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Rab11 Is Associated with Epidermal Lamellar Granules

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Short title: Rab11 association with Lamellar Granules

Abbreviations: CDSN, corneodesmosine; LEKTI, lymphoepithelial Kazal-type related inhibitor; LGs, lamellar granules; TGN, trans-Golgi network

Keywords: Immunoelectron microscopy; Keratinocytes; Recycling endosomes

Summary

Epidermal lamellar granules (LGs) are specialized organelles that transport and secrete various molecules, including lipids, proteases, protease inhibitors and structural proteins, thereby providing a protective barrier against the environment. Abnormalities in LG-related molecules result in severe skin diseases, but their transport mechanisms are poorly understood. We studied the distribution of Rab11, a common GTPase in recycling endosomes, in normal human epidermis. Confocal laser scanning microscopy detected Rab11 immunoreactivity in differentiated epidermal keratinocytes. Staining was strong at the apical side of each cell, a pattern commonly seen in LG-associated molecules. Around the nuclei, Rab11 was colocalized with TGN46, a trans-Golgi network marker. Rab11 was also colocalized with known LG-molecules, namely LEKTI, corneodesmosine, cathepsin D and glucosylceramides. Immunoelectron microscopy revealed that Rab11 was widely distributed along TGN and tubular-vesicular structures containing different LG molecules. The present results suggest that Rab11 plays a role in the intra-cellular trafficking of various types of LG-molecule from the TGN to the cell surface.

Epidermal lamellar granules (LGs) are the specialized organelles that transport various molecules, including lipids, proteases, protease inhibitors and structural proteins (Elias and Choi, 2005; Elias *et al*., 1998; Ishida-Yamamoto *et al*., 2004; Madison *et al*., 1998). LGs begin to appear in the upper spinous layer of the epidermis and are most prominent in the granular cell layer. LGs collect at the apical surface of the upper granular cells, fuse with the cell membrane, and extrude their contents into the extracellular space. LGs are crucial in the formation of the protective, water-impermeable stratum corneum that permits terrestrial life. Abnormalities in LG-related molecules result in severe skin diseases (Akiyama, 2006; Chavanas *et al.*, 2000; Sprecher *et al.*, 2005). A large body of evidence indicates that LGs originate from the trans-Golgi network (TGN) (Madison *et al.*, 1998; Elias *et al.*, 1998; Ishida-Yamamoto *et al.*, 2004). With regard to the post-Golgi trafficking routes of LGs, the involvement of caveolins (Sando *et al.*, 2003) and SNAP29 has been suggested (Sprecher *et al.*, 2005), but never been fully elucidated. Members of the small GTPase Rab protein family play important roles in membrane trafficking (van IJzendoorn *et al*., 2003). Among these is Rab11, which is found at high levels in recycling endosomes (Hoekstra *et al.*, 2004; van IJzendoorn, 2006) and expressed in epithelial tissues, including the epidermis (Goldenring *et al.*, 1996). In order to explore possible associations between Rab11 and epidermal LGs, we studied the distribution of Rab11 in normal human epidermal keratinocytes by confocal immunofluorescent microscopy and immunoelectron microscopy. We found that Rab11 was associated with the TGN and tubular-vesicular structures carrying a panel of LG-related molecules. Our results suggest a role of Rab11 in LG trafficking from TGN to the plasma membrane, and a close relationship between LGs and recycling endosomes.

Results and Discussion

Rab11 regulates the recycling process between recycling endosomes and the plasma membrane, and the exocytosis of secretory proteins from TGN (Chen *et al.*, 1998; van IJzendoorn *et al.*, 2003; Wilcke *et al.*, 2000). Its expression in the epidermis in a punctuate supranuclear pattern has been reported (Goldenring *et al.*, 1996); however, detailed subcellular localization has not been described. In the present study, we confirmed Rab11 expression in human epidermal keratinocytes by immunofluorescent microscopy (Fig. 1). It was detected from the middle spinous layer to the granular layer. Double staining with anti-Rab11 and anti-TGN46 (TGN marker) antibodies demonstrated partial colocalization in perinuclear areas. In order to determine its precise location in perinuclear areas, post-embedding immunoelectron microscopy was employed. The results showed that Rab11 was associated with TGN as well as other vesicular structures in keratinocyte cytoplasm

(Fig. 1g).

Because Rab11 immunostaining was strong at the apical side of the cells (Figs. 1a and 1d) (Goldenring *et al.*, 1996) and we have seen this type of expression in various LG-related molecules (Ishida-Yamamoto *et al.*, 2004; Ishida-Yamamoto *et al.*, 2005), an association between Rab11 and LGs was suggested. By double staining for Rab11 and various LG molecules, namely lymphoepithelial Kazal-type related inhibitor (LEKTI), corneodesmosine (CDSN), cathepsin D and glucosylceramides, we found substantial colocalization in the superficial epidermis (Fig. 2). To further characterize the relationship between Rab11 and LGs, we performed immunoelectron microscopy using cryo-ultra microtomy (superior method than post-embedding methods for preserving LG morphology). Rab11 was indeed associated with tubular-vesicular structures with partial lamellar internal structures, which are characteristic of LGs (Figs. 3-6). Rab11-positive LGs were carrying all the LG-molecules tested in this study, thus suggesting Rab11 involvement in the major trafficking system for LGs.

Recycling endosomes, also known as subapical compartments, govern the polarized distribution of proteins and lipids from endocytic and biosynthetic pathways (van IJzendoorn *et al.*, 2000; Hoekstra *et al.*, 2004; van IJzendoorn, 2006). In differentiated epidermal keratinocytes, various proteins and lipids are transported and secreted from the apical side by LGs, thus suggesting functional overlap with recycling endosomes. Recycling endosomes display a tubular-vesicular morphology (for a review, see van IJzendoorn, 2006). Our group and other groups have shown that LGs have a similar tubular-vesicular structure (Elias *et al.*, 1998; Ishida-Yamamoto *et al.*, 2004). Taken together with the present results, this suggests that LGs are organelles related to apical recycling endosomes.

Materials and Methods

Antibodies

As primary antibodies, monocolonal antibodies against LEKTI (Zymed Laboratories, South San Francisco, CA), cathepsin D (Oncogene, Boston, MA) and Rab11 (BD Biosciences Pharmingen, San Jose, CA), polyclonal rabbit antibodies against Rab11 (Zymed), CDSN (Descargues *et al.*, 2005) and glucosylceramides (Glycobiotech, Kukels, Germany), and sheep anti-TGN46 (Serotec, Oxford, UK) were used.

For immunofluorescence analysis, 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).

Immunostaining

Normal human skin was obtained during plastic surgery procedures with informed consent using protocols approved by the local medical ethics committee that complies with the Declaration of Helsinki Principles. Confocal microscopy was performed as described previously (Ishida-Yamamoto *et al.*, 2004). Frozen sections of human skin fixed with 2% paraformaldehyde in PBS for 1 hour at 4°C were permeabilized with the treatment with methanol for 15 min at 4°C and 0.1% Triton X-100 in PBS for 3 min at room temperature. For immunostaining of glucosylceramides, this permeabilization step was omitted. Fluorescence images were obtained using a Fluoview FV500 confocal laser scanning microscope (Olympus America Inc., Melville, NY). Immunoelectron microscopy using Lowicryl K11M resin (Chemische Werke Lowi, Waldkraiburg, Germany)-embedded samples and ultrathin cryosections was performed as described previously (Ishida-Yamamoto *et al.*, 2004). For all immunohistochemistry, negative controls included incubation in the presence of secondary antibody alone and incubation with unrelated primary antibodies. The medical ethical committee of Asahikawa Medical College approved all described studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure legends

Figure 1

Rab11 is strongly expressed in differentiated epidermal keratinocytes, and is partially colocalized with TGN46. (a – f) Confocal laser scanning microscopy. Lower (a – c) and higher (d – f) magnification views. Rab11 (rabbit polyclonal antibody, green) is expressed from the middle spinous layer to the superficial granular layer (a, d). Staining is more intense at the apical parts of each cell. TGN46 staining (sheep polyclonal antibody, seen in red) is seen around the nuclei in all cells (b, e). Merged images are shown in c and f. There is partial overlap between Rab11 and TGN46 staining. Differential interference microscopy images are superimposed upon immunofluorescent images. (g) Post-embedding immunoelectron microscopy staining of epidermal keratinocytes with Rab11 and TGN46 antibodies. TGN46 labels (10-nm gold particles) are localized on the trans-side of Golgi apparatus, while Rab11 labels (5-nm gold particles, arrows) are associated with TGN and other vesicular structures. Bar = 20 μm (a – c). Bar = 10 μm (d – f). Bar = 100 nm (g).

Figure 2

Rab11 expression is overlapped with that of various lamellar granule molecules. Confocal laser scanning microscopy. Rab11 (a, d, g, j) and LEKTI (b), corneodesmosin (CDSN) (e), cathepsin D (CathD) (h) and glucosylceramides (GlcCer) (k) are double stained. Merged images are shown in c, f, i and l. Differential interference microscopy images are superimposed on immunofluorescent images. Rab11 and lamellar granule molecules are co-localized in superficial spinous cells and granular cells. Note that Rab11 labels are less intense in figures d and j than in figures a and g, probably because a mouse monoclonal Rab11 antibody was used for the former while a rabbit polyclonal Rab11 antibody was uses for the latter. Bar = 20 μm.

Figure 3

Rab11 is associated with LEKTI-positive lamellar granules. Double staining of Rab11 (5-nm gold labels, small arrows) and LEKTI (10-nm gold labels, large arrows) using cryo-ultra microtomy. (a) shows a lower magnification view of the stratum corneum (C), the most superficial stratum granulosum (G1), the second (G2) and the third stratum granulosum (G3). The rectangular areas marked in Figures a and b are shown at higher magnification in Figures b and c, respectively. Note that Rab11 labels are widely distributed along the

tubular-vesicular structures with partial lamellar internal structures extending from the proximity of the nucleus (Nu) to the cytoplasm. Bar = 2 μm (a). Bar = 500 nm (b). Bar = 100 nm (c).

Figure 4

Rab11 is associated with corneosesmosin-positive lamellar granules. Double staining of Rab11 (5-nm gold labels, small arrows) and corneodesmosin (10-nm gold labels, large arrows) using cryo-ultra microtomy. (a) shows a lower magnification view of the stratum corneum (C), the most superficial stratum granulosum (G1), the second (G2), the third (G3) and the fourth stratum granulosum (G4). The rectangular area marked in Figure a is shown at higher magnification in Figure b. Nu, nucleus. Bar = 2 μm (a). Bar = 100 nm (b).

Figure 5

Rab11 is associated with cathepsin D-positive lamellar granules. Double staining of Rab11 (5-nm gold labels, small arrows) and cathepsin D (10-nm gold labels, large arrows) using a cryo-ultra microtomy method. (a) shows a lower magnification view of the most superficial stratum granulosum (G1), the second (G2) and the third (G3) stratum granulosum. The rectangular area marked in Figure A is shown at higher magnification in Figure b. Bar = 1 μm (a). Bar = 100 nm (b).

Figure 6

Rab11 is associated with glucosylceramide-positive lamellar granules. Double staining of Rab11 (5-nm gold labels, small arrows) and glucosylceramides (10-nm gold labels, large arrows) using cryo-ultra microtomy. The rectangular area marked in Figure a is shown at higher magnification in Figure b. d, desmosome. Bar = 500 nm (a). Bar = 100 nm (b).

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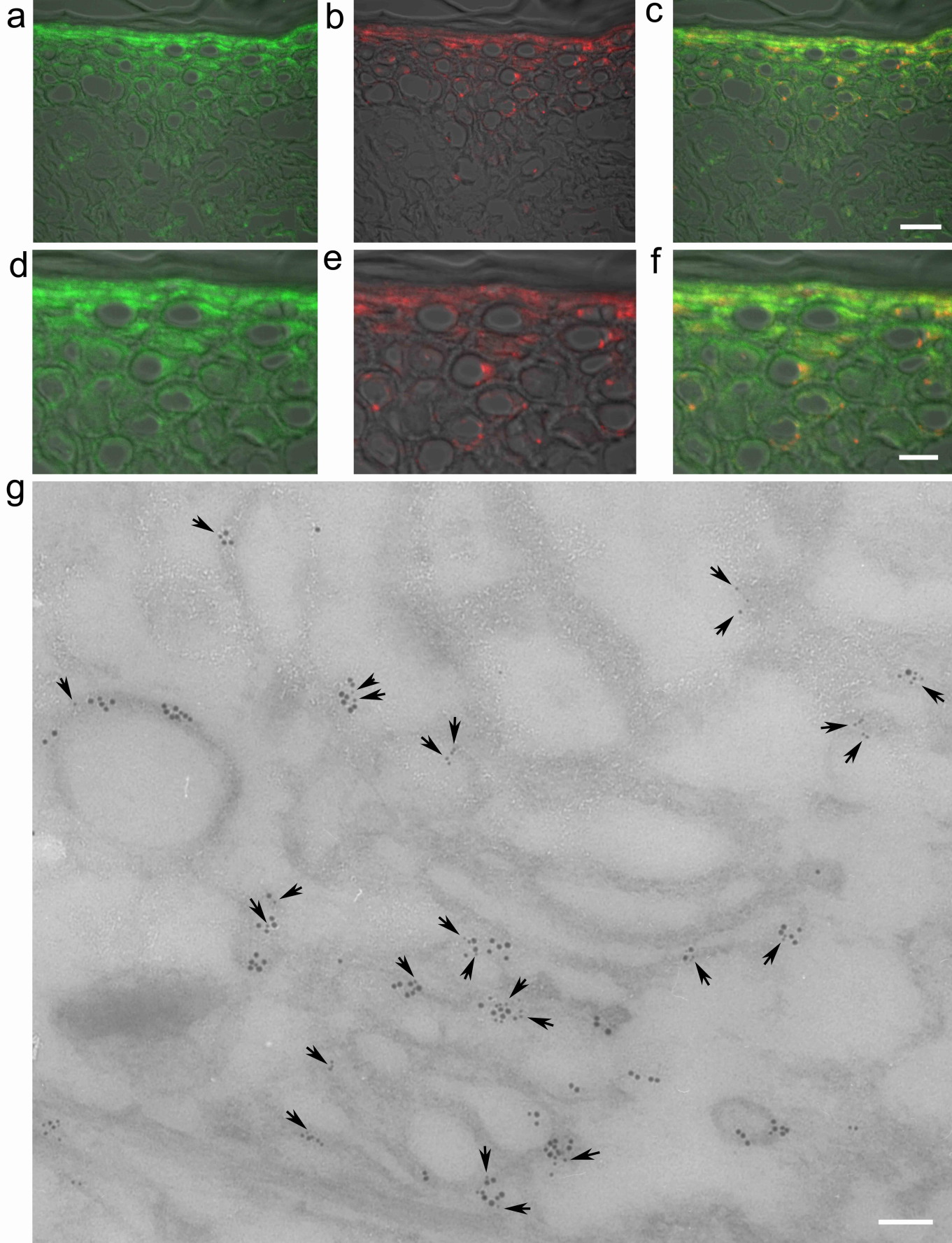
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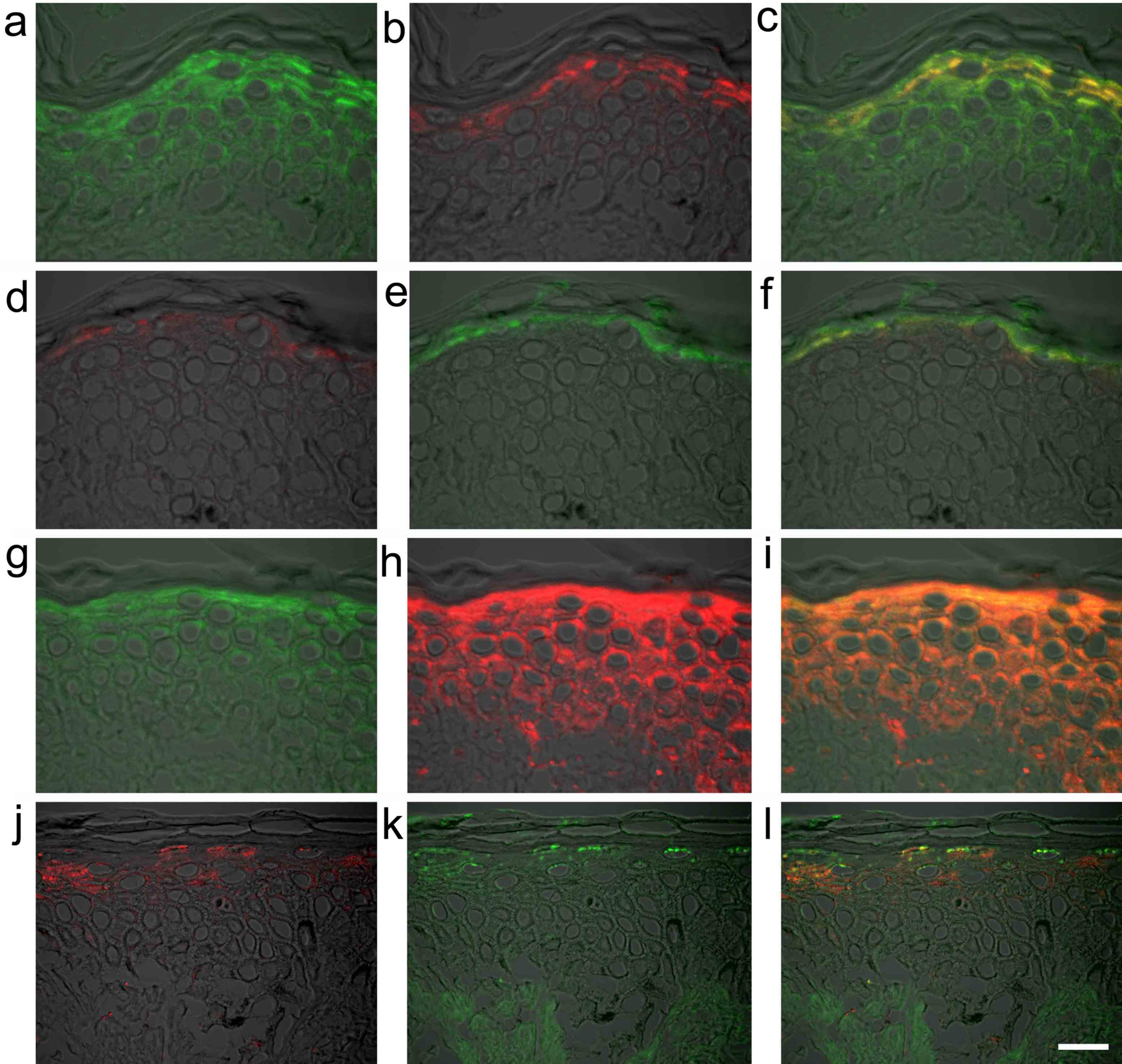
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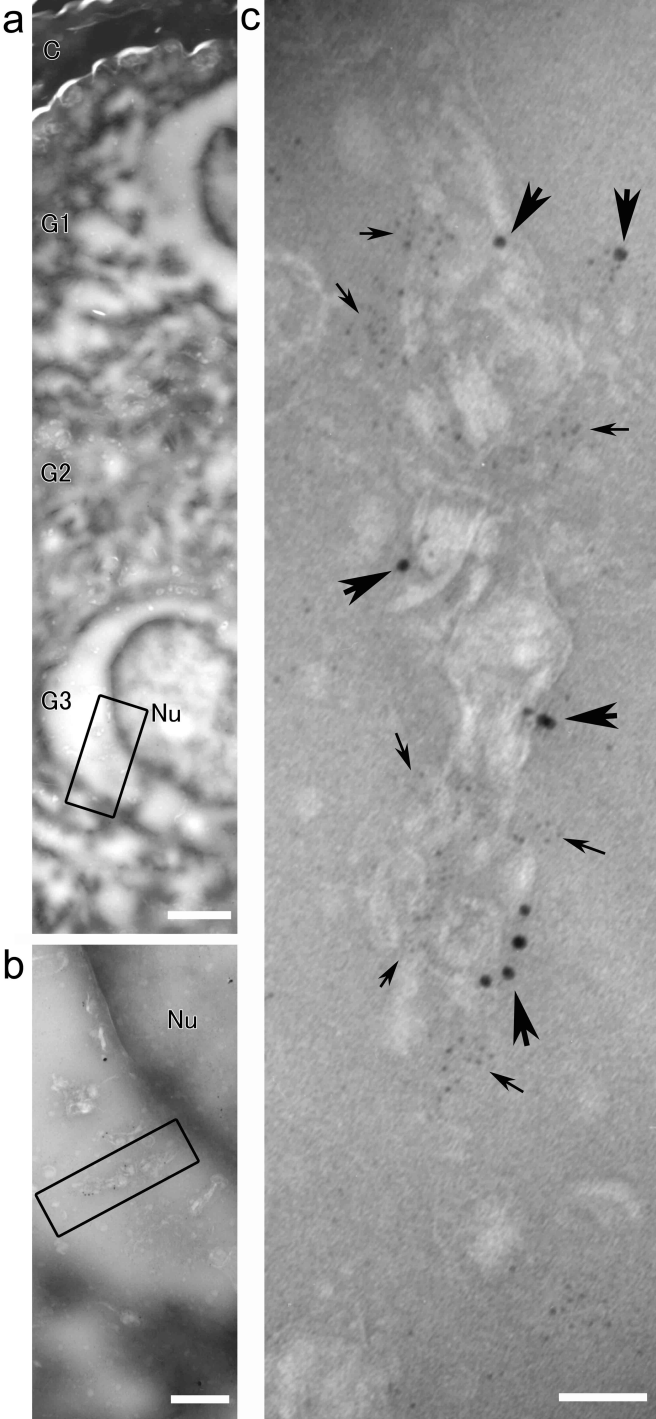
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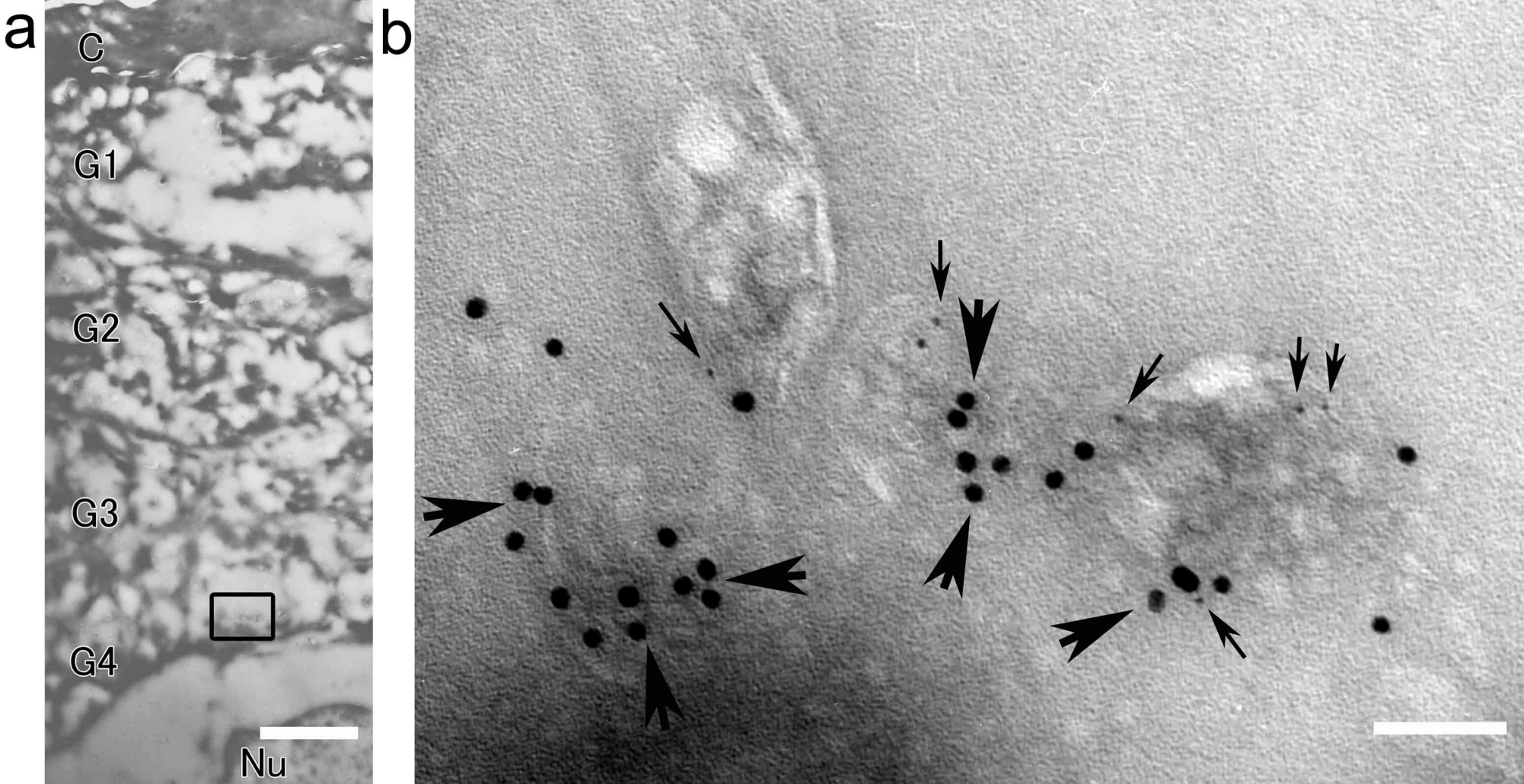
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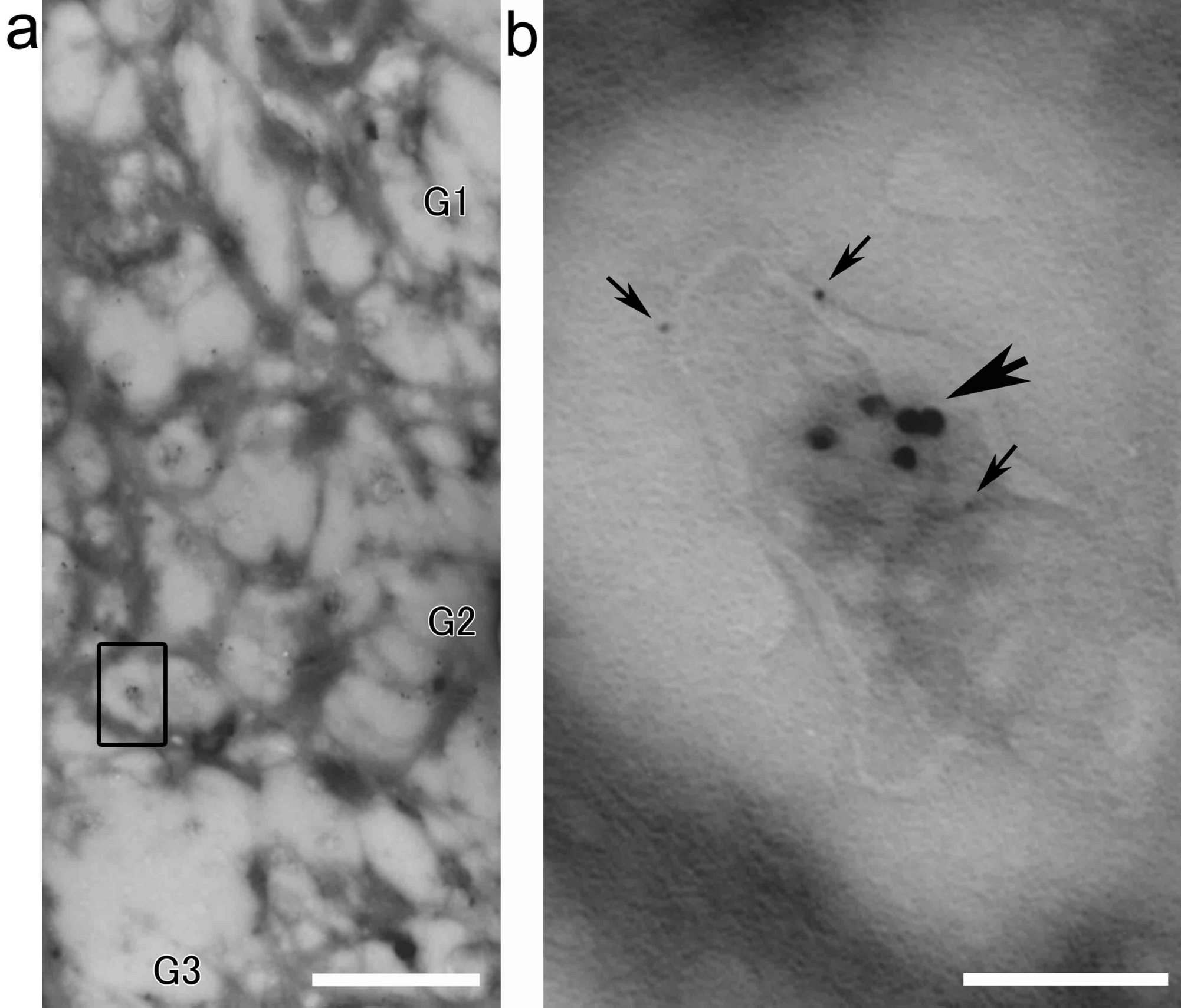
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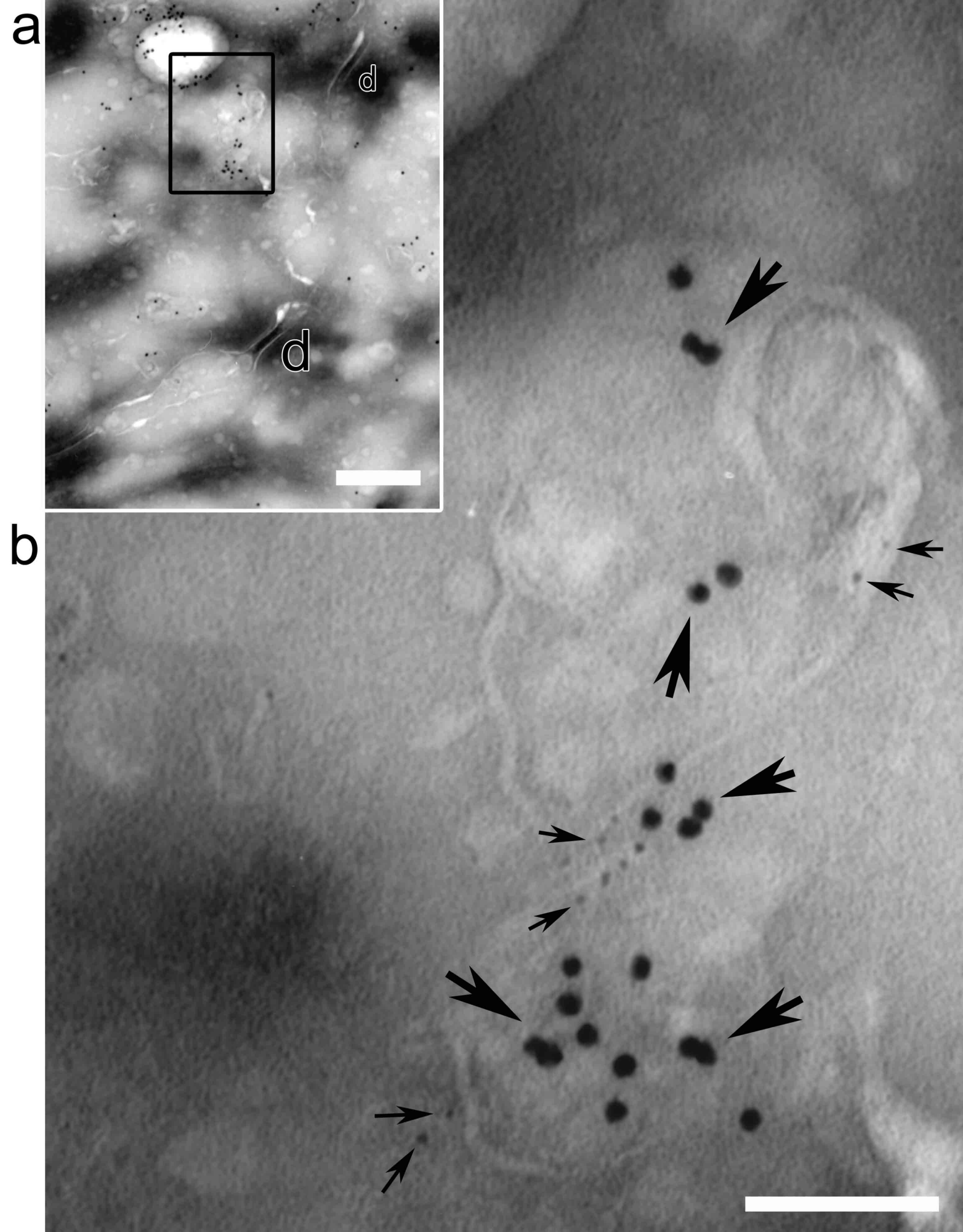
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