SHORT COMUNICATION

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Low temperature (15ºC) induces COPII dissociation from membranes and slow exit from the endoplasmic reticulum in HeLa cells

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Abstract

Low temperature induces a transport blockade at the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) in cultured cells. Our previous studies support that the primary effect of low temperature is the detachment of COPI complexes from membranes. In the present study, we have used immunofluorescence and cryoimmunoelectron microscopy to investigate the effects of low temperature on both COPII and clathrin coat complexes in HeLa cells. Strikingly, COPII proteins moved from membranes to the cytosol at 15ºC, accumulating into electron-dense areas. In agreement with this observation, we also showed that ER exit is delayed in cells cultured at this temperature. By contrast, clathrin coat is not affected. Together, our results demonstrate that low **temperature** induces COPII dissociation from membranes and slow exit from the endoplasmic reticulum.

Introduction

Membrane transport throughout the secretory pathway is a highly regulated process (Farquhar and Hauri, 1997). The selective package of cargo and the formation of donor vesicles depend on coat complexes (Bonifacino and Glick, 2004). Three coats (i.e. COPI, COPII and clathrin) have been unambiguously identified and their molecular composition characterized. Two types of coats morphologically indistinguishable, COPI and COPII, operate at the early secretory pathway. COPII is composed of the small GTPase Sar1 and the complexes Sec23-Sec24 and Sec13-Sec31 (Barlowe et al., 1994). It is accepted that this coat is the unique responsible of the anterograde transport from the endoplasmic reticulum (ER). COPII-coated vesicles quickly lost their coats and fuse to form the ERGIC (ER-Golgi intermediate compartment) from which cargo is directed to the Golgi complex (Bannykh and Balch, 1998; Klumperman, 2000). COPI coat consists of the small GTPase Arf1 and the coatomer, a complex formed for seven subunits which are pre-assembled at the cytosol (Rothman, 1994). COPI is involved in the retrograde transport from the Golgi stack and ERGIC (Letourneur et al., 1994). This coat might be also involved in the anterograde transport from ERGIC and along Golgi stack (Orci et al., 1997). Although COPI is present in late compartments (TGN and endosomes) (Martínez-Menárguez et al., 1996; Aniento et al., 1996), the main coat complex operating at the trans-Golgi area is clathrin. Three light and three heavy clathrin chains assembled into triskelions which polymerize forming a basket-like network (Schmid, 1997). The association of clathrin with membranes and the selective package of cargo are mediated by different adaptors complexes (Robinson and Bonifacino, 2001). Clathrin is involved in the transport of cargo from **both** the TGN and the plasma membrane to the endosomes.

The analysis of the secretory pathway is a difficult task because of the complex morphology of the compartments involved, the complexity of molecular machinery operating and, the fact that

all routes are bidirectional. Different strategies have been applied to approach its study. One of these approaches is the modification of the temperature conditions of cultured cells. It is possible to induce a blockade of the secretory pathway in a specific compartment by lowering the temperature. Thus, 15ºC has been widely used to study components of the ERGIC (Saraste and Kuismanen, 1984; Lotti et al., 1992; Klumperman et al., 1998). Recently, our group has used this methodology to study Golgi tubulation (Martínez-Alonso et al., 2005, 2007). However, despite of the extensive use of this methodology, the molecular basis of this transport stop is not well characterized. In our previous study, we showed that COPI dissociates from membranes at 15ºC (Martínez-Alonso et al., 2005). It may explain the selective block of at the ERGIC. Therefore, low temperature may affect the association of coats with membranes. In the present study, we **tested** this hypothesis studying COPII and clathrin distribution in HeLa cells cultured at low temperature.

Materials and Methods

Antibodies and reagents

COPII was detected using a polyclonal antibody against sec13 provided by Dr. W.J. Hong (Institute of Molecular and Cellular Biology, Singapore). Monoclonal antibody against N-acetyl-galactosaminyltransferase (GalNAcTr) T2 was obtained from Dr H. Clausen (University of Copenhagen, Copenhagen, Denmark). Monoclonal antibody against the heavy chain of clathrin was from Abcam (Cambridge, UK). FITC/TRITC-conjugated secondary antibodies and the rabbit anti-mouse antibody were from Dako A/S (Glostrup, Denmark). Protein A-gold was obtained from the Department of Cell Biology at Utrecht University (Utrecht, The Netherlands). Cell culture media were obtained from Gibco BRL Life Technologies (Paisley, Scotland, UK). Brefeldin A and unspecified chemical reagents were purchased from Sigma.

Cell culture and temperature lowering

HeLa cells were cultured under standard tissue culture conditions (37ºC, 5% CO_2) in D-MEM supplemented with 10% FBS, 100 i.u. /ml penicillin/streptomycin and 100 μg/mL glutamine. For the temperature experiments, cells were cultured in a temperature controlled incubator at 15ºC for 3 h. Where indicated, cells where treated with 2 μg/mL brefeldin A for 30 min before drug removal.

Immunofluorescence microscopy

Cells grown on coverslips were quickly fixed with 4% paraformaldehyde in PBS or cold (-20ºC) methanol, permeabilized with 0.1% saponine and processed for indirect immunofluorescence. Polyclonal and monoclonal antibodies were visualized with TRITC or FITC-conjugated anti-rabbit or anti-mouse. Samples were examined with a Zeiss Axiophot fluorescent microscope or under a Leica DMIRE2 confocal microscope. Images were assembled using Adobe Photoshop version 6.0.

Quantitative cryoimmunoelectron microscopy

This methodology has been described extensively in our previous works (see Martínez-Menárguez et al, 1999, 2001; Martínez-Alonso et al., 2005, 2007). Briefly, HeLa cells cultured at 37ºC or 15ºC were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The cells were pelleted by centrifugation, embedded in 10% gelatin and infused with 2.3 M sucrose and, finally, frozen in liquid nitrogen. Ultrathin cryosections were incubated with rabbit polyclonal antibodies followed by protein A-gold. Rabbit anti-mouse immunoglobulins antibodies were used as bridging when monoclonal antibodies were used. After

labelling, the sections were counterstained with uranyl acetate pH 7 and embedded in methyl cellulose-uranyl acetate pH 4 (9:1).

To study the relative distribution membrane/cytosol of the coat **proteins** sec13 (indicative for COPII) **and clathrin** in the Golgi area (i.e. the stack of **cisternae** and **surrounding** tubule-vesicular elements), the highest quality sections with unambiguous identification of membranes were selected. The percentage of the total gold particles allocated to membranes (gold particles touching any membrane profile at the Golgi region i.e. cisternae and tubule-vesicular elements) and cytosol (gold particles without contact with membranes) was calculated. 20 Golgi areas were counted for each antibody and temperature condition.

Results and Discussion

COPII and COPI multiprotein coat complexes are involved in anterograde and retrograde transport at the ER/Golgi interface, respectively. In our previous study we showed that COPI proteins dissociate from membranes at 15ºC (Martínez-Alonso et al., 2005). Therefore, we examined whether low temperature culture conditions perturb the COPII membrane dynamics. Sec13 was selected as COPII marker. This protein is likely a structural scaffold involved in coat formation, membrane curvature and cargo recruitment (Bonifacino and Glick, 2004). By immunofluorescence, sec 13 was located in small dots distributed throughout the cell, showing the highest density at the perinuclear Golgi-like area (Figure 1A). When HeLa cells were cultured at 15ºC, the labelling pattern changed. Temperature lowering induced the appearance of larger dots in the Golgi area as well as the peripheral cytoplasm (Figure 1B). At 15ºC the number of immunolabelled dots decreased but their size increased when compared to control cells. **Thus, the number of reactive dots per cell shifted from 160 ± 13 to 100 ± 5 (mean ± SEM, n=30) after temperature lowering.** This pattern is identical to that previously described for ERGIC

markers such us ERGIC-53 and the KDEL receptor (Schweizer et al., 1990; Klumperman et al., 1998). So far, all ERGIC markers are itinerant proteins cycling throughout ER/ERGIC/Golgi (Hauri and Schweizer, 1992; Hauri et al., 2000). Thus, a block of ERGIC exit induces the accumulation of these proteins which results on bigger spots. However, COPII vesicles are very close but they are not *bona fide* ERGIC components (Bannykh et al., 1996). Given that COPII proteins do not cycle, the enlargement of immunolabelled spots at 15ºC may be cause by other factors.

We next studied COPII distribution by cryoimmunoelectron microscopy. We observe that, in accordance to previous studies (Martínez-Menárguez et al., 1999), the COPII component sec 13 was associated with buds at the ER transitional elements as well as small vesicles in control cells (Figure 2A). No significant labelling was observed at the stack. However, one third (37.7 ± 2.7 % of the total labelling in the Golgi area, **Table I)** of sec13 was not clearly associated to membranes. **Most of this** labelling **likely** represents un-assembled cytosolic COPII proteins. **However, we can not exclude that a percentage of this labelling may be due to tangentially sectioned coated vesicles where membranes are not visualized.**

A detailed description of the Golgi complex of HeLa cells cultured at low temperature has been previously published (Martínez-Alonso et al., 2005; 2007). Briefly, this organelle appeared more complex than control cells and show abundant tubules (see also Figure 2B). At this temperature, COPII labeling was also observed in small ER buds and vesicles. Interestingly, we observed a three-fold increase of the non membrane bound labelling in cells cultured at 15ºC (82.0 ± 3.5 % of the total labelling at the Golgi area, **Table I**) (Figure 2B,C). **Most of this cytosolic labelling (96.8%)** was seen within cytosolic electron dense areas. **These areas often appeared round with a diameter of 260 ± 22 nm (mean ± SEM, n=12).** These structures have been previously described in other cells types such us exocrine pancreatic cells (Martínez-

Menárguez et al., 1999). These areas likely represent pools of COPII proteins ready to be mobilized when the traffic increases. They were observed in the Golgi area as well as distributed throughout the cell. Occasionally, they were closely opposed to the membranes of the ER (Figure 2C). The labelling density for COPII in these areas was very high and likely, they are the main component of the big spot observed by immunofluorescence. After a detailed observation we could also observe membrane-free electron dense areas in control cells cultured at 37ºC (not shown). However, these areas were scarce and small (79 ± 6 nm, mean diameter ± SEM, n= 16). The percentage of COPII labelling associated with these areas in control cells was very low (14.0 ± 4.1 % of the cytosolic labelling). Therefore, these data strongly suggest that transport blockade at 15ºC produces a significant dissociation of COPII coats from ER/Golgi interface endomembranes.

As COPII mediates ER exit (Barlowe et al., 1994), the dissociation of these complexes from membranes must affect transport from the ER to latter compartments. Low temperature does not block ER exit but it is not know whether it is somehow affected (Klumperman et al., 1998; Appenzeller-Herzog and Hauri, 2006). To test this hypothesis we synchronized cargo exit from the ER by using the drug brefeldin A (Figure 3). It is well know that after 30 min of treatment with the drug, Golgi components got into the ER (Lippincott-Schwartz et al., 1990; Klausner et al., 1996). Thus, the Golgi marker N-acetyl-galactosaminyl transferase (Rottger et al., 1998) shifted from a typical perinuclear to a diffuse pattern which is typical of the ER. After drug removal, we expect that the enzyme move first to the ERGIC in their way to the Golgi complex. Thus, when these experiments were carried out under standard condition of temperature (37ºC) we observed immunolabelled dots indicative of ERGIC at short period of time (15 min) (Figure 3B). Golgi recovery was complete after 60 min (see the typical perinuclear labelling in figure 3D-F). However, when recovery was carried out at 15ºC, the diffuse ER-like pattern remained longer

(note the typical nuclear envelope labeling characteristic of ER in figure 3G-I). Thus, typical ERGIC labeling (i.e. immunolabeled dots distributed throughout the cell) was not observed until 120 min. Under this condition of temperature, perinuclear labelling was not reached because ERGIC-to-Golgi transport is blocked. These results demonstrate that ER exit is significantly delayed, but not blocked, in cells cultured at low temperature. COPII **dissociation** from membranes could explain this delay.

To study **whether** all coat complexes are affected in cells cultured at low temperature, we next investigated the effect of temperature modifications on clathrin. It is well know that clathrin is associated to plasma membrane and TGN (Figure 4A). Low temperature did not affect the distribution of the coat. Thus, clathrin remained associated to membranes at 15ºC (Figure 4B, **Table I**). Therefore, the effect of low temperature on coat complexes seems to be **specific**.

Together, our results support that low temperature **affects** membrane traffic at the early secretory pathway by modifying the binding to membranes of both COPI (Martínez-Alonso et al., 2005) and COPII (the present study). The effect of low temperature on COPII proteins explains the delay of ER exit observed under this condition.

Acknowledgments

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Figure legends

Fig. 1. Immunofluorescence analysis of the distribution of sec 13 in cells cultured at 37ºC and 15ºC. (A) COPII gives a punctate labelling pattern in control cells with the highest density at the perinuclear region. (B) At 15ºC, COPII show less but bigger spots which concentrate at the Golgi region. *Bars*= 20 µm

Fig. 2. **Cryoimmunoelectron microscopical** analysis of the distribution of COPII in cells cultured at 37ºC and 15ºC. This coat complex was identified by using polyclonal antibody against Sec 13 and followed by 10 nm protein A-gold complex. (A) COPII labelled membranes adjacent to the stacks (G) and transitional endoplasmic reticulum (ER). (B) At low temperature, COPII proteins are very abundant in electron-dense membrane-free areas (asterisk) within the Golgi region. (C) The figure shows a membrane-free area located at the cell periphery which seems connected to the endoplasmic reticulum (arrow) G= Golgi stack, ER= endoplasmic reticulum. *Bars*=250 nm

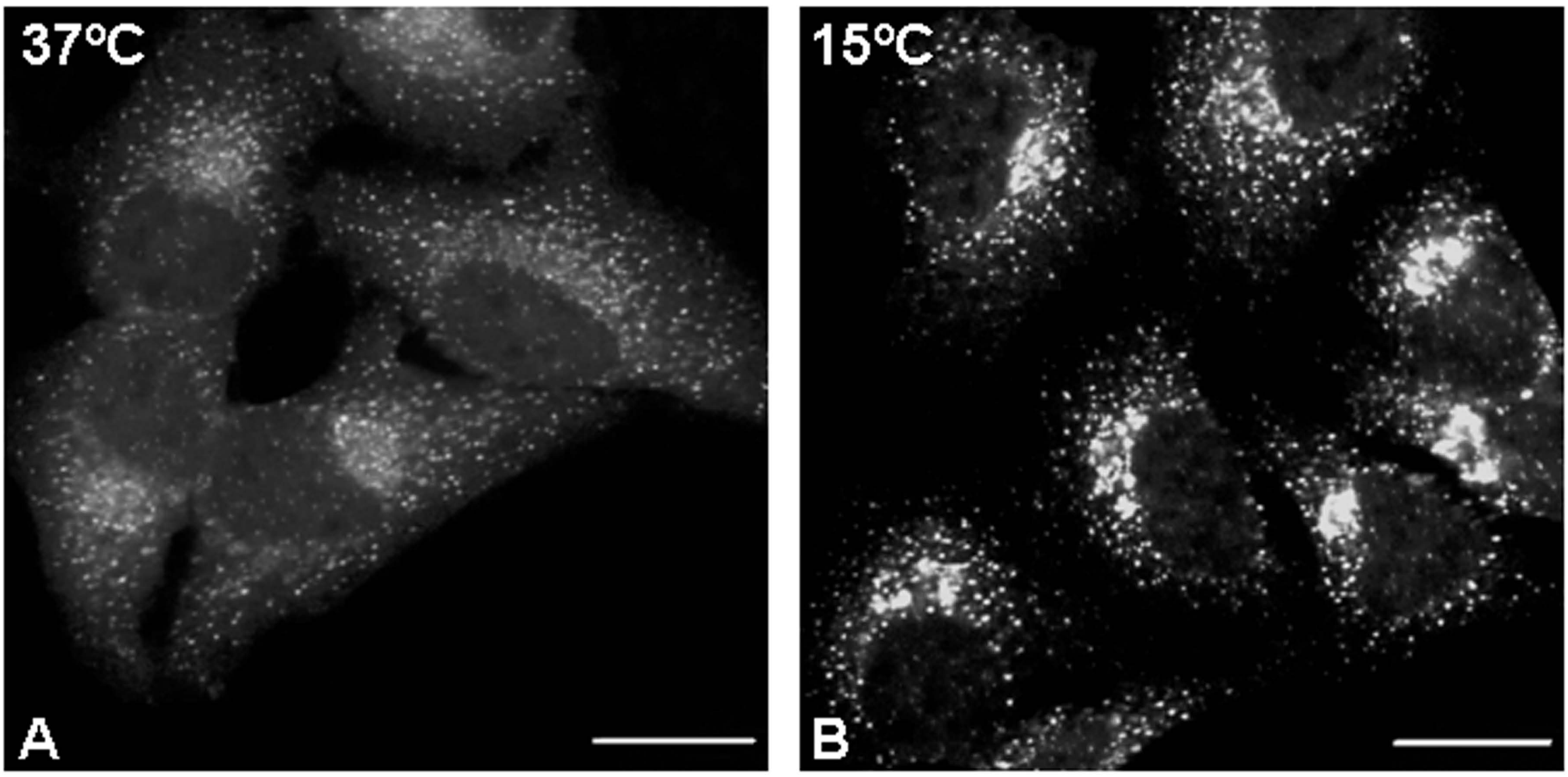
Fig. 3. Analysis of ER exit after brefeldin A treatment at 37ºC and 15ºC. Cells were treated with brefeldin A for 30 minutes. After drug removal, cells were immunolabelled for N-acetylgalactosaminyl transferase during different time points (0-120 minutes). Parallel experiments were done at 37ºC (B-F) and 15ºC (G-K). (A) Control. The Golgi enzyme showed typical ER labeling pattern after 30 min of treatment with brefeldin A. (B-F). After drug removal at 37ºC, the diffuse labeling pattern changes to reactive dots and latter concentrate at the perinuclear area. (G-K) At low temperature, the ER labeling pattern remains until 60 minutes. At 120 minutes (K), immunolabelled dots (i.e. ERGIC) are observed. *Bar*=20 µm

Fig. 4. Distribution of clathrin in cells cultured at 37ºC (A) and 15ºC (B). This coat complex was identified by using a monoclonal antibody against the heavy chain of clathrin followed by a rabbit anti-mouse antibody and 10 nm protein A-gold complex. (A) In control cells, clathrin is observed in both the trans-Golgi and coated pit at the plasma membrane (inset). (B) In cells cultured at low temperature, clathrin remained associated to the membrane of coated vesicles. Note that all reactive vesicles wear a thick coat characteristic of clathrin. G= Golgi stack, N= nucleus, PM= plasma membrane. *Bar*=250 nm

Table I. Percentage of labelling for coat proteins which is non-associated to membranes

	37ºC	15ºC
COPII	37.7 ± 2.7	82.0 ± 3.5
Clathrin	6.6 ± 3.3	6.7 ± 1.5

Numbers represent the percentages (mean ± SEM) of the total labelling in the Golgi area which is not associated to membranes (see Material and Methods). 20 Golgi areas were counted for each antibody and temperature condition.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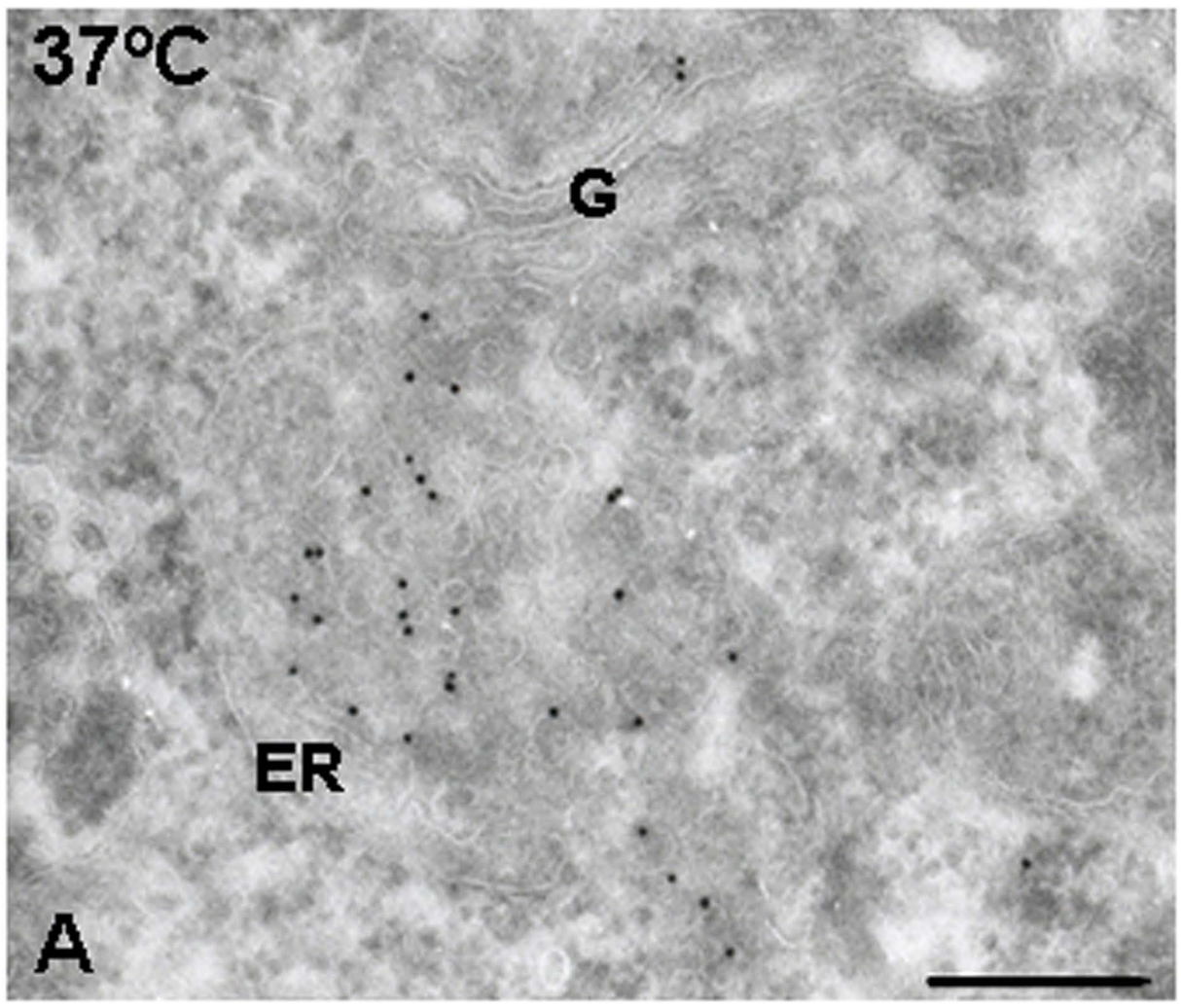
15°C
A
B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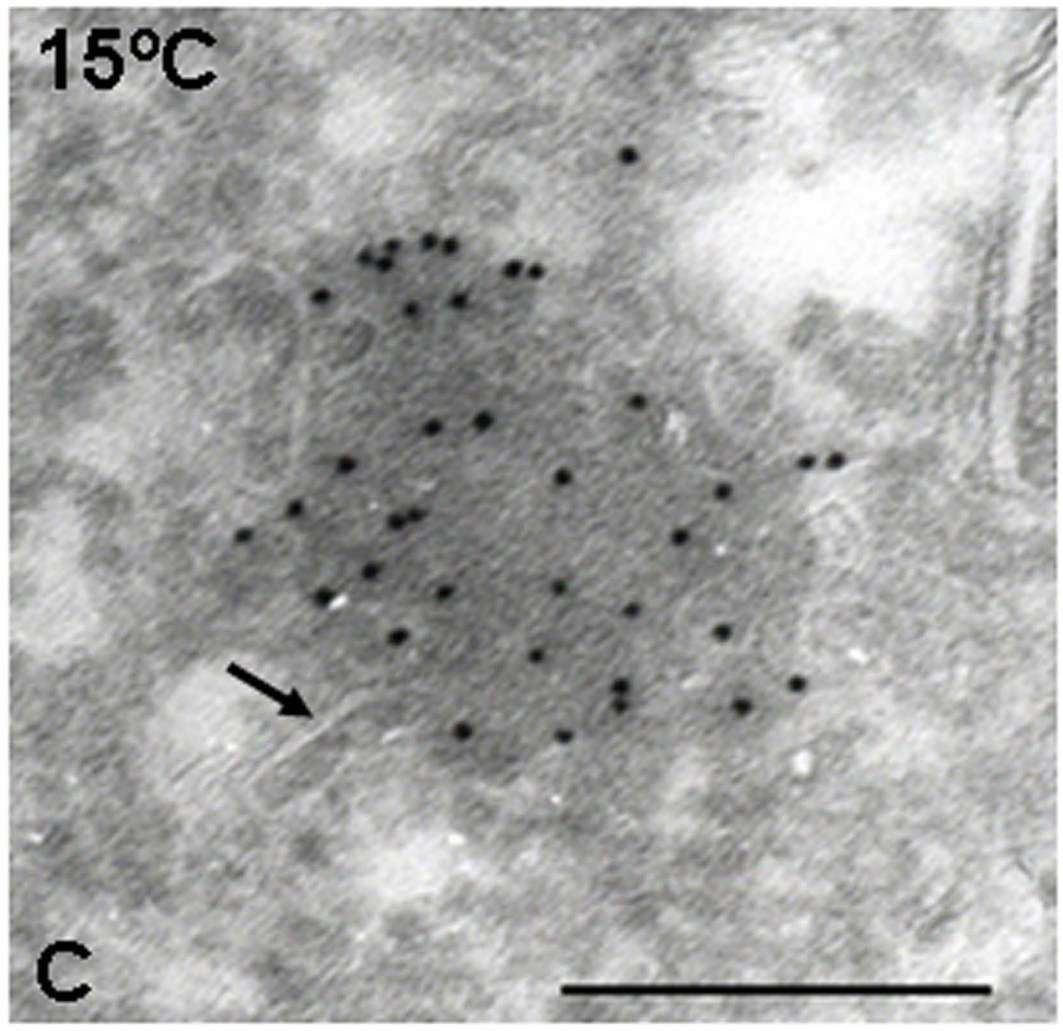
15°C
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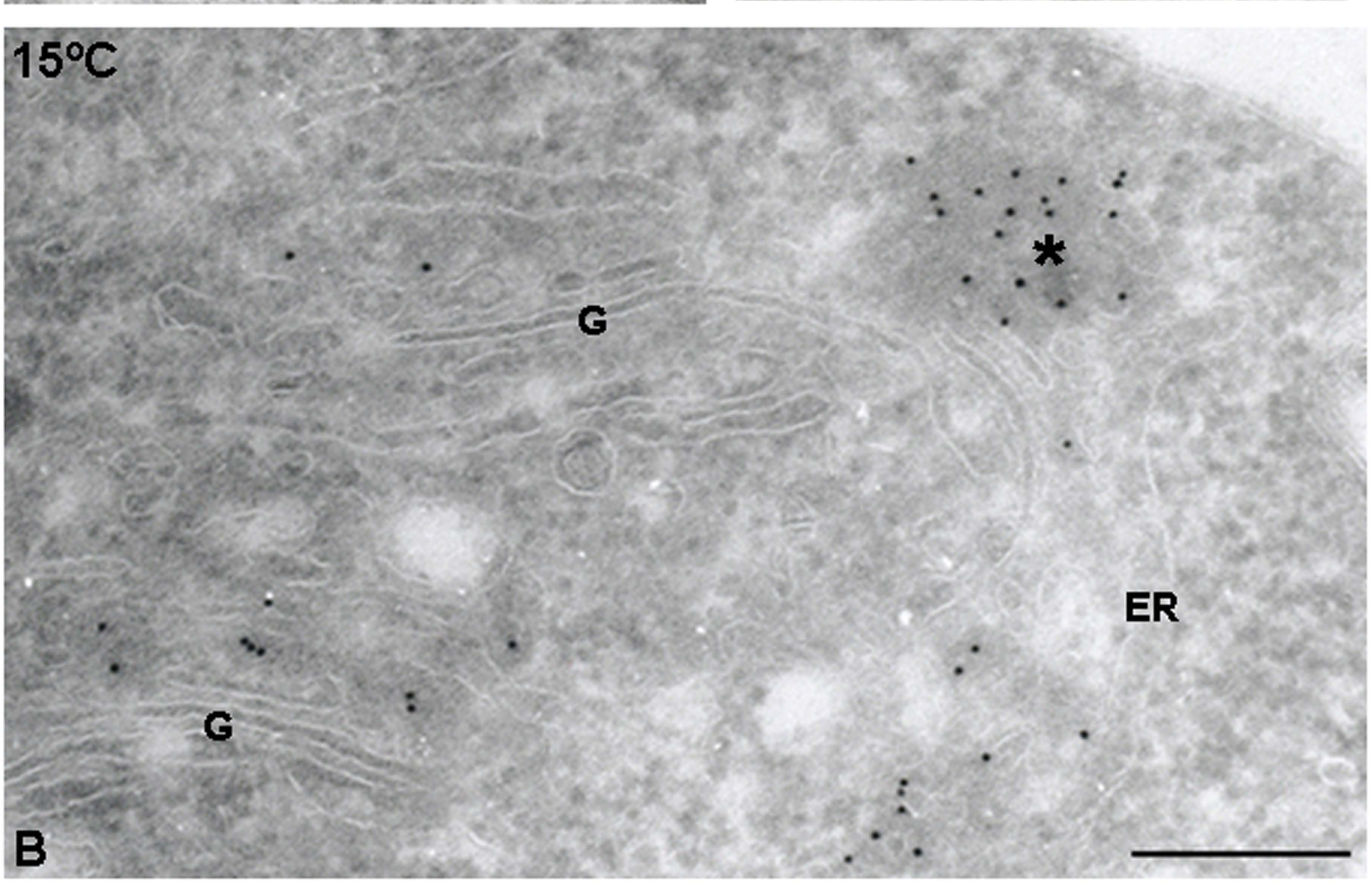
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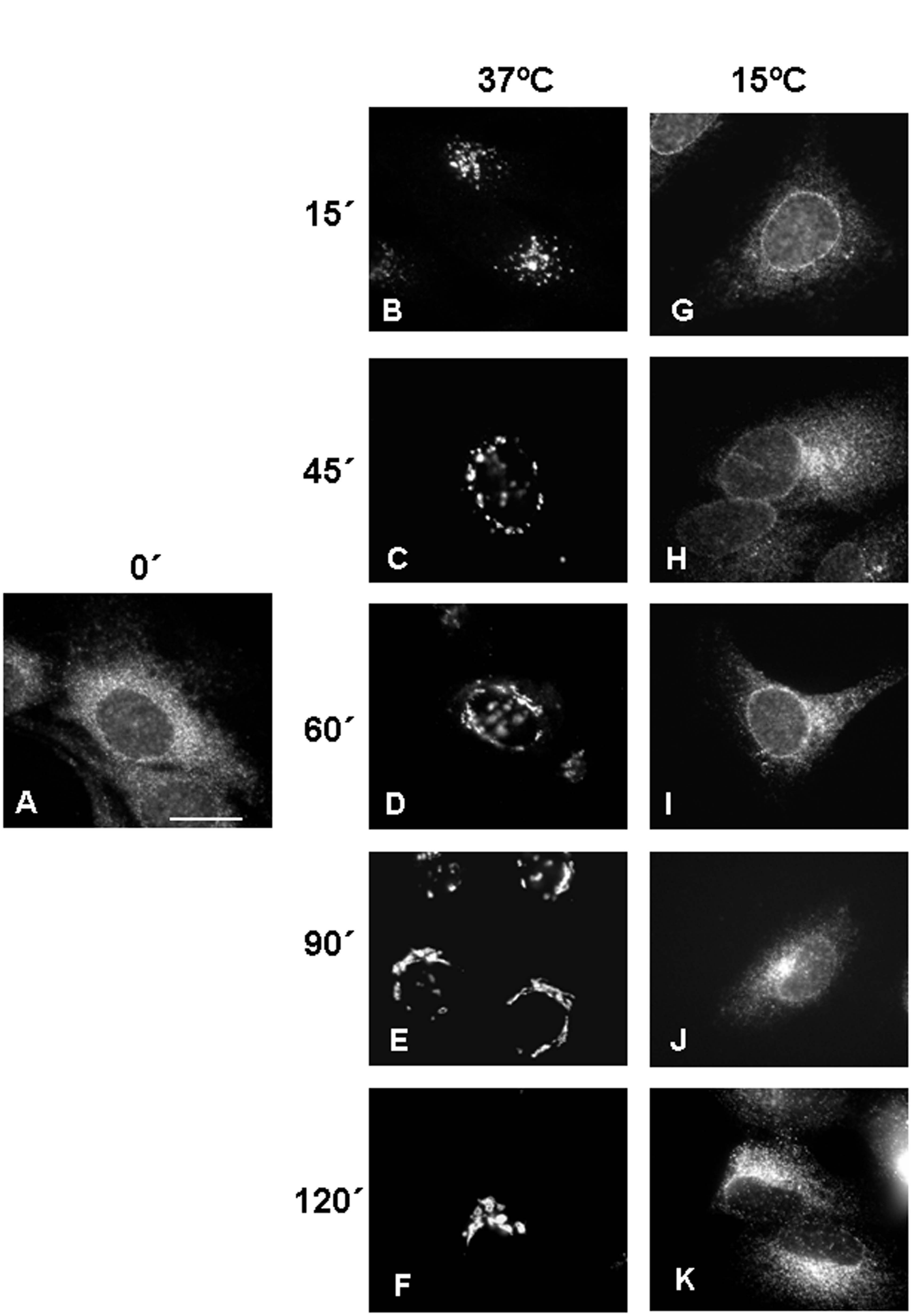
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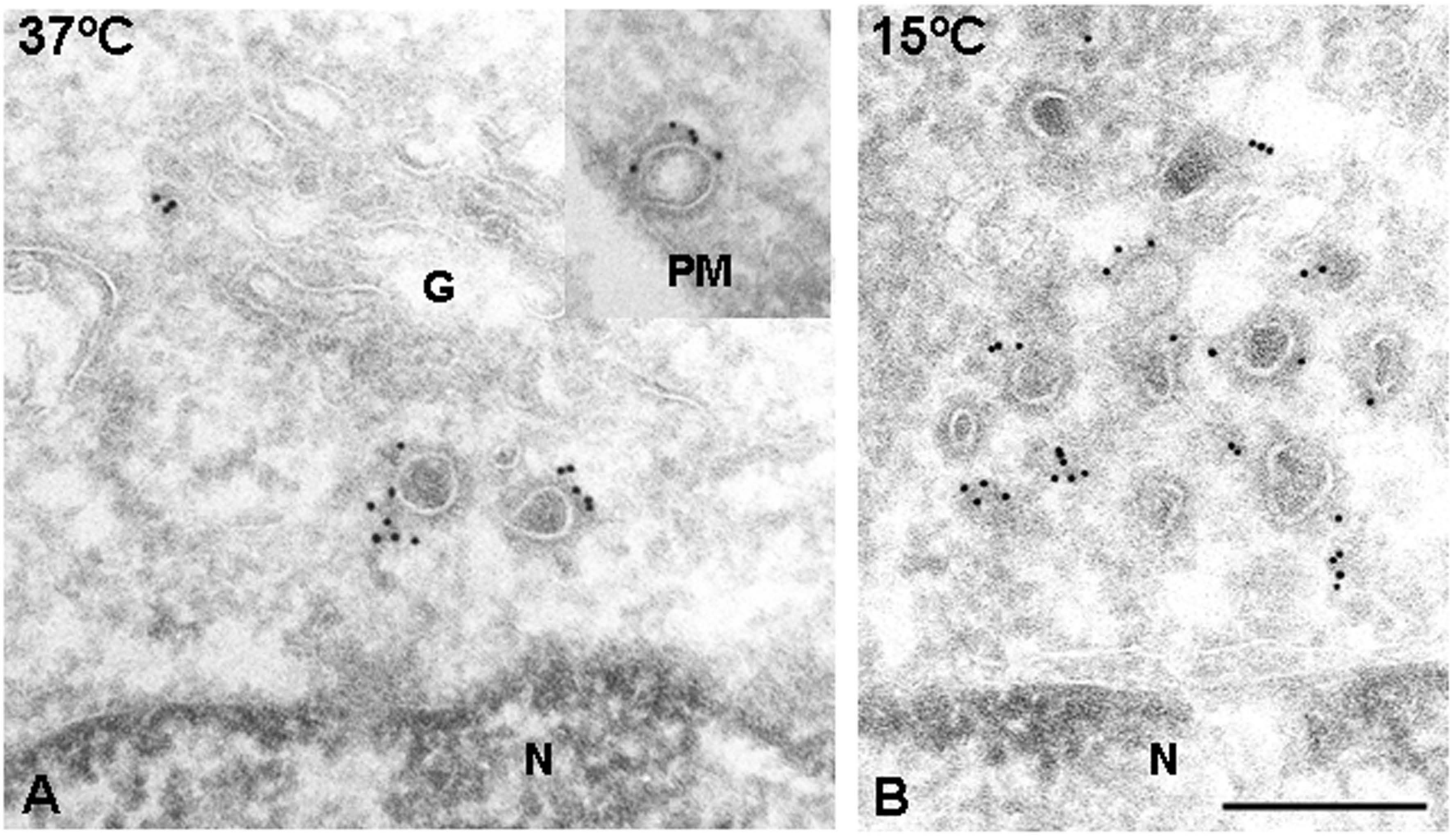
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