Supplementary Material

Fine structure of the "PcG body" in human U-2 OS cells established by correlative light-electron microscopy

Jana Šmigová, Pavel Juda, Dušan Cmarko, Ivan Raška

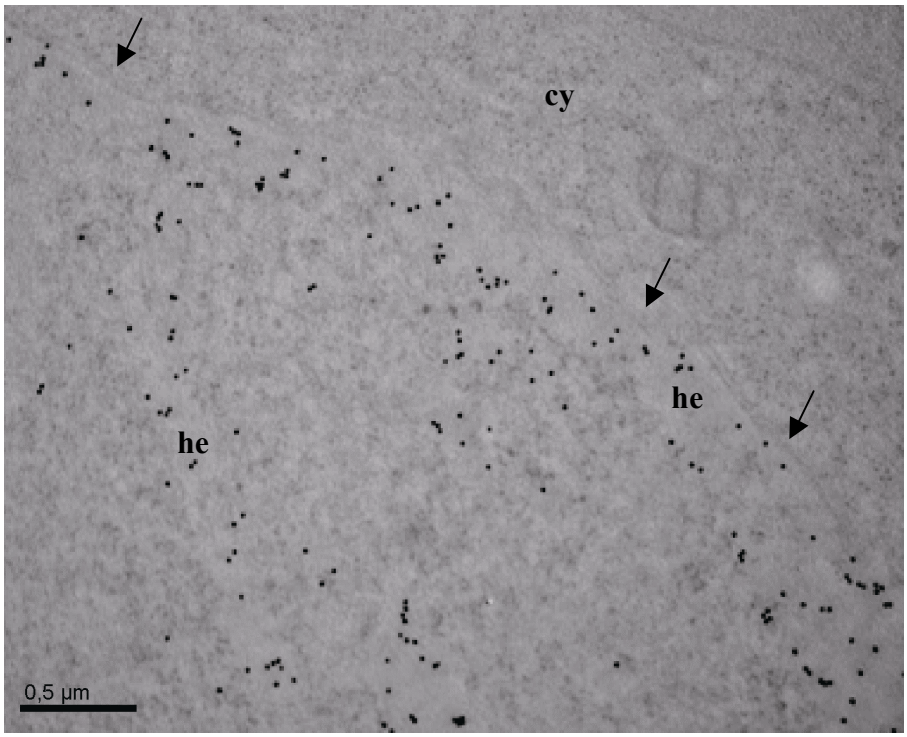

Figure S1. BMI1 protein immunogold labeling of non-transfected U-2 OS cells. High-pressure frozen and cryosubstituted cells were on-section labeled with the primary monoclonal mouse anti-BMI1 antibody and the secondary goat anti-mouse antibody-gold complex (18 nm gold particles) as described in the main article. The heterochromatin, which is specifically enriched in the immunogold label, appears to be bleached. Due to the bleached heterochromatin phenomenon, sometimes observed after HPF and cryosubstitution (Paul Verkade, personal communication), the gold particles are clearly visible. Note that gold particles are often observed towards the periphery of the heterochromatin structures (he). Cytoplasm (cy), nuclear envelope (black arrows).

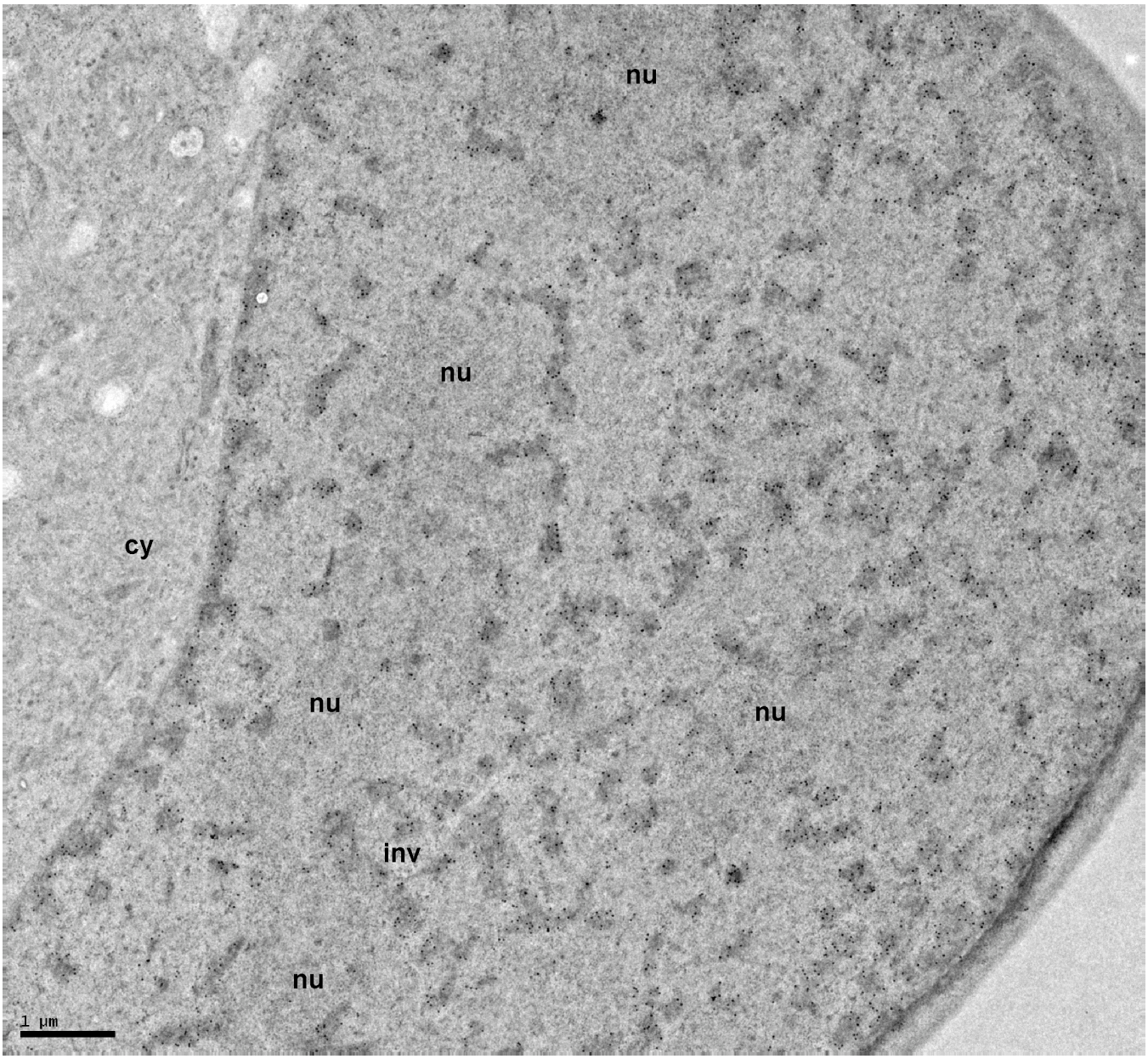

Figure S2. BMI1 protein immunogold labeling of transfected U2-OS cells. Heterochromatin structures (electron-dense areas in the nucleus) are specifically enriched in the immunogold label (15 nm gold particles) all over the thin sectioned nucleus. Note a variety of preserved structures depicted in the cytoplasm (cy) after high-pressure freezing/cryosubstitution of cells. Nucleoli (nu); invagination of the nuclear envelope (inv).

Figures S3-S5 are included in the Supplementary Material. With respect to Figs. 2 and 3 of the main article, they describe in more detail the identification of the nuclear domain corresponding to the "PcG body."

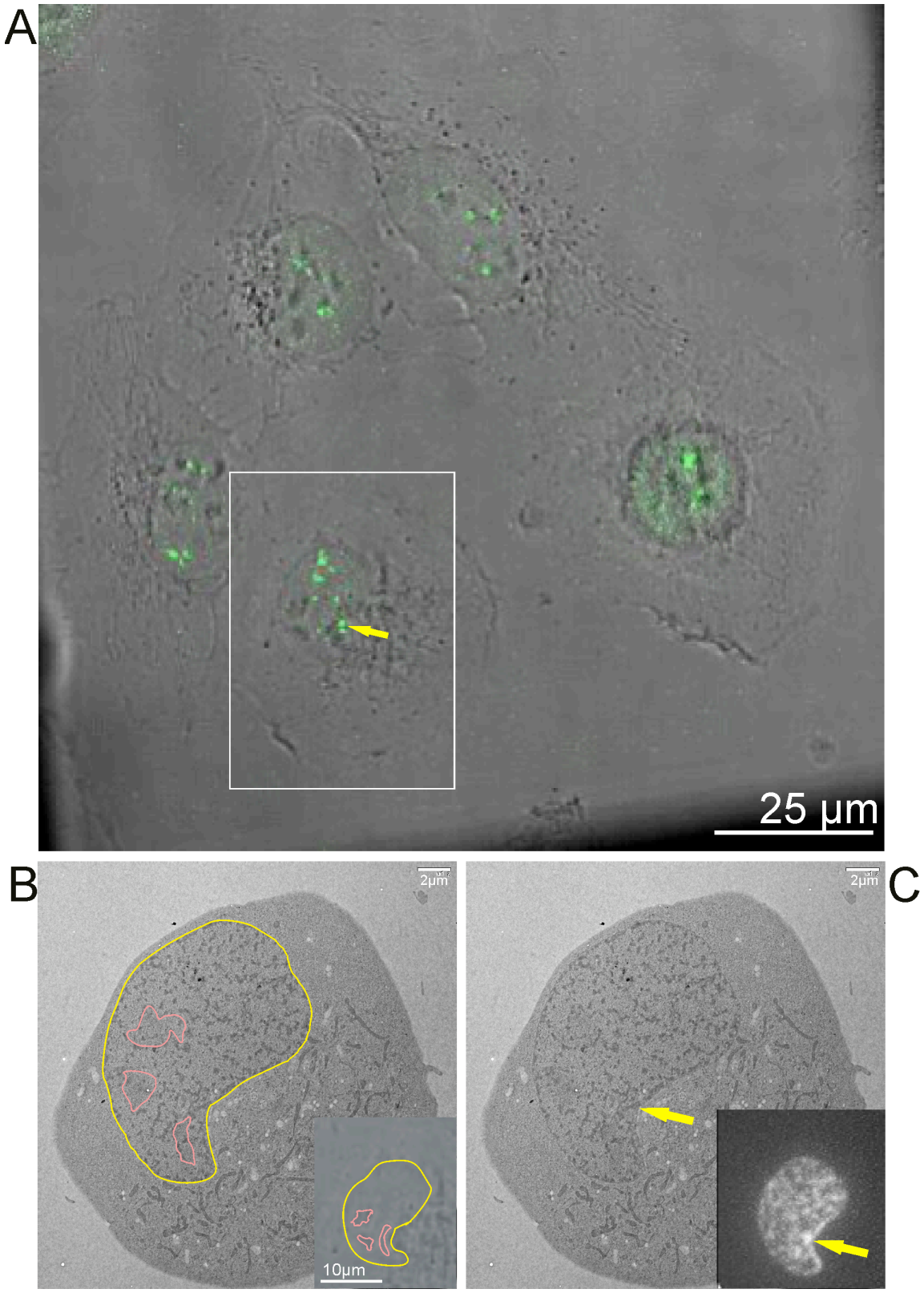

Figure S3. An overview of the correlation from the main article. (A) An overview image (merge of fluorescence and phase contrast) of the cell used for CLEM; the maximum intensity projection of fluorescence is shown. The cell of the interest is delineated in the rectangle. (B) The same, but embedded and thin sectioned cell (shown already in Fig. 3B of the main article). The nucleus is delineated by a yellow line and the nucleoli with pink lines. In the insert, the cell shown in Fig. S3A is shown in phase contrast only. The nucleus also is delineated with a yellow line and the nucleoli by pink lines. (C) The same, but embedded and thin sectioned cell (shown already in Fig. 3B of the main article). Yellow arrow points to the nuclear region (domain) that corresponds to a section through the "PcG body." This thin section was first used for on-section immunofluorescence mapping of the BMI1 protein (insert); the same insert is already shown in Fig.3B of the main article, with the yellow arrow pointing to the section through the fluorescent "PcG body."

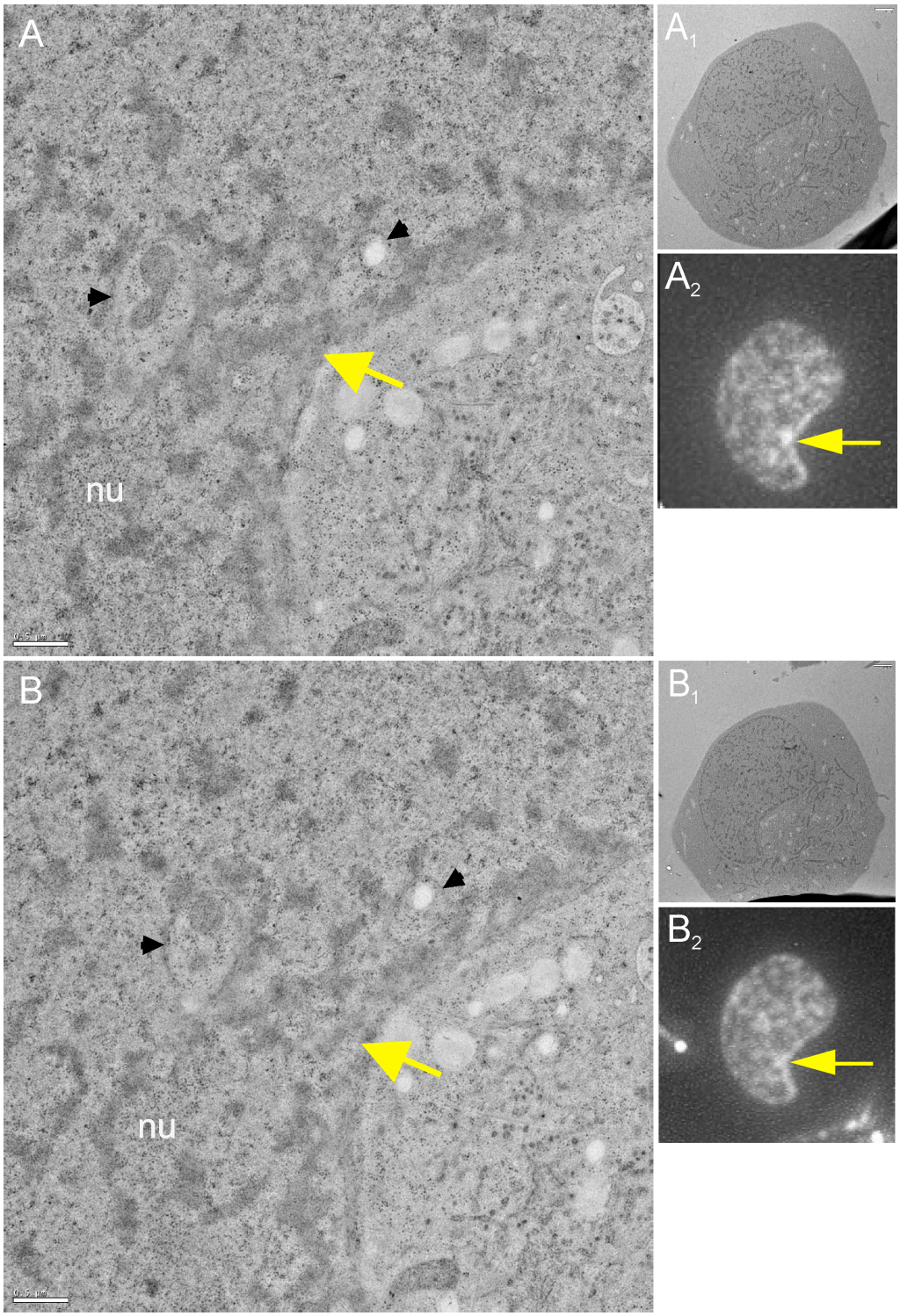

Figure. S4. Higher magnification of Figs. 3B and 3C from the main article. (A, B) The yellow arrow points to the nuclear region/domain corresponding to the "PcG body" seen in the electron microscope in two consecutive serial sections. Nucleolus (nu), invaginations of the nuclear envelope (black arrowheads). (A1, B1) The low magnification of the thin sections shown in Figs. S4A and B. (A2, B2) The on-section 2-D immunofluorescence labeling of the

BMI1 protein in the two serial thin resin sections. Fig.S4A2 was already shown in Figs. 3B and S3C, Fig. S4B2 in Fig.3C.

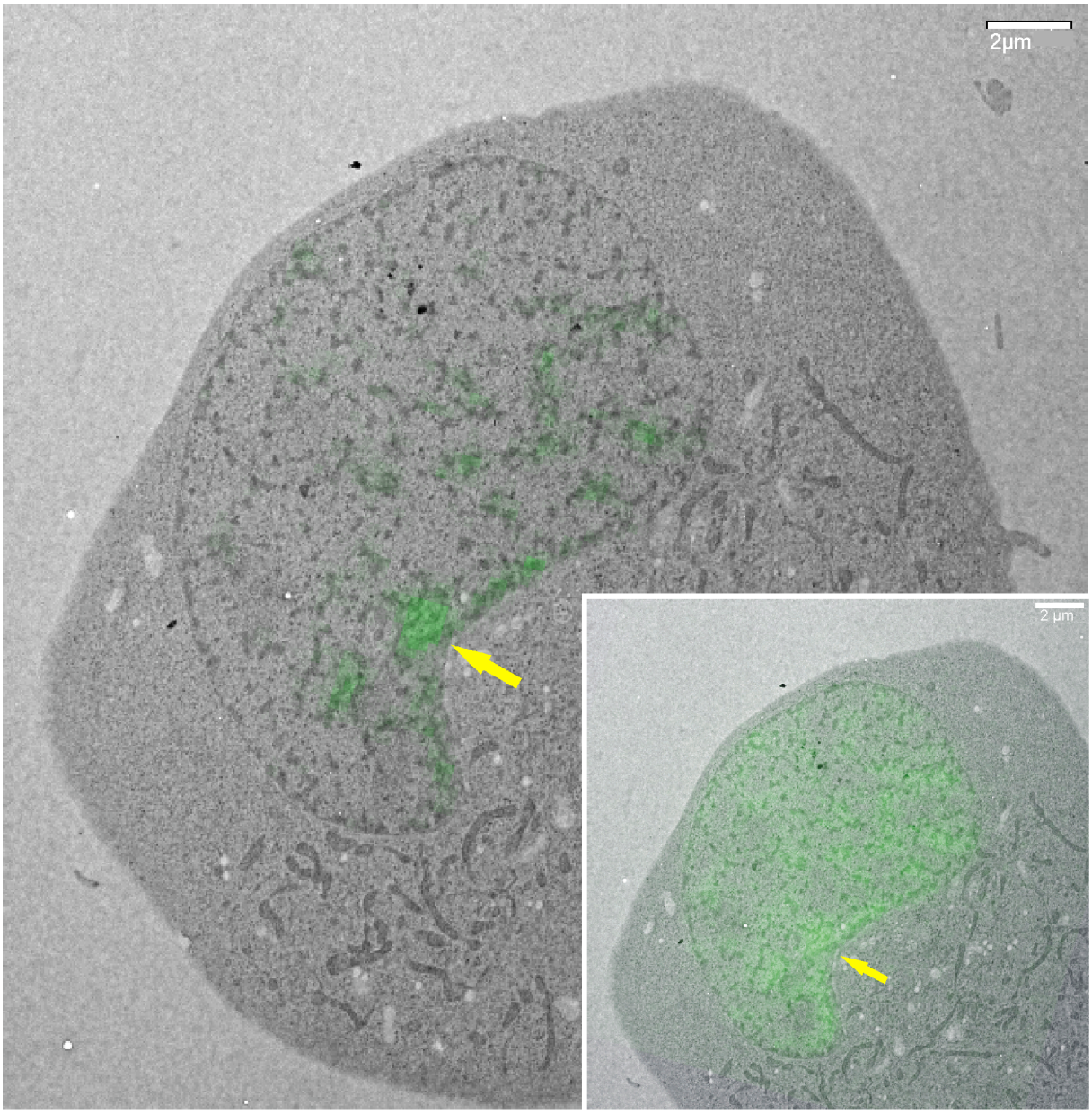

Figure S5. Overlay of immunofluorescence (in green) and electron microscopic image on the same resin section shown in Figs. 3B, S3C and S4A. In the lower magnification image, the ovelay is performed using original, unadjusted fluorescence image (e.g. ref. 1). The yellow arrow points to the nuclear region/domain corresponding to the "PcG body. In the higher magnification image, we applied a threshold such that only the highest intensities are displayed. Yellow arrow points to (a section through) the "PcG body" as identified by green fluorescence. The "PcG body" corresponds to a local accumulation of heterochromatin

fascicles. A higher magnification of the region/domain corresponding to this "PcG body" is provided in Fig. S4A.

Supplementary Results

To further expand our findings with the CLEM in which HPF and freeze substitution was implemented, we provide here two other CLEM approaches (Figs. S6, S7) that lead, with a help of the pre-embedding labeling, to the identification of the "PcG bodies."

CLEM with pre-embedding labeling

To mimic the immunofluorescence procedure of the cell processing[2], and thus to also achieve the 3-D immunolabeling of cells, we immunolabeled cells prior to their embedding into resin (Fig. S6). Because of the weak (and relatively slow) fixation, subsequent permeabilization/extraction, numerous washing steps and rather abrupt dehydration, the ultrastructure of cytoplasm and nuclei was largely affected.

However, via CLEM, and despite the presence of a non-specific silver label, the "PcG bodies" could be identified in a straightforward way through their very intense labeling (Figs. S6B-D). Silver particles apparently completely labeled three distinct nuclear domains of the three "PcG bodies" (Fig. S6), with two "PcG bodies" being associated with nucleoli. However, the "PcG bodies" were compacted and their fine structure was ruined. Interestingly enough, the implementation of CLEM would likely not be necessary for the identification of the "PcG bodies" in this pre-embedding approach as the "PcG bodies" are in the sectioned resin embedded cells noticed immediately in a straightforward way.

CLEM with pre-embedding labeling of cells extracted prior fixation

In the next approach to identify "PcG bodies" at the ultrastructural level, we used even a more invasive method with respect to the previous approach - extraction of cells prior fixation (Fig. S7). This approach was originally introduced in order to visualize rather stable structures like cytoskeleton, with many other cellular components being extracted. [3]

Here, the living cells were extracted with a hyperosmotic cytoskeleton CSK buffer containing detergent prior fixation and pre-embeding labeling (Fig. S7). In such processed cells, the preservation of the cellular ultrastructures was even more affected than with the previous pre-embedding approach. The "PcG bodies" were, however, still easily identified through the more intense silver label (Fig. S7), but their fine structure was extensively affected.

The labeling pattern observed differed from that of the previous approach. Here, silver particles decorated the periphery of the "PcG bodies", indicating a high aggregation/compaction of the heterochromatin structures giving rise to such a "body." Immunoprobes apparently did not penetrate such compacted structures as other heterochromatin structures also appeared to be labeled on the outer surface.

Supplementary Discussion

We identified the "PcG body" in the two other CLEM approaches in which a chemical fixation of cells was used. These two approaches led to an identification of the "PcG bodies" at the ultrastructural level due to a higher labeling density, one throughout the "PcG body" and the other decorating the outer surface of the "PcG body." These approaches, especially the second one, revealed the presence of more aggregated/compacted heterochromatin structures correlating with the respective fluorescent PcG foci.

It has to be emphasized here that chromatin is very complex and highly dynamic, and its structure exhibits extraordinary sensitivity to environmental factors.[4-9] The molecular composition of chromatin, that necessarily involves numerous modifications of its components, is of course related to the highly dynamic *in situ* structure of chromatin in living cells. At the same time, the composition of ions, particularly of the polyvalent ions, and osmolarity are very important factors. The weak (and relatively slow) chemical fixation, change in ionic conditions, change of osmolarity, permeabilization/extraction, various washing procedures, and abrupt dehydration do affect the structure of chromatin. This is apparently due to both extraction of molecules and pronounced structural changes, particularly aggregation of molecules, including their sticking to pre-existiting structures.[5,7,8] To establish the fine structure of the large scale chromatin organization in a convenient way, it is crucial to minimize such effects that lead to structural changes. And even though such changes are not resolved by light microscopy (Fig. 1), they are put in evidence by electron microscopy. In the context of this study, we consider only the results obtained in the HPF and cryosubstitution approach (Figs. 2, 3) as appropriate for both the identification, and for the description of the fine stucture of the "PcG bodies."

Materials and Methods

For all CLEM approaches using chemical fixation, the U-2 OS cells stably expressing BMI1-GFP were grown either on CELLocate coverslips (Eppendorf) or on gridded Petri dishes (MatTek Corporation). Before electron microscopy procedure (prior fixation if not mentioned otherwise), Z series from the inverted confocal microscope (Leica TCS SP5) were obtained.

After the EM embedding procedure, a pyramid was made to only leave the small area with the cells of the interest. 70 nm sections were then cut and collected on formvar carbon coated nickel slot grids. The sections were counterstained using 3.7% uranyl acetate for 15 minutes and lead citrate[10] for 2 minutes. The ultrastructural images were recorded with a FEI Tecnai G2 Sphera or Zeiss EM 900 electron microscopes.

CLEM with pre-embedding labeling procedure

For pre-embedding labeling, the cells were first fixed with the 2% formaldehyde in 0.2M PIPES (pH 7.2) for 15 minutes and permeabilized with 0.1% TritonX-100 in PBS for 10 minutes. Prior to antibody incubation, the cells were blocked with 5% NGS in PBS for 30 minutes. The BMI1 monoclonal antibody (diluted 1:50 in BSA/PBS/Tween 20) was followed by the goat anti-mouse ultrasmall gold conjugate (diluted 1:50 in 1%BSA/PBS). After the immunogold labeling the cells were washed and postfixed with 2.5% glutaraldehyde in PBS. Finally, several important washing steps with distilled water were performed and the cells were incubated with Aurion R-Gent Silver Enhancement Kit (equal parts of the developer, enhancer and gum arabic mixed just before applying) for 23 minutes. The cells were again washed extensively with distilled water, dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100%) and flat embeded into Araldite, Embed 812 (Epon-812) (Electron Microscopy Sciences).

CLEM with pre-embedding labeling of cells extracted prior fixation

U-2 OS BMI1-GFP cells were permeabilized in CSK buffer (100mM NaCl, 300mM sucrose, 3mM $MgCl_2$ and 10Mm PIPES; pH 6.8) with 0.1% TritonX-100 for 10 minutes.[2] Then the cells were fixed with 2% formaldehyde in CSK for 30 minutes at RT and the CELLocate coverslip was transferred (face down) into a fluorodish with PBS. After acquiring

serial confocal sections, the cells were processed for EM, sectioned and viewed as in the previous pre-embedding approach.

Figures

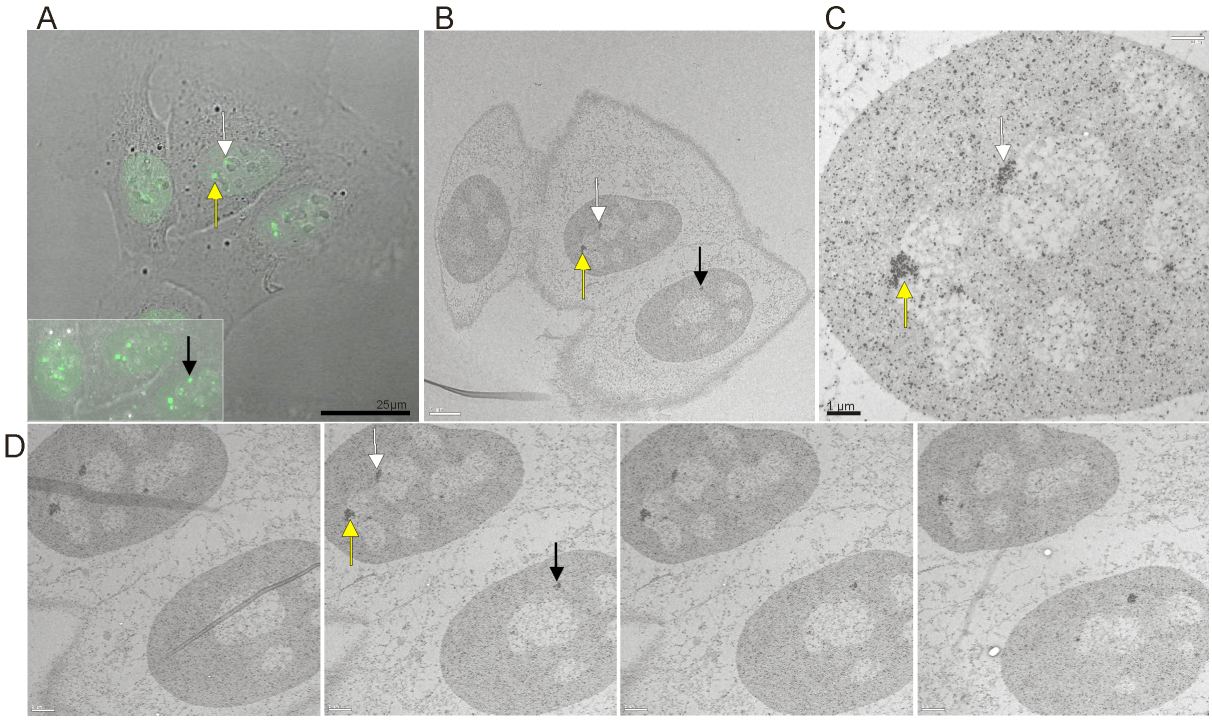

Figure S6. CLEM with pre-embedding procedure. U-2 OS cells were imaged for BMI1-GFP signals on the gridded Petri dishes. Yellow, white and black arrows point to the signals corresponding to three "PcG bodies." (A) One optical section and in the insert maximum intensity projection of all optical sections through the cells. Merge of fluorescence and phase contrast is shown. (B) Electron micrograph of the same group of cells after the pre-embedding procedure (C) Detailed view showing accumulations of silver enhanced ultrasmall gold particles (in the preembedding labeling of BMI1 protein) corresponding to the fluorescent PcG foci. (D) Four consecutive serial sections depicting "PcG bodies" (arrows pointing to the three "PcG bodies" are drawn in the second serial section). The pre-embedding CLEM allowed, at the EM level, to identify the "PcG bodies" as detected by fluorescence.

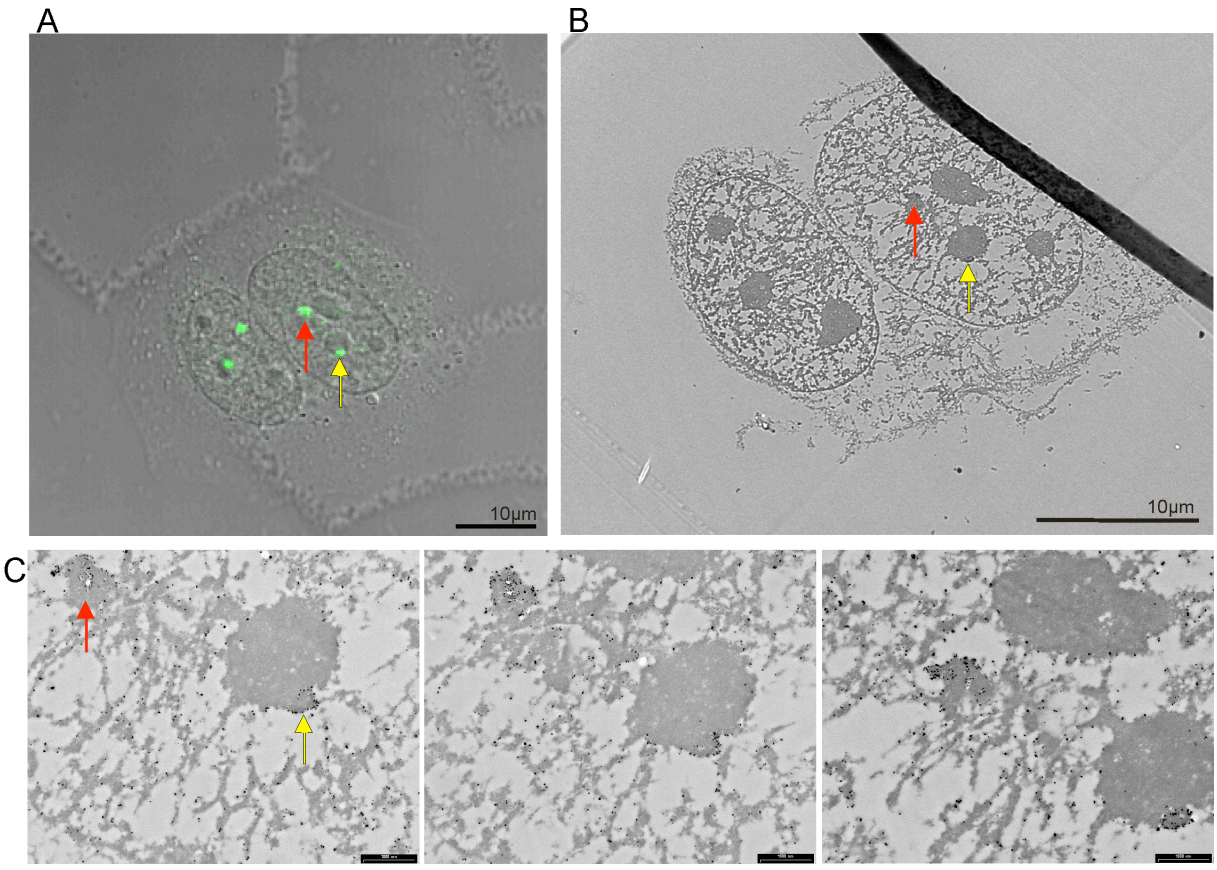

Figure S7. CLEM of extracted cells prior fixation and pre-embedding labeling.

U-2 OS BMI1-GFP cells were, after extraction with CSK buffer containing detergent and fixation with 2% formaldehyde, imaged on CELLocate coverslips in the fluorodishes. Yellow and red arrows point to two "PcG bodies. " (A) Merge of GFP fluorescence and phase contrast images; one optical section. (B) The same cells as imaged by the electron microscope. (C) The structures corresponding to the fluorescence PcG bodies (yellow and red arrows in the first serial section) in the three serial ultrathin sections immunolabeled for BMI1 protein (pre-embedding, silver enhancement). The heterochromatin corresponding to the "PcG body" fluorescence signal is found associated with the nucleoli.

References

1. Koberna K, Malinsky J, Pliss A, Masata M, Vecerova J, Fialova M, et al. Ribosomal genes in focus: new transcripts label the dense fibrillar components and form clusters indicative of "Christmas trees" in situ. J Cell Biol 2002; 157:743-8.

2. Raska I. Oldies but goldies: searching for Christmas trees within the nucleolar architecture. Trends Cell Biol 2003; 13:517-25.

3. Fey EG, Krochmalnic G, Penman S. The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. J Cell Biol 1986; 102:1654-65.

4. Armbruster BL, Wunderli H, Turner BM, Raska I, Kellenberger E. Immunocytochemical localization of cytoskeletal proteins and histone 2B in isolated membrane-depleted nuclei, metaphase chromatin, and whole Chinese hamster ovary cells. J Histochem Cytochem 1983; 31:1385-93.

5. Dubochet J, Sartori Blanc N. The cell in absence of aggregation artifacts. Micron 2001; 32:91-9.

6. Hancock R. Internal organisation of the nucleus: assembly of compartments by macromolecular crowding and the nuclear matrix model. Biol Cell 2004; 96:595-601.

7. Richter K, Nessling M, Lichter P. Experimental evidence for the influence of molecular crowding on nuclear architecture. J Cell Sci 2007; 120:1673-80.

8. Maeshima K, Hihara S, Eltsov M. Chromatin structure: does the 30-nm fibre exist *in vivo*? Curr Opin Cell Biol 2010; 22:291-7.

9. Matsuda A, Shao L, Boulanger J, Kervrann C, Carlton PM, Kner P, et al. Condensed mitotic chromosome structure at nanometer resolution using PALM and EGFP- histones. PloS one 2010; 5:e12768.

10. Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 1963; 17:208-12.