Lipid-dependent and -independent regulation of nuclear envelope disassembly

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The nuclear envelope (NE) is an organelle of unique complex and dynamic structure, which serves not only as a physical barrier between the nucleus and the cytoplasm, but also as a regulator of many cellular events. During open mitosis, the NE is disassembled in prophase in order to allow the mitotic spindle to access the chromosomes [1]. NE breakdown entails the disassembly of all the NE components and is associated with a series of phosphorylations of proteins of the inner nuclear membrane [2], nuclear pore complex [3] and lamina [4], the latest promoting depolymerisation of the lamin filaments. The A-type lamins are then solubilised in the cytoplasm, whereas B-type lamins disperse in the endoplasmic reticulum membranes [5]. Multiple kinases, including cyclin-dependent kinase 1 (CDK1) and diacylglycerol-activated protein kinase Cs (PKCs), have been suggested to play a role in lamin phosphorylation and disassembly in various models and organisms, mostly in cell-free systems [6–17]. In *Caenorhabditis elegans*, a homologue of lipin, a lipid phosphatase which converts phosphatidic acid to DAG, is required for lamin disassembly during NE breakdown [18, 19]. To investigate mitotic lamin B1 disassembly, Mall et al. have developed a high-throughput live-imaging assay [20]. The data obtained in HeLa cells using this assay confirm previous results suggesting the involvement of CDK1, PKCs and lipins in lamin disassembly.

This assay is based on the automatic tracking and classification into mitotic phases of hundreds of individual mitotic cells expressing the chromatin marker H2B and lamin B1 chimerae, monitored by wide-field microscopy. Briefly, nuclei were detected in the H2B-mCherry channel,

classified as previously described [21] and the chromatin was segmented. A ring around the chromatin was defined to measure GFP-lamin B1 fluorescence intensity and deduce the assembled or disassembled (in the cytoplasm) state of lamin B1 throughout mitosis. Using a mathematical model, the authors determined the durations of lamin disassembly in each condition. Mitotic progression from prophase to anaphase and chromosome congression (mean duration of prometaphase and metaphase) were also calculated.

Results obtained with transiently transfected wild-type or mutant lamin B1 reporter using this assay or higher resolution images acquired by confocal microscopy showed that mutation of the PKC consensus phosphorylation site did not alter lamin disassembly, whereas mutation of the CDK1 phosphorylation site alone or combined to the mutation of the PKC motif significantly delayed lamin B1 disassembly compared to controls. However, the differences in the mean duration of lamin disassembly between wild-type and CDK1- or CDK1/PKC-phosphorylation site mutants were subtle, although significant (7.51 ± 0.87 and 8.24 ± 0.79 min $p=0.0146$ or 8.86 ± 0.80 min, $p<0.0001$, respectively). The weakness of the mutants might be attributed to insufficient levels of expression compared to endogenous proteins (Western blots omitted) and inability to form a network of mutant proteins which would resist lamina disassembly. Another possibility is that these phosphorylation sites, only play a minor role in lamin disassembly. Relatively high doses (compared to IC₅₀) of kinase inhibitors, i.e. classical PKC inhibitor Gö6976 and CDK1 inhibitor roscovitine alone or combined, caused significant perturbations of lamin disassembly and mitotic progression, although the two events were not correlated, and induced the apparition of ‘cytoplasmic fragments’ of lamin B1. Those high intensity ‘cytoplasmic fragments’, which were also observed by confocal microscopy with the CDK1- or CDK1/PKC-site mutants, could correspond to aggregates of lamin B1 in

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the endoplasmic reticulum. Colocalisation experiments with markers of the different subcellular compartments would have brought useful information on the lamin B dynamics in these conditions. The delays on mitotic progression, especially with both inhibitors combined, seemed mostly related to a dramatic increase in the duration of chromosome congression, which might be due to unspecific inhibition of other kinases controlling the mitotic spindle checkpoint. RNAi of PKC- α or - β also resulted in delayed lamin (B1 and A) disassembly and mitotic progression. The mild effects of mutation of lamin B1 phosphorylation sites and the not complete block of lamin disassembly by PKC and CDK1 inhibitors or RNAi of PKC suggest that although important, those two kinases are not essential for lamin disassembly, but may act in concert with further kinases, as was suggested by previous studies [4, 6, 7], or that compensation mechanisms for the lack of CDK1 or PKC might have occurred.

As activation of classical PKCs requires DAG [22] and nuclear DAG levels peaks in G2/M transition [12], the role of PLC, which hydrolyses PtdIns(4,5) P_2 to form DAG, was assessed with the inhibitor U73122 and PLC- β 1 RNAi. A slight effect on mitotic progression was observed in both conditions, but without significant delay in lamin B1 disassembly. Although RNAi against only one isoform of PLC and only one relatively low concentration (compared to IC₅₀) of inhibitor were tested, the authors concluded that PLC is not involved in lamin disassembly, and that other factors such as lipins are responsible for the production of PKC-activating DAG. However, similar results (not shown), i.e. no delay in lamin B1 disassembly, were obtained with RNAi against each individual lipin, suggesting a redundancy of the lipins which may also occur with PLC isoforms. On the contrary, RNAi against all three lipin isoforms delayed both lamin (B1 and A) disassembly and mitotic progression, as did high doses of propanolol, inhibitor of phospholipase D-derived DAG formation (and probably PKC at this concentration [23]) or, to a lesser extent, RNAi of Dullard, a phosphatase activating lipins at the NE [24]. However, although not discussed here, these RNAi also induced an increase in PKC- β RNAs, suggesting the alterations of the other signalling pathways and questioning the specificity of the effects observed here. Finally, addition of DAG in these conditions restored fully lamin B1 disassembly and partially mitotic progression, suggesting that DAG is responsible for the mitotic effect of lipins, although confirmation of the change in DAG or its incorporation in the cell membranes, e.g. via the use of fluorescently labelled DAG, was omitted. Furthermore, as DAG was added to the cells in DMSO, which is known to interfere with membrane morphology [25], the specificity of their observations will need to be confirmed.

Mall et al. have developed a high-throughput wide-field microscopy assay, that may provide a rigorous method to

quantify lamin disassembly and the events associated with NE breakdown, the mechanisms of which remain unclear. Evidence for a collaborative role in lamin disassembly of CDK1 and PKC was obtained by the use of lamin B1 chimaeras mutated on their phosphorylation sites or kinase inhibitors. RNAi experiments confirmed the involvement of classical DAG-dependent PKCs in lamin disassembly and suggested, as did the use of a PLD pathway inhibitor and the addition of DAG, the involvement of lipins, as a source of DAG necessary for PKC activation. However, most of the results obtained here rely on the automated analysis of GFP-lamin B1 fluorescence intensity around the chromatin followed by wide-field microscopy throughout mitosis. Limitations of this assay include the low resolution of the images and the requirement of H2B-mCherry to define the mitotic stage and the region corresponding to the chromatin, implying inaccurate quantification of GFP-lamin fluorescence. Co-expression of compartment-specific markers, e.g. monitoring the translocation of lamin B1 to the endoplasmic reticulum, could represent a more precise readout of lamin disassembly and minimise the potential artefacts due to for example the proximity of the endoplasmic reticulum and chromatin or to aggregates of over-expressed proteins. Another limitation of this work relates to the global approach, i.e. inhibitors or RNAi, used to investigate the regulation mechanisms of lamin disassembly. Of particular concern is the use of relatively high concentrations of inhibitors known to affect several targets, which may interfere with multiple signalling pathways in the cell and induce artefacts or misinterpretations of the effects observed. It could also be argued that the use of RNAi, although more specific than inhibitors, could, due to the long incubation needed to deplete the gene of interest (48 h here), perturb the cell metabolism or allow the cell to adapt and develop compensation mechanisms which may affect other pathways and induce artefacts. Examples of potential artefacts are the increase in PKC- β transcripts upon lipin RNAi treatment or the important differences, i.e. in the same order of magnitude as the specific effects observed with RNAi, in lamin disassembly or mitotic progression between the controls of each type of experiment. An alternative approach would exploit the rapalogue dimerisation tool which allows the recruitment of lipid-modifying enzymes to specific endomembranes to *rapidly and inducibly* deplete DAG.

By targeting DAG kinase or SKIP phosphatase to specific membranes, we recently showed that acute and inducible DAG depletion resulted in failure of the NE to reform at mitosis and reorganisation of the ER into multi-lamellar sheets as revealed by correlative light and electron microscopy and 3D reconstructions. Depleted cells divided without a complete NE, and unless rescued by DAG soon died [26]. In the context of this study, the spatiotemporal control of levels of DAG using this tool could allow a deeper

understanding of the sequence of events involved in NE breakdown, of the relative roles of key proteins such as PKC, CDK1 or lipins in lamina disassembly, and of the relationship between membrane composition and downstream signalling in NE dynamics.

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