmosome. Sizes of gold particles are indicated in each figure. Bar: 1μm in the inset in b, 100 nm in the other figures.

Fig 8. Localization of CatD and Cdsn. (a) The apical surface of a granular cell just beneath the stratum corneum (SC). LGs containing CatD (white arrow) are open to the cell surface. The area shown in figure a corresponds to the area marked with a square in the inset. (b, c)CatD labels are associated with small vesicles (white arrows) at the trans face of Golgi apparatus (Golgi) near the nucleus (Nu). (d, e) CatD and Cdsn labels are seen in aggregates in LGs. Note the thin cord-like interconnection (*) between the Cdsn-positive granule and the CatD-positive granule in e. D, desmosome. 10-nm gold labeling for CatD (a, b). 10-nm gold is used for CatD and 5-nm for Cdsn in c and d. 5-nm gold is used for CatD and 10-nm for Cdsn in e. Bar: 1μm in the inset in figure a, 100 nm in the others.

Fig 9. Localization of Cdsn and KLK7. (a) Cdsn and KLK7 labels are associated with distinct aggregates in the LGs. The area shown in figure a corresponds to the area marked with a square in the inset. (b) At the stratum granulosum (G)-stratum corneum (C) junction, Cdsn labels are rich at the desmoglea of desmosomes (d), but KLK7 labels are seen outside of desmosomes. 5-nm gold is used for Cdsn and 10-nm gold for KLK7. Bar: 1μm in the inset in figure a, 100 nm in the others.

Fig 10. Hypothetical scheme of LG-molecular trafficking in keratinocytes. LGs are branched tubular structures continuous with the TGN. KLK7, KLK8, Cdsn and GlcCer are individually transported as

aggregates directly form bulbous protrusions of the TGN. CatD are sorted into small vesicles from the TGN. Some of the vesicles may subsequently join with LGs. Alternatively; some CatD may escape small vesicle-targeting and be secreted from LGs. All the LG-molecules are released from the apical surface of the flattened most-superficial granular cells.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20 μm
KLK7
Cdsn
b
10μm
10 μm
KLK8
Cdsn
c
20 μm
Glc Cer
d
Glc Cer
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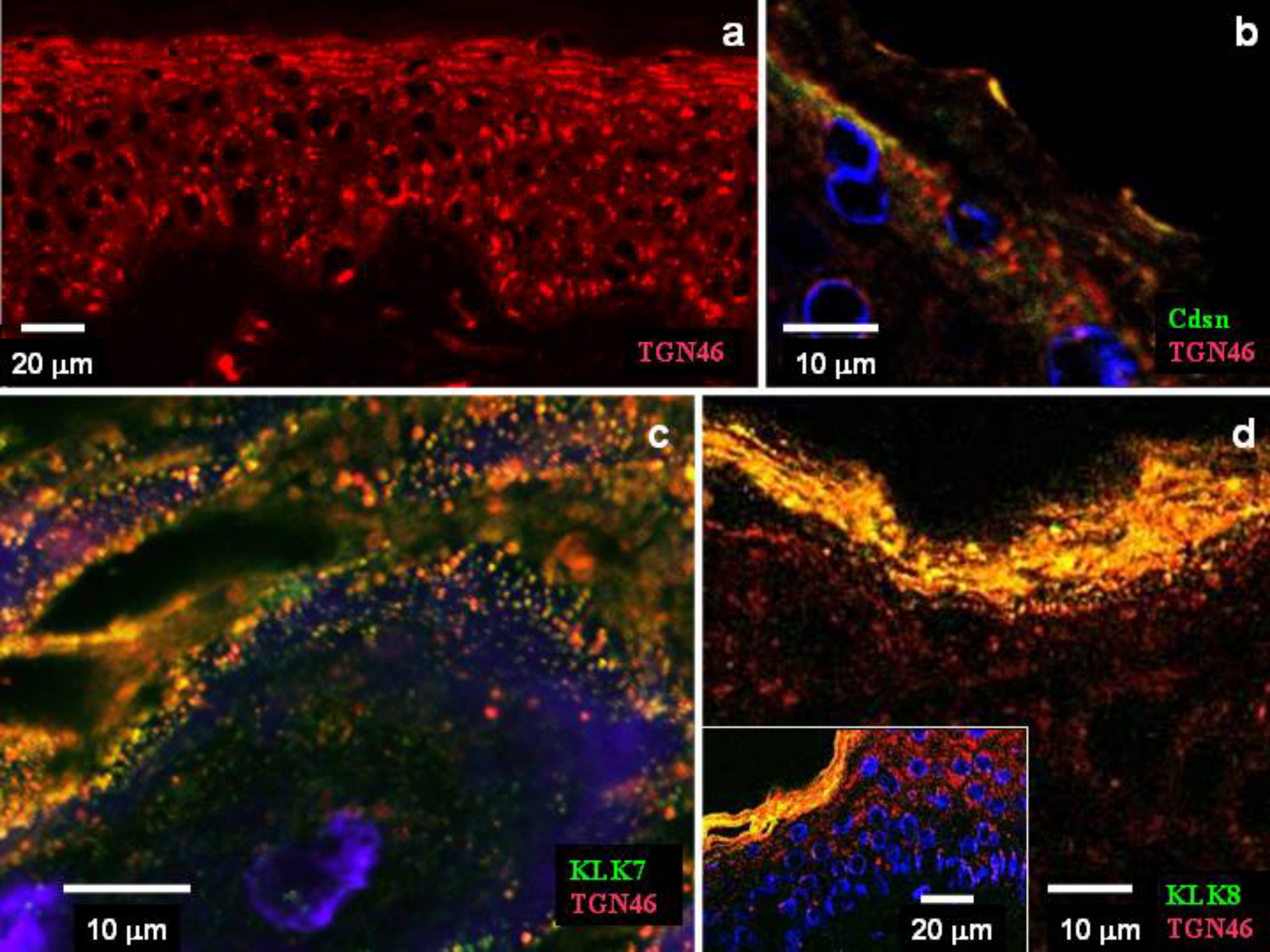
e
Glc Cer
Cdsn
10 μm
f
CatD
10 μm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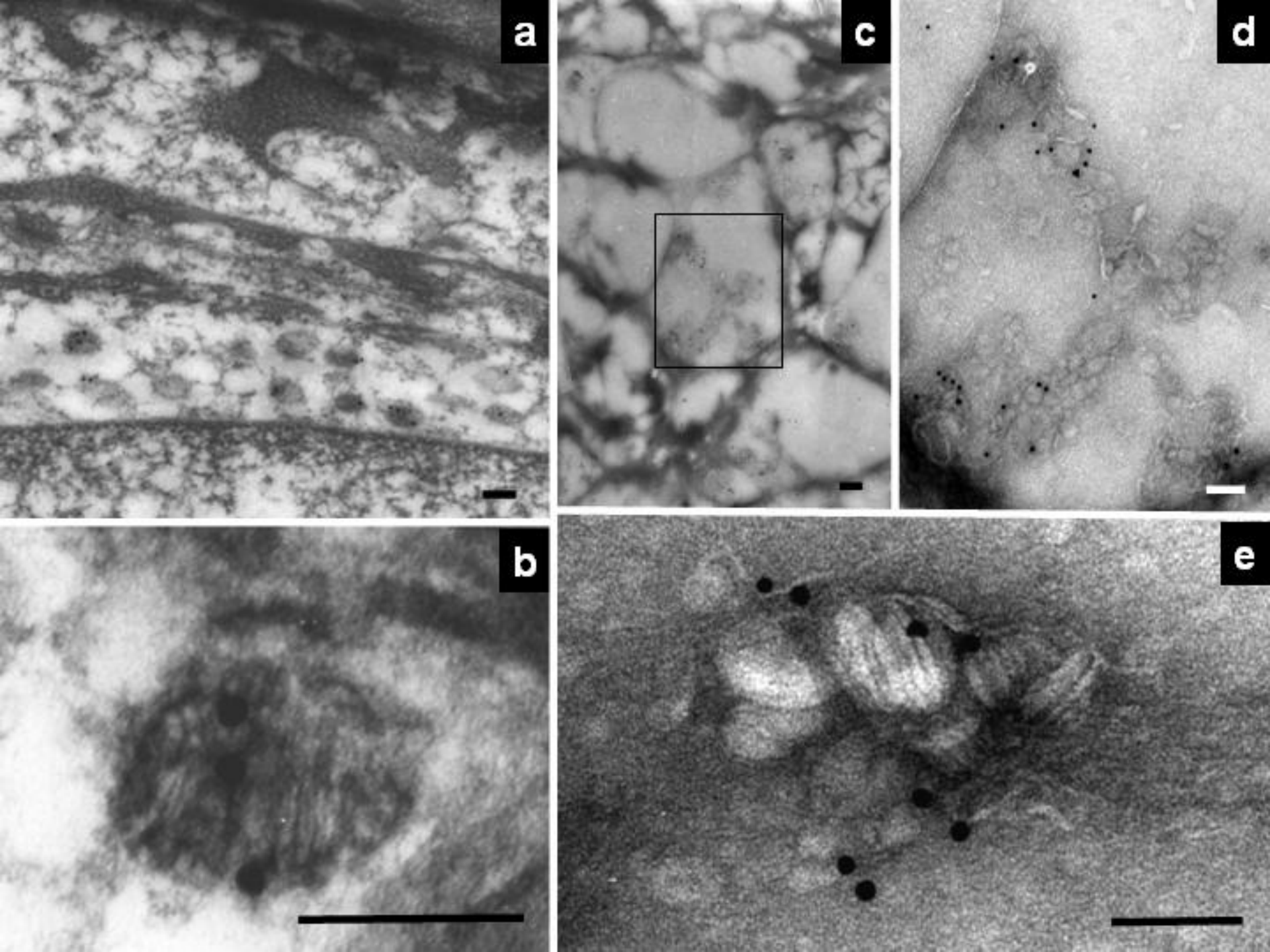
20 μm
TGN46
b
10 μm
Cdsn
TGN46
c
10 μm
KLK7
TGN46
d
20 μm
10 μm
KLK8
TGN46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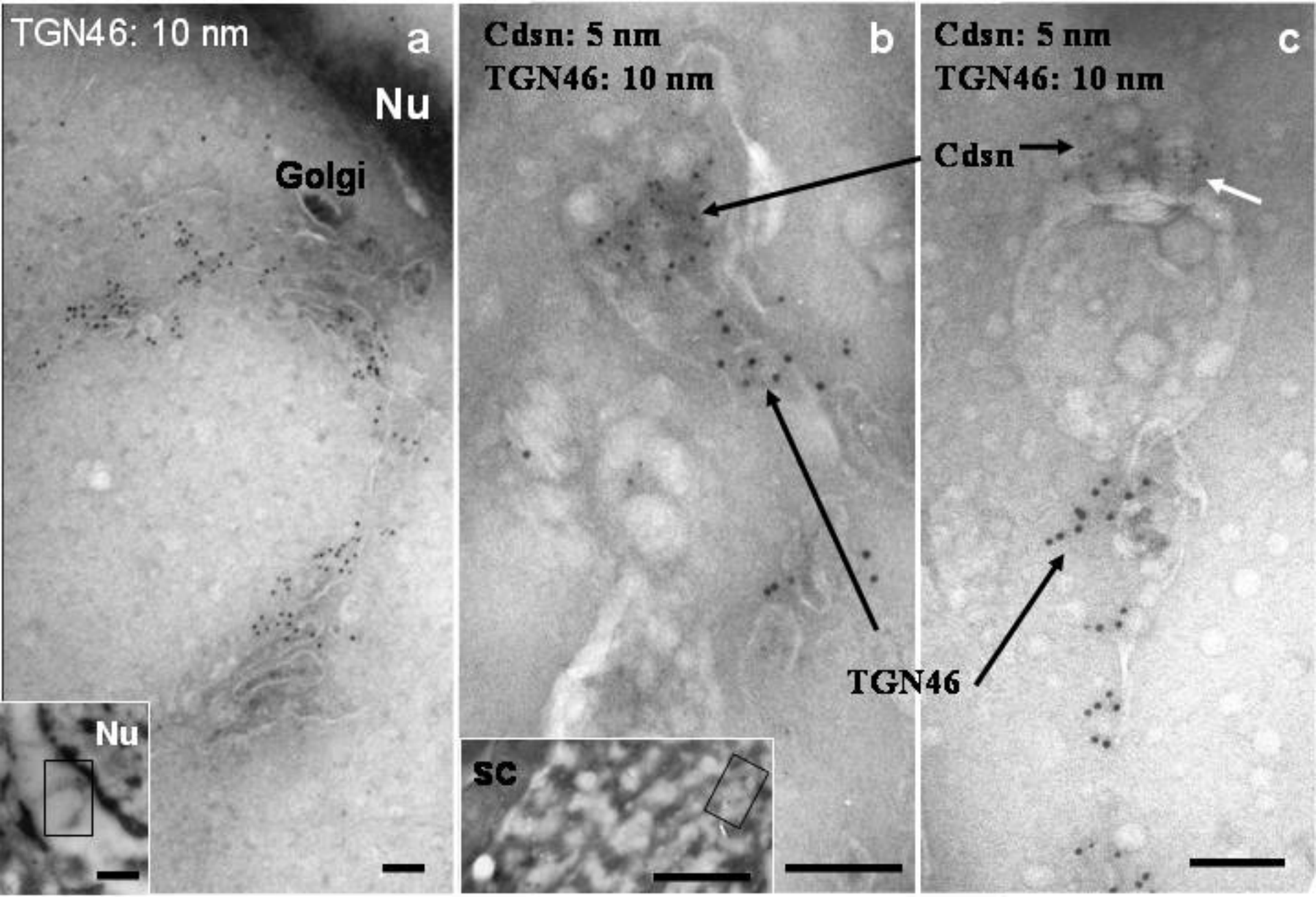
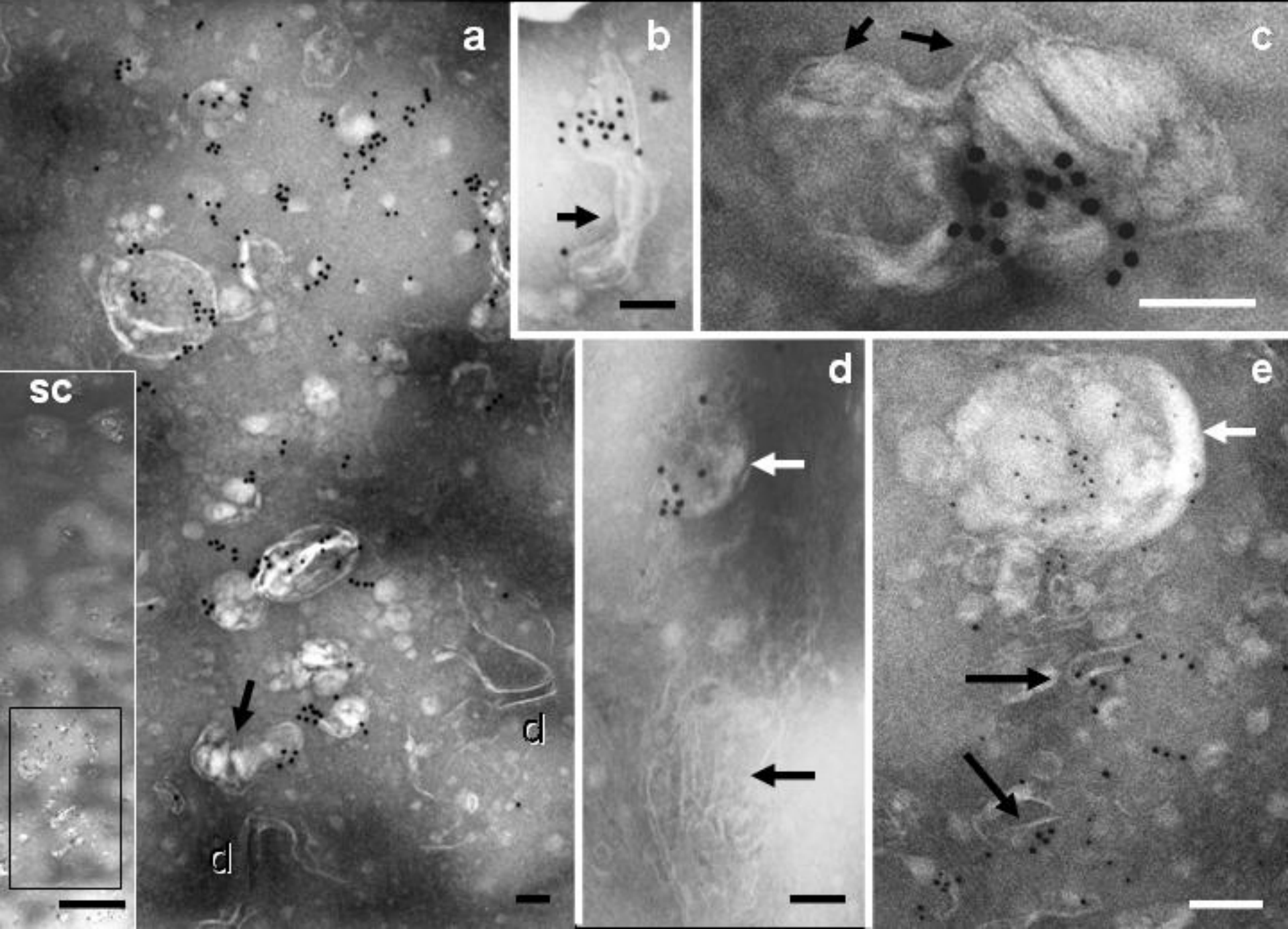
b
c
d
e


TGN46: 10 nm
a
Nu
Golgi
Nu
Cdsn: 5 nm
TGN46: 10 nm
b
Cdsn
TGN46
SC
Cdsn: 5 nm
TGN46: 10 nm
c
Cdsn
TGN46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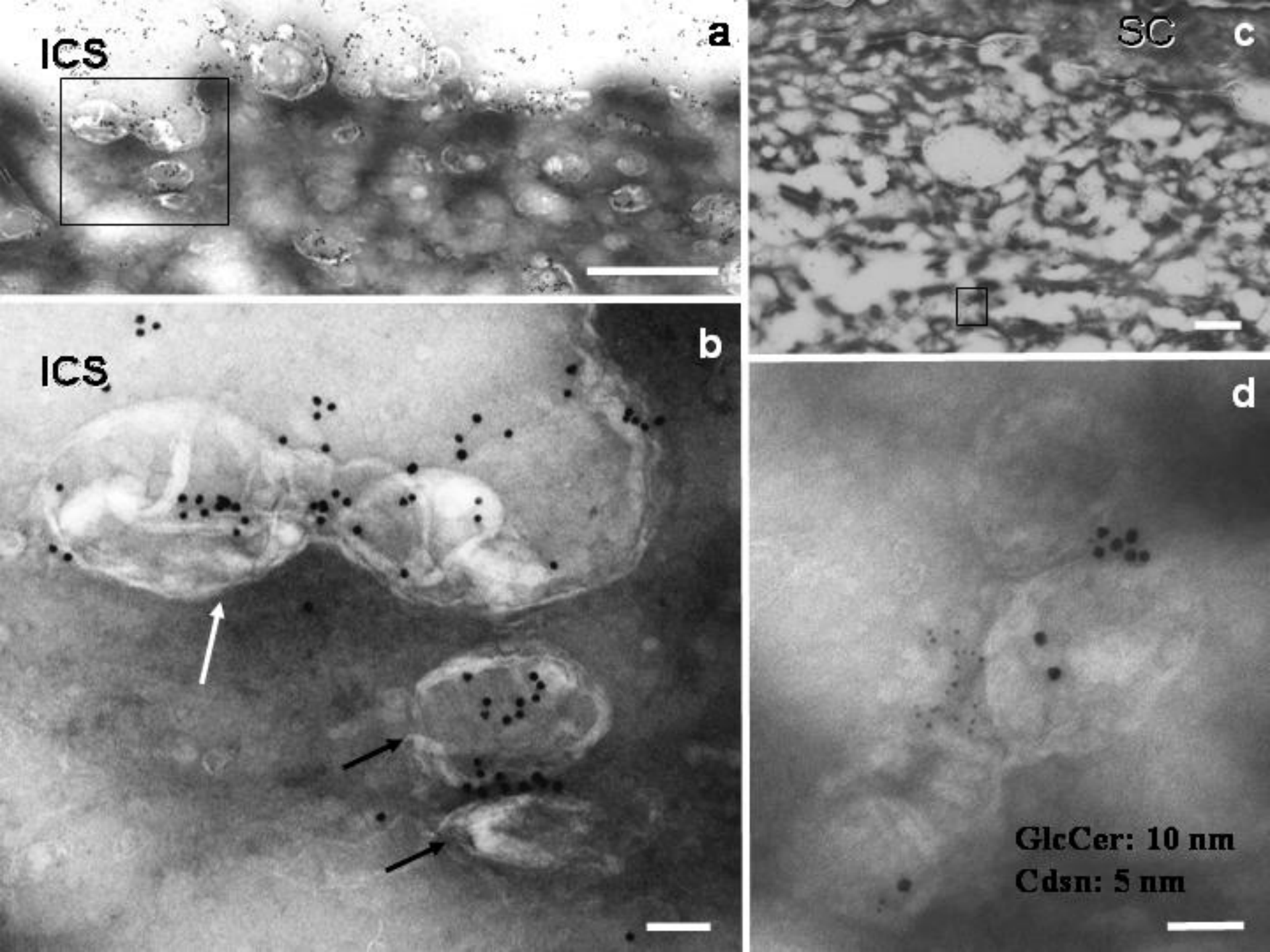
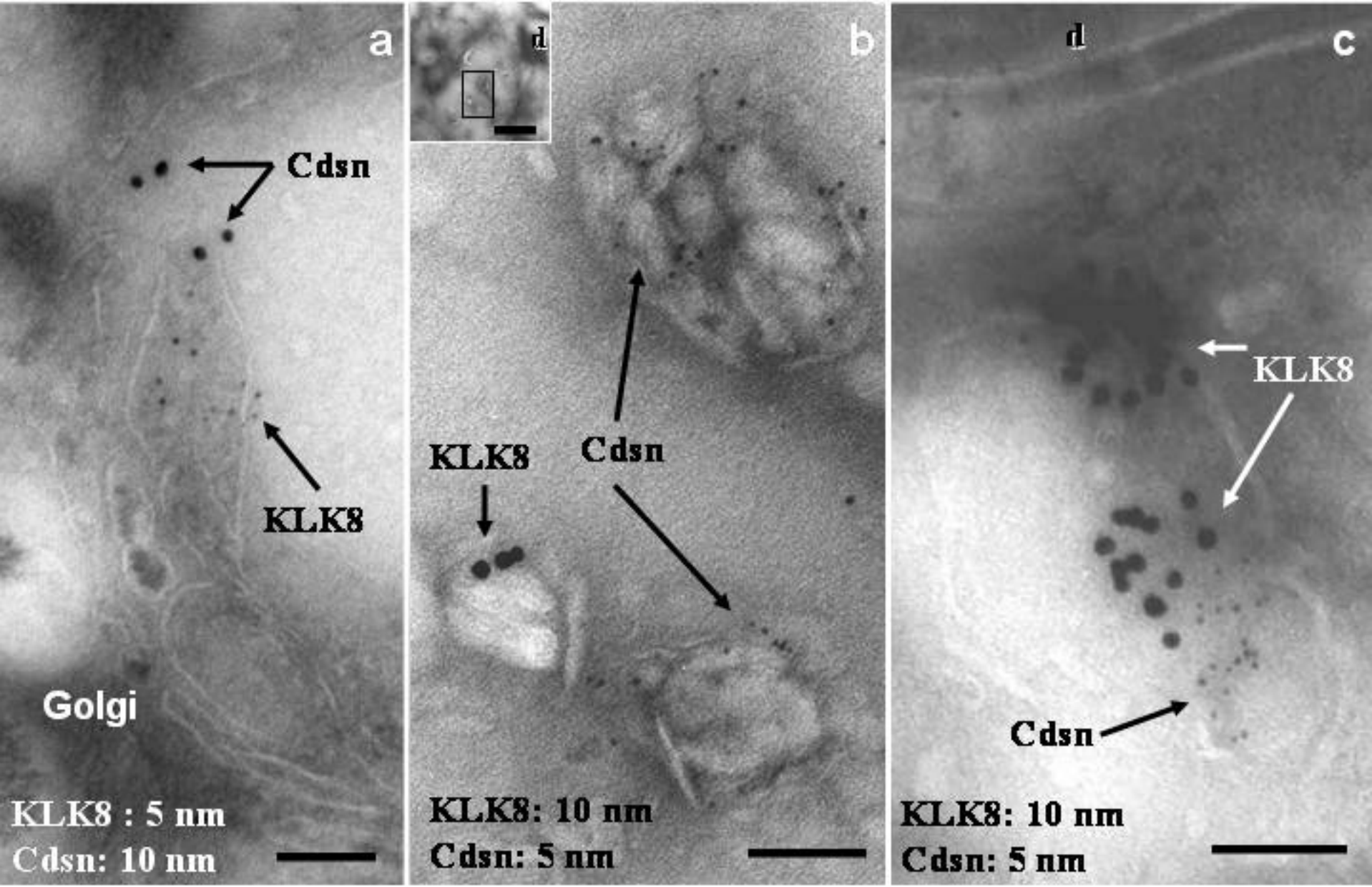
b
c
sc
cl
cl
d
e


ICS
a
ICS
b
SC
c
d
GlcCer: 10 nm
Cdsn: 5 nm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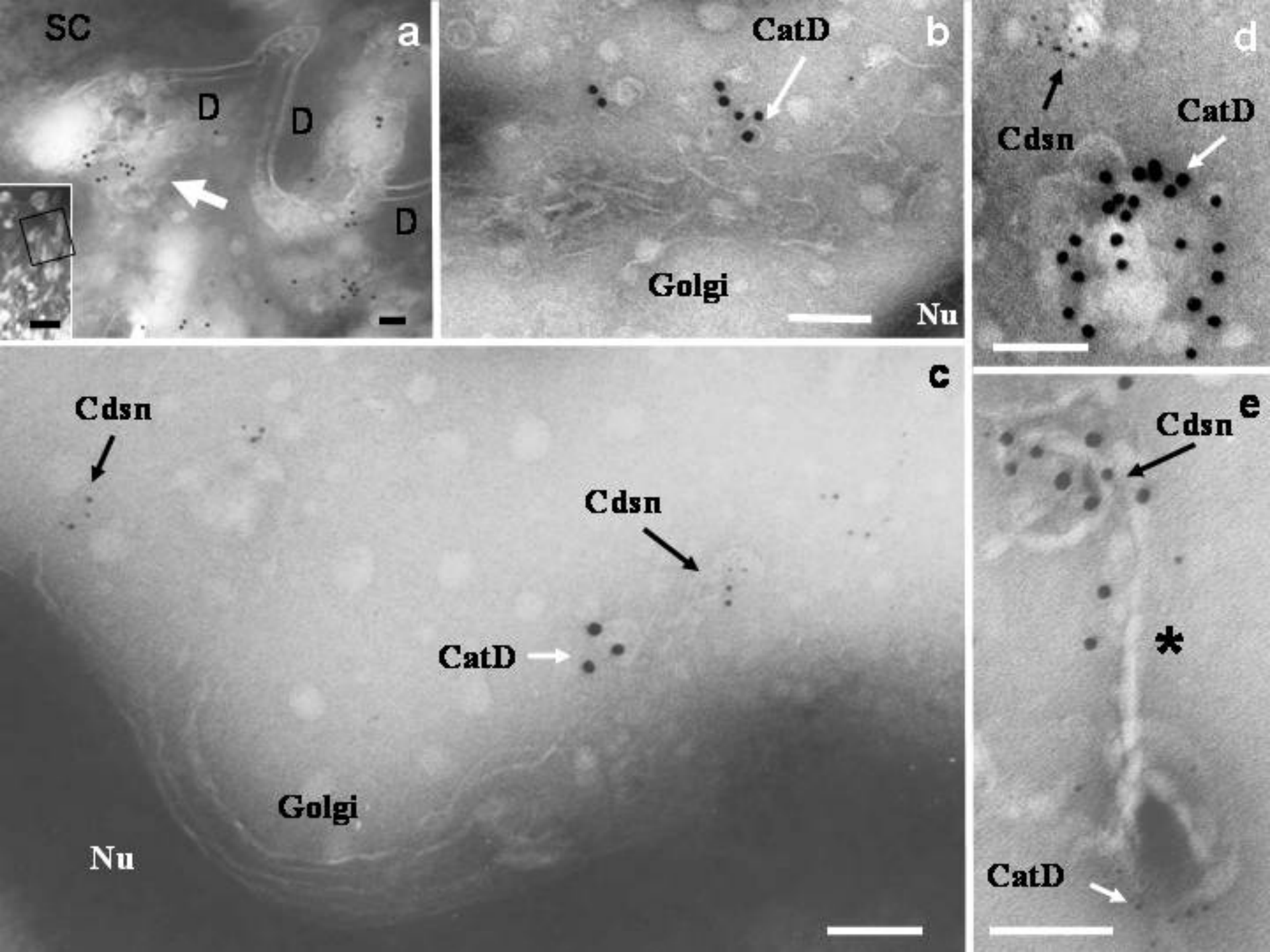
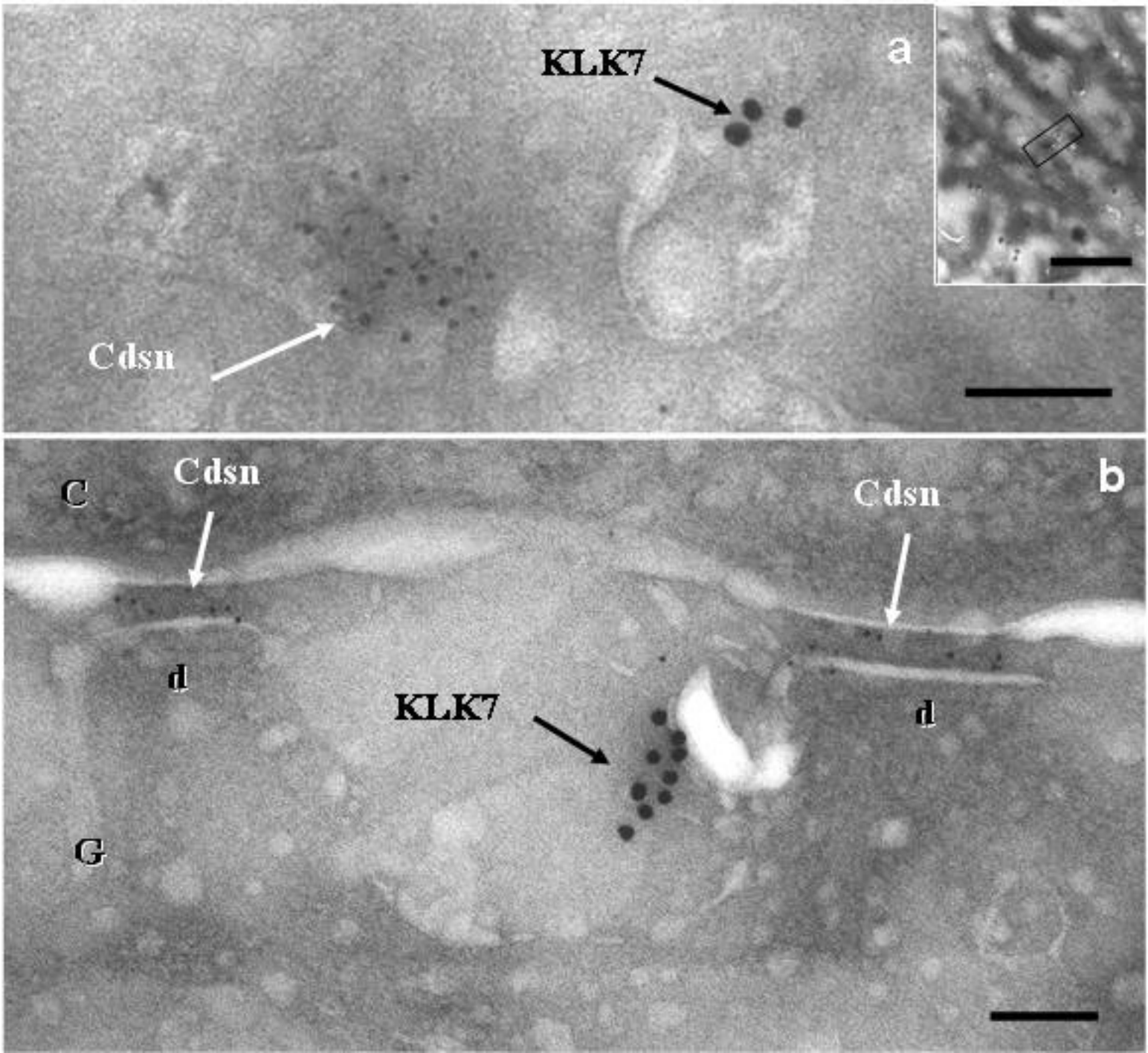
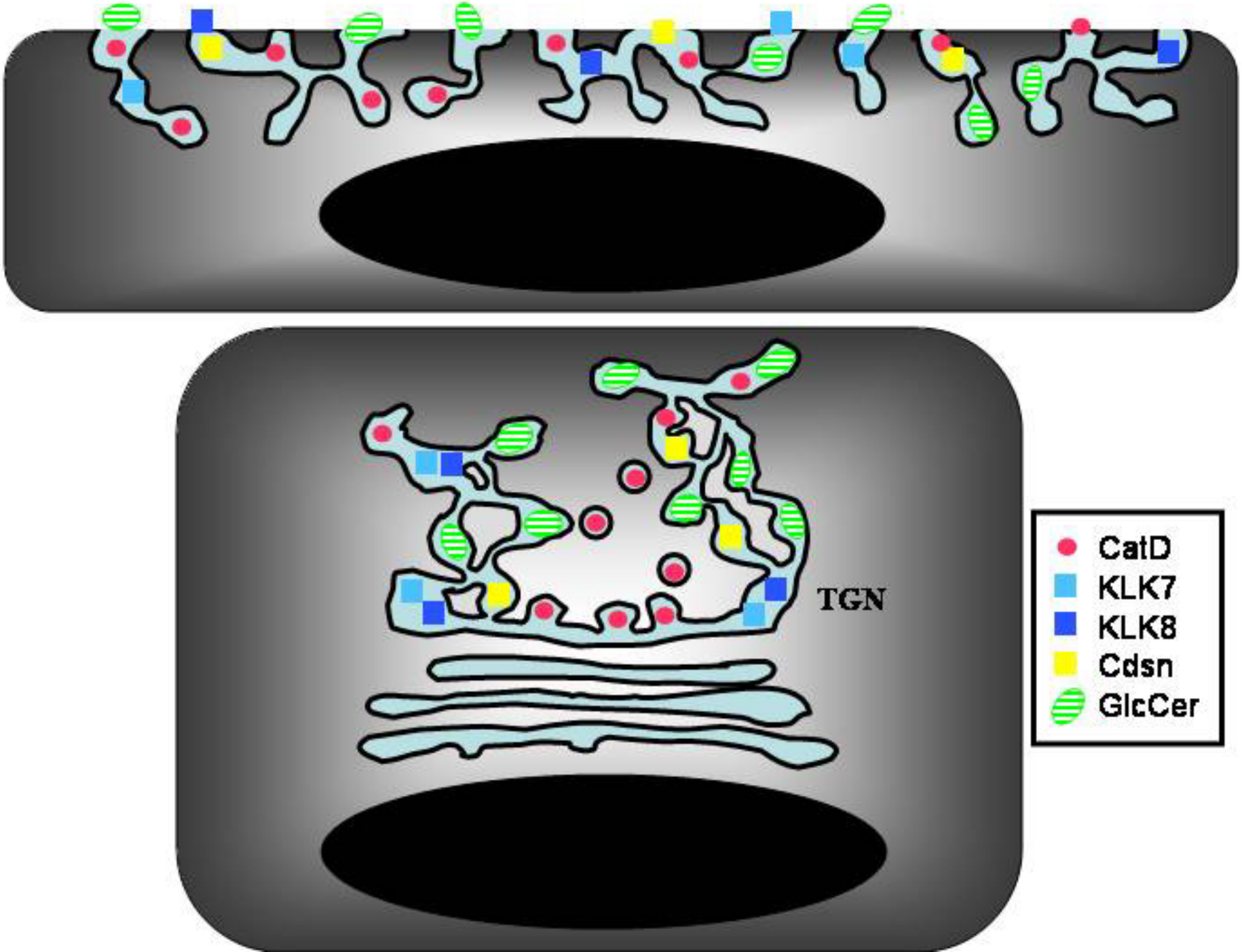
Cdsn
KLK8
Golgi
KLK8 : 5 nm
Cdsn: 10 nm
d
b
KLK8
Cdsn
KLK8: 10 nm
Cdsn: 5 nm
d
c
KLK8
Cdsn
KLK8: 10 nm
Cdsn: 5 nm


SC
a
D
D
D
CatD
b
Golgi
Nu
d
Cdsn
CatD
c
Cdsn
Cdsn
CatD
Golgi
Nu
e
Cdsn
*
CatD


KLK7
a
Cdsn
C
Cdsn
Cdsn
b
d
KLK7
d
G


TGN
CatD
KLK7
KLK8
Cdsn
GlcCer