

# Nuclear envelope organization in *Dictyostelium discoideum*

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**ABSTRACT** The nuclear envelope consists of the outer and the inner nuclear membrane, the nuclear lamina and the nuclear pore complexes, which regulate nuclear import and export. The major constituent of the nuclear lamina of *Dictyostelium* is the lamin NE81. It can form filaments like B-type lamins and it interacts with Sun1, as well as with the LEM/HeH-family protein Src1. Sun1 and Src1 are nuclear envelope transmembrane proteins involved in the centrosome-nucleus connection and nuclear envelope stability at the nucleolar regions, respectively. In conjunction with a KASH-domain protein, Sun1 usually forms a so-called LINC complex. Two proteins with functions reminiscent of KASH-domain proteins at the outer nuclear membrane of *Dictyostelium* are known; interaptin which serves as an actin connector and the kinesin Kif9 which plays a role in the microtubule-centrosome connector. However, both of these lack the conserved KASH-domain. The link of the centrosome to the nuclear envelope is essential for the insertion of the centrosome into the nuclear envelope and the appropriate spindle formation. Moreover, centrosome insertion is involved in permeabilization of the mitotic nucleus, which ensures access of tubulin dimers and spindle assembly factors. Our recent progress in identifying key molecular players at the nuclear envelope of *Dictyostelium* promises further insights into the mechanisms of nuclear envelope dynamics.

**KEY WORDS:** nuclear envelope, *Dictyostelium*, lamin, NET, centrosome, centromere

## Introduction

Every eukaryotic cell encases its genetic material within a defined organelle, the nucleus. The spherical nucleus of vegetative *Dictyostelium discoideum* amoebae has a diameter of approximately 2  $\mu\text{m}$ . Its nuclear envelope consists of the outer and the inner nuclear membrane (ONM and INM; Fig. 1). Both are interconnected at the nuclear pores and contiguous with the ER. Nonetheless, ER, ONM and INM each are enriched in specific nuclear envelope transmembrane proteins (NETs) and possibly also specific lipids (Fig. 1). The nuclear pores are built by the nuclear pore complexes (NPCs), which mediate specific nuclear

import and export. On its nuclear side, the INM is associated with a fibrous, so-called nuclear lamina. Among the NETs, the Sun-domain proteins in the INM (e.g. Sun1) and KASH-domain proteins in the ONM (e.g. nesprin) have to be highlighted, as they form the so-called LINC complex, which spans the whole nuclear envelope (Lee and Burke, 2018; Razafsky and Hodzic, 2009; Starr and Fridolfsson, 2010). LINC complexes physically couple the nuclear interior to the cytosolic cytoskeleton, since Sun-proteins bind to the nuclear lamina and KASH-domain proteins are linked directly or indirectly to all main cytoskeletal elements (microtubules, actin filaments and intermediate filaments). The lamina, the NETs and the NPCs determine the specific functions of the

*Abbreviations used in this paper:* BAF, barrier to autointegration factor; BioID, proximity-dependent biotin identification; CDK1, cyclin-dependent kinase 1; cryo-ET, cryo-electron tomography; DAPI, 4',6-diamidin-2-phenylindol; EM, electron microscopy; ER, endoplasmic reticulum; ExM, expansion microscopy; feSEM, field-emission scanning electron microscopy; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; HeH, helix-extension-helix; IF, intermediate filament; INM, inner nuclear membrane; KASH, Klarsicht, ANC-1, Syne Homology; LBR, lamin B receptor; LECA, last eukaryotic common ancestor; LEM, lamina-associated polypeptide 2 (LAP2), Emerin, and MAN1; LINC, linker of the nucleoskeleton and the cytoskeleton; MCAK, mitotic centromere-associated kinesin; MEF, mouse embryonic fibroblast; NET, nuclear envelope transmembrane protein; NLS, nuclear localization sequence; NPC, nuclear pore complex; ONM, outer nuclear membrane; STED, stimulated emission depletion.

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nuclear envelope as a specialized subcompartment of the nucleus.

## The nuclear lamina

### Historic perspective

Beginning in the mid last century, several ultrastructural analyses disclosed that the inner nuclear membrane is tightly associated with a fibrous lamina. This held true not only for vertebrate (Fawcett, 1966) and invertebrate cells (Gray and Guillery, 1963), but also for non-metazoan cells such as gregarines (Beams *et al.*, 1957) and *Amoeba proteus* (Pappas, 1956). In 1978, Gerace, Blobel and co-workers succeeded in isolating three major protein components of this nuclear lamina from rat liver nuclei (Gerace *et al.*, 1978), for which they later coined the names lamin A, B and C for the 70, 67 and 60 kDa components, respectively (Gerace and Blobel, 1980). Soon after that Goldman and co-workers realized that these lamins are keratin-like proteins, in other words cytoskeletal components of the intermediate filament family (Goldman *et al.*, 1986). At that time, three mammalian lamin genes, *LMNA*, *LMNB1* and *LMNB2* were also identified (see (Gruenbaum and Foisner, 2015) and references therein). While *LMNA* encodes lamin A and the splice variant lamin C, *LMNB1* encodes lamin B1 and *LMNB2* the lamin B2 protein and its splice variant lamin B3 (= lamin LIII). While at least one B-type lamin is expressed in all mammalian cells, A-type lamins are mainly restricted to differentiated cells. In contrast to mammals, most invertebrate animals possess only one lamin gene, encoding a B-type polypeptide (Gruenbaum and Foisner, 2015).

It is clear that the nuclear lamina consists not only of lamins but also of many lamin associated proteins at the nuclear membrane collectively called NETs (Czapiewski *et al.*, 2016; Wilson and Foisner, 2010). However, as its major protein components, lamins and the structures they form are key to our understanding of nuclear lamina function. Ground-breaking work conducted in the eighties on *Xenopus* oocyte nuclear envelopes by Ueli Aebi and colleagues revealed details of the structural organization of lamins (Aebi *et al.*, 1986). In freeze dried/metal shadowed EM preparations they disclosed a fibrous network at the surface of inner nuclear membranes, in which regularly spaced individual fibers

were arranged in an orthogonal pattern as in a window screen. Filaments displayed a thickness of ~10.5 nm at a spacing of ~52 nm. Yet, our picture of the nuclear lamina mainly consisting of a rather regular and stable assembly of filaments was challenged in the 21st century (see below).

Despite various indications for the presence of a nuclear lamina in non-metazoan organisms, no obvious lamin genes had been identified in genomes other than from animal cells for many years. On the contrary, nuclear lamina proteins able to assemble into filamentous coiled-coil structures were identified in higher plants and trypanosomes (Gindullis *et al.*, 2002; Harder *et al.*, 2000; Rout and Field, 2001), but exhibited no evolutionary relationship to the intermediate filament protein family. Thus, the paradigm that intermediate filaments are part of a metazoan-specific protein toolkit was remained, until a lamin-like protein called NE81 was identified in *Dictyostelium* (Krüger *et al.*, 2012). NE81 not only showed protein sequence similarity to metazoan lamins, it also behaved as a lamin from a functional point of view (Batsios *et al.*, 2012; Grafe *et al.*, 2019; Krüger *et al.*, 2012). The identification of a lamin in one of the non-opisthokont branches of the eukaryotic tree of life and the improvement of bioinformatics tools facilitated identification of lamin-related protein sequences in organisms representing Archaeplastida, Excavata and SAR (Stramenopile, Alveolata, Rhizaria) (Kollmar, 2015; Preisner *et al.*, 2018). Nowadays, there can be no doubt anymore that lamins belonged to the protein equipment of the LECA (Gräf *et al.*, 2015; Koreny and Field, 2016).

### The Amoebozoan lamin

The *Dictyostelium* lamin NE81 was found by incident in a proteome analysis of isolated centrosomes, which were contaminated with small patches of nuclear envelope (Reinders *et al.*, 2006; Schulz *et al.*, 2009b). When the identified centrosomal candidates were expressed as GFP-fusion proteins, NE81 was found exclusively associated with the nuclear envelope instead of centrosomes. Despite its relatively low amino acid sequence identity to lamins, the predicted NE81 domain organization was strikingly similar. As in metazoan lamins, an  $\alpha$ -helical, central rod domain consisting of 370 amino acid residues is preceded by a head domain including

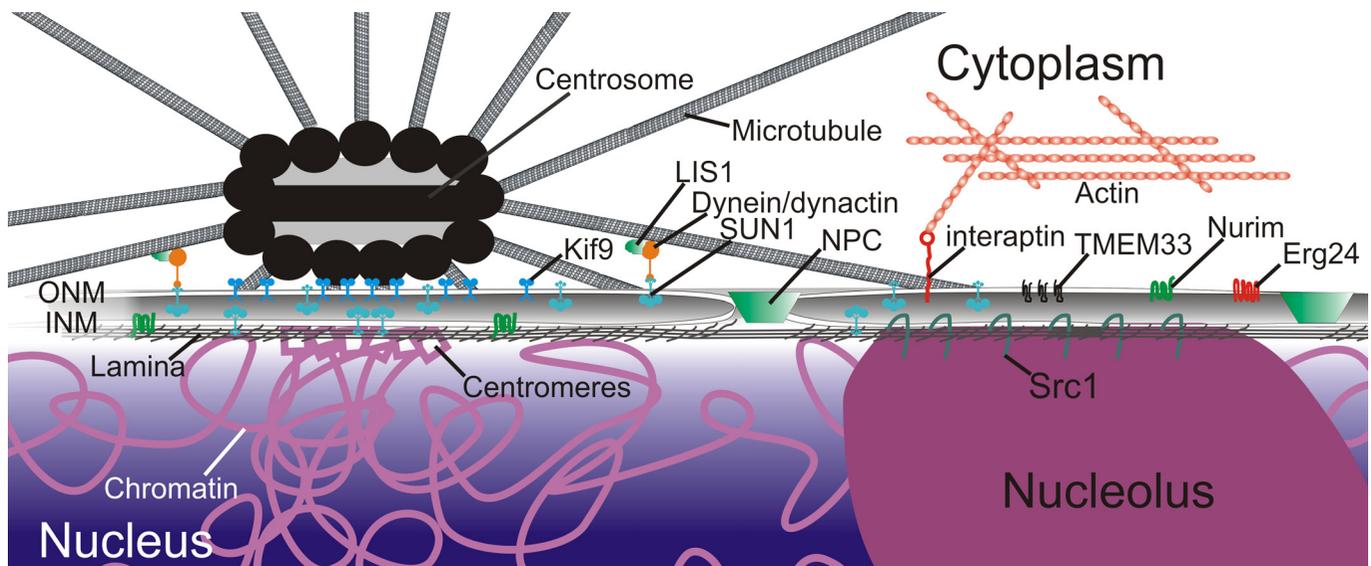


Fig. 1. Schematic view of *Dictyostelium* nuclear envelope organization.

a CDK1 phosphorylation consensus sequence and followed by (in this order) a tail domain featuring a NLS, a conserved lamin tail domain, and a CaaX-box (= cysteine, two aliphatic aa and X = residue specifying the type of isoprene moiety) for prenylation at the C-terminal end (Krüger *et al.*, 2012). Furthermore, microscopic and molecular studies confirmed that NE81 should be considered as a *bona fide* lamin due to its localization, functions, and protein interactions (Batsios *et al.*, 2012; Batsios *et al.*, 2016a; Batsios *et al.*, 2016b; Grafe *et al.*, 2019; Krüger *et al.*, 2012). For example, immunogold-electron microscopy revealed an exclusive distribution of NE81 along the inner nuclear envelope. The NE81 CaaX-box, a typical feature of isoprenylated proteins, appears to be processed in the same way as mammalian B-type lamins. Here, in a conserved sequence of posttranslational modifications, the cysteine residue is isoprenylated through farnesyl transferase, after which the last three amino acid residues (aaX) are cleaved off either by Ras-converting enzyme 1 or ZMPSTE24 protease. Next, the cysteine residue becomes methylated by isoprenylcysteine carboxyl methyltransferase (Gruenbaum and Foisner, 2015). The resulting C-terminal lipid anchor is required, albeit not sufficient, for B-type lamin association with the nuclear envelope (Adam *et al.*, 2013). Unlike B-type lamins, A-type lamins are further processed through an additional cleavage by ZMPSTE24, whereby the last 15 aa including the farnesyl anchor are removed. In *Dictyostelium*, lamin B-like CaaX box processing is required for proper NE81 function as well. Disruption of the *icmA* gene encoding isoprenylcysteine carboxyl methyltransferase resulted in mis-localized NE81 clusters in the nuclear interior (Batsios *et al.*, 2012). Along this line, disruption of CaaX-box function also resulted in typical disarrangements of NE81. Instead of an even, two-dimensional distribution along the nuclear envelope corresponding GFP-NE81 fusion proteins (GFP-NE81 $\Delta$ CLIM = CaaX-box deleted, and GFP-NE81SLIM = CaaX-box cysteine replaced by serine) formed three-dimensional clusters within the nucleus, most probably due to the lack of a lipid anchor that usually enforces a two-dimensional distribution (Batsios *et al.*, 2012; Krüger *et al.*, 2012). These mutants were also hypersensitive to mechanical stress, a typical phenotype upon lamin dysfunction also observed in mammalian cells (Gruenbaum and Foisner, 2015).

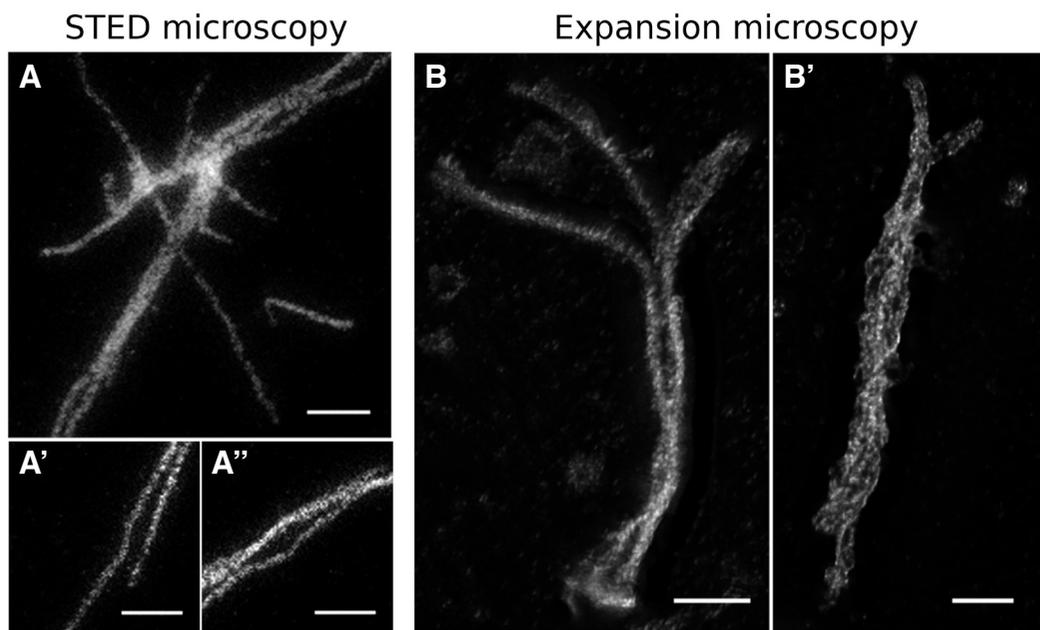
Like metazoan lamins, NE81 undergoes cell cycle-dependent assembly and disassembly cycles. In animals, CDK1 phosphorylation of lamins at the onset of mitosis initiates disassembly of the nuclear lamina and nuclear envelope breakdown. Despite the absence of a nuclear envelope breakdown during semi-closed mitosis in *Dictyostelium*, the nuclear envelope needs to become more flexible, since its rigidity has to be low enough to allow the constriction between two daughter nuclei during karyokinesis. Indeed, FRAP experiments with GFP-NE81 cells revealed a high mobility from early to mid mitosis of the otherwise immobile fusion protein (Krüger *et al.*, 2012). The observed mobility pattern fitted perfectly to the activity pattern of CDK1. The GFP-NE81 $\Delta$ CLIM strain independently confirmed this mitotic behavior, as the nuclear GFP-NE81 $\Delta$ CLIM clusters mentioned above disassembled at the onset of mitosis and the GFP-fusion protein, which contains no lipid anchor, freely diffused throughout the cytoplasm. A further point mutation of the serine 122 to alanine within the CDK1 recognition site led to stable nuclear clusters of the fusion protein that were unable to undergo mitotic disassembly (Krüger *et al.*, 2012). These results strongly indicated that NE81 disassembly is

regulated by CDK1.

Functional similarities to metazoan lamins also became obvious upon overexpression as well as by knockout of NE81 (Krüger *et al.*, 2012). Both, resulted in disorganized chromatin and misshapen nuclei. Knockout of NE81 additionally produced aberrant centrosome numbers and disrupted attachment of the centrosome to the nucleus. All of these phenotypes make sense in the context of lamin function, because beyond their mechanical functions (Isermann and Lammerding, 2017) lamins are known as regulators of heterochromatin formation, gene expression, and as the nuclear anchor for the LINC complex (see above). Among other functions (see below) LINC complexes connect the centrosome to the nuclear envelope, which explains the observed centrosomal aberrations in the *Dictyostelium* NE81 mutants. Indeed, a protein-protein interaction between NE81 and Sun1 could be shown by proximity-dependent biotin identification (BioID) (Batsios *et al.*, 2016a). Furthermore, deployment of this method also proved an interaction of NE81 with Src1. The latter represents the only LEM-family protein in *Dictyostelium* (see below and Fig. 1) (Batsios *et al.*, 2016b). Interactions between these protein families are conserved at least in amoebozoans and opisthokonts suggesting that they are very ancient and were possibly already a feature of the LECA.

The domain structure of NE81 mentioned above strongly suggested that the protein is capable of forming filaments. Recently, Grafe and co-workers have been successful in visualizing supramolecular NE81 filament assemblies by stimulated emission depletion microscopy (STED), Expansion microscopy (ExM) (Wassie *et al.*, 2019) (Fig. 2), transmission EM and feSEM (Grafe *et al.*, 2019). They expressed Flag-tagged NE81 in *Xenopus* oocytes and showed by feSEM that NE81 assembles into filamentous structures with an overall appearance highly reminiscent of *Xenopus* lamin B2. NE81 filaments were ~8.5 nm thick, i.e. slightly thicker than *Xenopus* lamin B2 (~7.3 nm) and thinner than *Xenopus* lamin LIII (~11.7 nm) in the same assay (Goldberg *et al.*, 2008). Assembly patterns of NE81 and lamin B2 were less regular than those originally published by Aebi and co-workers using freeze-dried metal-shadowed *Xenopus* oocyte specimens without forced expression of autologous or heterologous proteins (Aebi *et al.*, 1986). However, these differences may lie in the nature of these different preparations and also in species-dependent variations in filament thickness and order. For example, *Caenorhabditis elegans* lamin filaments display a thickness of only 5-6 nm in cryo-ET and show no clear orthogonal order (Grossman *et al.*, 2012). In 2017, Medalia and co-workers showed in vimentin-null MEFs by cryo-ET that lamins form filaments of only ~3.5 nm, i.e. about the size of a typical IF protofilament, with a relatively irregular arrangement beneath the inner nuclear membrane (Turgay *et al.*, 2017). Many insights in lamin assembly came from *in vitro* experiments conducted mainly with recombinant *C. elegans* lamin expressed in *E. coli* (Karabinos *et al.*, 2003). The *Dictyostelium* lamin could not be expressed in bacteria, however, a soluble cytosolic NE81 variant with an N-terminal HisMyc-tag (NE81 $\Delta$ NLS $\Delta$ CLIM = without CaaX-box and functional NLS) could nicely be expressed and purified from *Dictyostelium* extracts. Like its metazoan lamin counterparts, this NE81 protein formed filamentous structures *in vitro* under low ionic strength conditions (Fig. 2A–A''), while filaments disassembled at high ionic strength.

Both light and electron microscopy revealed reticular assemblies resembling those published for lamins in MEFs (Grafe *et al.*, 2019;



**Fig. 2. HisMyc-NE81 $\Delta$ NLS $\Delta$ CLIM filaments in super-resolution fluorescence microscopy. (A–A'')** STED microscopy: filaments were stained with anti-NE81/anti-rabbit-Atto 647N. Scale bars = 1  $\mu$ m. (B, B') ExM with deconvolution: filaments were stained with anti-NE81/anti-rabbit-AlexaFluor 488. Maximum-intensity projections are presented. The scale bar (2  $\mu$ m) refers to the original size of the specimen. From (Grafe et al., 2019, <https://doi.org/10.3390/cells8020162>).

Shimi et al., 2015). Deconvolved ExM images revealed a close alignment of individual filaments in parallel arrangements of two to four filaments (Fig. 2B and B').

Taken together, the structural data from multiple sequences and lamins from a variety of experimental systems indicate a general ability of lamins to form filaments of varying thickness and order depending on the individual isoforms, cell types and organisms. Now, with established protocols for structural lamin studies and a well-characterized nuclear envelope including most relevant protein components known from animal cells, *Dictyostelium* has joined the club of model systems for functional nuclear envelope studies as the first non-mammalian model organism so far.

### The nuclear pore complexes

In animal cells, lamins interact directly not only with NETs but also with NPCs (Xie et al., 2016). Although direct interactions between these structures remain to be demonstrated in *Dictyostelium*, it is likely that they are conserved due to the overall similarities in nuclear lamina and NPC organization. NPCs generally show an eight-fold symmetry and consist of roughly 30 different proteins (Beck and Hurt, 2017) called Nups (nuclear pore complex proteins). *Dictyostelium* NPCs have been thoroughly characterized by cryo-ET and were shown to form two different structural states, most likely representing different stages during cargo transport through the pore (Beck et al., 2004). Thus, from a structural point of view the *Dictyostelium* NPC is among the best studied NPCs so far. A search for human and yeast nucleoporins in *Dictyostelium* revealed similar protein composition in these three species (Beck and Medalia, 2008). Many conserved Nups have been identified from the *Dictyostelium* genome project (Basu et al., 2013) by sequence homology. A couple of them have already been verified by expression of GFP fusion proteins (e.g. Nup43, Nup62, Sec13L and GLE2), and localized to the nuclear envelope in interphase cells (Beck and Medalia, 2008; Xiong et al., 2008)

### Nuclear transmembrane proteins

#### HeH-family proteins

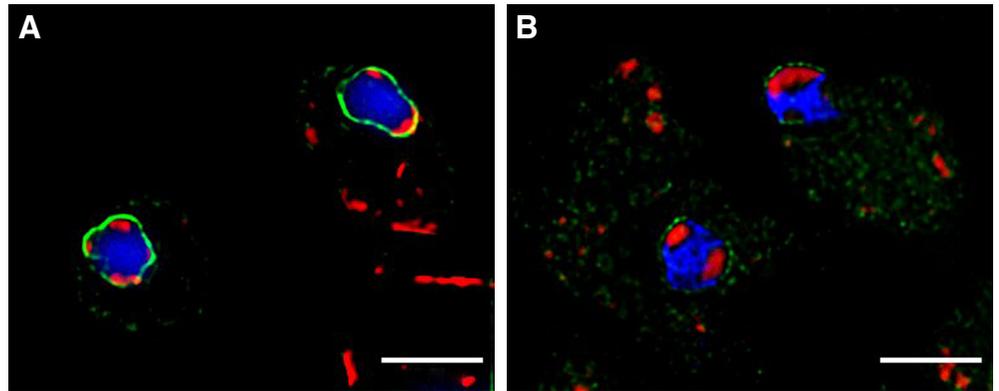
The NETs within the INM are required for nuclear structure, chromosome organization, DNA repair, epigenetic gene regulation and transcriptional control (Gruenbaum and Foisner, 2015). One of the common families of NETs is the HeH superfamily of DNA-binding proteins, which include the LEM-domain proteins, named for a shared, conserved domain found in lamina-associated polypeptide 2 (LAP2), Emerin, and MAN1. LAP2 and Emerin are anchored to the INM with one transmembrane domain, with the N-terminus facing the nucleoplasm. MAN1 contains two transmembrane domains and as such, both the N- as well as the C-terminus face the nucleoplasm. The LEM-domain, a typical feature in higher eukaryotes, binds the chromatin-associated protein BAF. In *Dictyostelium* the only HeH-family protein, the MAN1-like Src1, has been thoroughly characterized. Src1, as revealed by light and electron microscopy, is an integral INM protein that interacts with the lamin NE81 in BioID and mis-localization assays (Batsios et al., 2016b). Like several other HeH-family members in unicellular eukaryotes, Src1 contains no LEM-domain, probably due to the lack of its binding partner BAF (barrier to autointegration factor), which links the chromatin to the nuclear lamina (Shumaker et al., 2001). In yeasts, and probably also in *Dictyostelium*, chromatin interactions appear to be mediated directly by the HeH-domain (Brachner and Foisner, 2011). In *Dictyostelium*, Src1 was not evenly distributed around the nuclear envelope, but concentrated at sites of nucleolar attachment (Fig. 3) suggesting a nucleolar function.

#### Nucleolar proteins

With a focus on vertebrate cells, a review about the nuclear envelope would typically not include a discussion of the nucleolus. However, in many non-metazoans (e.g. *Dictyostelium*, *Saccharomyces*, *Aspergillus*, *Daphnia*, and *Neurospora*) the nucleolus is distinctly associated with the inner nuclear envelope. The signifi-

**Fig. 3. Localization of NE81 and Src1 relative to the position of nucleoli.** While the lamin NE81 is evenly distributed at the nuclear envelope, Src1 is concentrated adjacent to nucleoli. Fluorescence deconvolution microscopy of wildtype cells stained with

(A) anti-NE81/anti-rabbit-AlexaFluor 488 (green) and (B) anti-Src1/anti-rat-AlexaFluor 488 (green) and DAPI (blue). Inverted phase contrast highlighting the dark nucleoli is shown in red. Single image slices are shown. Scale bars 5  $\mu$ m.



cance of this linkage remains largely unknown. The *Dictyostelium* nucleolus consists of 2-4 dense patches that are tightly adhered to the inner nuclear envelope (Fig. 3). These patches are more or less homogeneous structures lacking the characteristic fibrillary center, dense fibrillary component, and granular component typical of higher eukaryotic nucleoli (Catalano and O'Day, 2013). The Nucleolar Proteome Database (Ahmad *et al.*, 2009) predicts more than 4500 nucleolar proteins in the human nucleolus. By contrast, there have been relatively few proteins identified in the *Dictyostelium* nucleolus, and most of these provide no insight into the relationship between the nucleolus and the INM. Interestingly, these do not all show uniform localization throughout the nucleolus, suggesting the presence of subnucleolar organization including fibrous matrices with ribosome-like granules (O'Day, 2019). The few *Dictyostelium* proteins of potential relevance (based on subnucleolar localization) to our discussion here include FhkA, Hsp32, and Src1 (Batsios *et al.*, 2016b; Catalano and O'Day, 2013).

FhkA (CHK2 in humans) does show some enrichment at the nucleolar periphery at the nuclear envelope, but the significance of this localization is not yet known. Interestingly, this is also the region of the nucleolus where rDNA resides. In *Dictyostelium*, rDNA is found on 88 kb extrachromosomal palindromes (100 copies) arranged in beaded ring-like structures around the periphery of each nucleolus (Simon and Olins, 1994). Hsp32 also has a similar localization pattern as that of FhkA, the significance of which also remains elusive. This localization is upregulated during stress (Moerman and Klein, 1998) and it therefore becomes interesting to speculate that the localization of both FhkA and Hsp32 is more related to cellular stress response and less to association of nucleolar patches with the nuclear envelope. Src1 may play a more significant role in the association of nucleolar patches with the nuclear envelope as it is concentrated there at the contact sites of nucleoli (Fig. 3B) (Batsios *et al.*, 2016b).

Expression of truncated GFP-Src1 versions, in which the C-terminal part including both transmembrane domains are deleted, result in a uniformly nucleolar distribution (Laroche lab, unpublished data). Src1 contains seven predicted nucleolar localization signals (NoLS) in the N-terminal half of the protein. Interestingly, in a BioID search for additional Src1 interacting proteins Hsp32 was identified (in addition to NE81), thus providing a plausible linkage between the INM and rDNA of the nucleolus (Ren, Batsios and Laroche lab, unpublished data). HeH1, the budding yeast homolog of Src1, is required to sequester rDNA in the nucleolar periphery. HeH1 deletion results in an amorphous distribution of rDNA and is correlated with increased repeat instability, implicating HeH1

in nucleolar organization in budding yeast (Mekhaill *et al.*, 2008). Perhaps Src1 plays a similar role in *Dictyostelium*, tethering the nucleolar patches to the INM by creating a linkage to rDNA within the nucleoli and embedded in the INM, anchored through its transmembrane domains and its interaction with NE81. It will be exciting to elucidate whether Src1 uses the nucleoli as a guide to insert into those regions of the INM or whether Src1 serves as an anchor tethering the nucleolar patches to the INM.

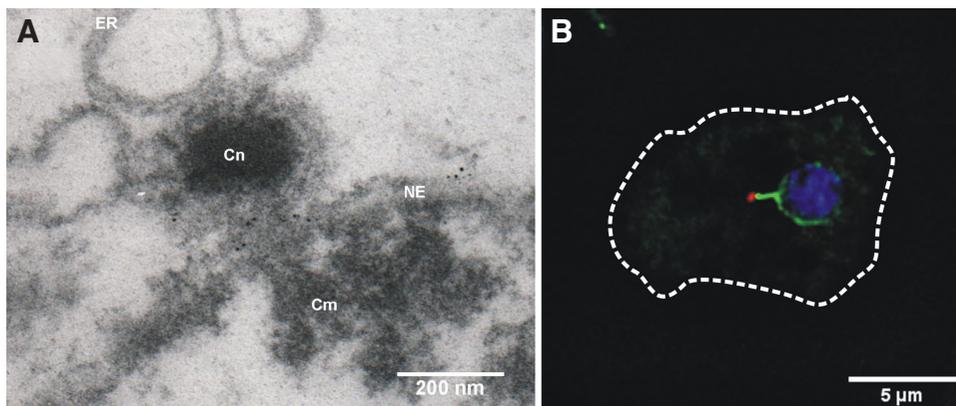
#### Ima1

A further protein associated with the inner nuclear envelope that merits discussion is Ima1 (Integral Membrane Protein 1; DDB\_G0292450). Ima1 belongs to a family, whose first member, the mammalian orthologue Net1, was identified in a large scale proteomic analysis of nuclear envelope proteins in rat liver cells (Schirmer *et al.*, 2003). Since then, numerous orthologs in many organisms including humans (Samp1), *C. elegans* (Samp1), and the fission yeast *Schizosaccharomyces pombe* (Ima1) have been discovered. Interestingly, no apparent ortholog could be identified in *Saccharomyces cerevisiae* so far (King *et al.*, 2008). Although sequence homology among the family members varies significantly, most share a similar domain architecture which includes an amino terminal hydrophobic stretch followed by an Ima1 domain characterized by four CXXC motifs, thought to participate in the formation of two zinc fingers. These motifs are typically followed by four membrane-spanning domains, which anchor the protein in the INM. Ima1 is still poorly characterized in *Dictyostelium*, however, a number of functions have been attributed to its orthologs in other organisms. The situation is complicated, since there is likely functional redundancy among the proteins that are found in the nuclear envelope. For example, in fission yeast Ima1, Lek2, and Man1 may play partially redundant roles in the control of mitotic cell growth and nuclear membrane morphology, since loss of anyone appears to have little or no effect. Strong phenotypes are observed only when all three are deleted (Hiraoka *et al.*, 2011). Consequently, the idea that these proteins function in nuclear organization much like links in a chain is an imperfect analogy, at best, because all links in a chain may have a similar function.

In fission yeast, Ima1 has been described to form a macromolecular linkage to the centrosome over the LINC complex via direct binding to the Sun-ortholog Sad1 and to be responsible for centromere clustering at the nuclear envelope (King *et al.*, 2008). This centromere cluster is positioned close to the INM adjacent to the spindle pole body, similar to *Dictyostelium* centromeres, which also cluster in the pericentrosomal region of the nuclear matrix

**Fig. 4. Centrosome-Nucleus-Centromere cluster.**

**(A)** Immunoelectron microscopy image showing one section of an isolated nucleus with the attached centrosome. Nuclei were labeled with an antibody against *Dictyostelium* Sun1 and nanogold conjugated anti-rabbit antibodies. The centrosome (Cn), the centromeric cluster (Cm), the nuclear envelope (NE) and the endoplasmic reticulum (ER) are indicated (image by Prof. Otto Baumann). **(B)** Immunofluorescence microscopy image of a Sun1-GFP knock-in cell (green) stained with an antibody against the centrosomal core protein CP91 and anti-rabbit-AlexaFluor 568 conjugates (red) and DAPI (blue). The cell edges are outlined by a dashed line.



(Fig. 4A) (Eichinger *et al.*, 2005). Knockout of *Ima1* in fission yeast (*ima1Δ*) disrupts nuclear envelope organisation in the centromere/spindle pole body attachment region including extension of the nuclear envelope towards the spindle pole body (King *et al.*, 2008). Furthermore, knockdown in human cells results in detachment of the centrosome from the nuclear envelope (Buch *et al.*, 2009). Although a direct connection between *Ima1* and the LINC component Sun1 in *Dictyostelium* has not been established, the similarity of the fission yeast *ima1Δ* phenotypes to those observed both for depletion or dominant-negative expression of Sun1 (Schulz *et al.*, 2009a; Xiong *et al.*, 2008) and knockout of CenB (Mana-Capelli *et al.*, 2009) in *Dictyostelium* are suggestive of a role for *Ima1* in maintaining the centrosome-nucleus connection. Perhaps related to this is the role *Ima1* plays in transferring force from the cytoplasm to the nucleoskeleton during nuclear migration in *C. elegans* (Bone *et al.*, 2014). Some of this force may be buffered through interactions of *Ima1* and its homologs with chromatin. Although it was known that *Ima1* is connected to centromeric heterochromatin (King *et al.*, 2008), Steglich *et al.* (2012) found *Ima1* to link two types of chromatin to the nuclear periphery in fission yeast. More recently, using fluorescence ratiometric imaging of chromatin (FRIC), Bergqvist *et al.* (2019) observed reduced heterochromatin in the nuclear envelope periphery when Samp1 was depleted in human cells. Conversely, the opposite was observed when Samp1 was overexpressed. Interactions of *Ima1* with chromatin could help buffer microtubule forces during closed mitosis. Whether *Ima1* in *Dictyostelium* has similar functions waits to be elucidated.

**Putative lamin B receptor orthologues**

Another common member of the INM is the lamin B receptor (LBR). The LBR is related to ergosterol biosynthesis proteins. Two members, Erg4/Erg24-like, encoded by DDB\_G0284407 and DDB\_G0267448 (*Dictybase* gene IDs) respectively, have been identified in the *Dictyostelium* genome and both are predicted to have eight transmembrane domains similar to LBR. Yet, they lack the N-terminal lamin B binding site and the Tudor-domain important to bind methylated histones at the nucleoplasmic site. Upon expression of DDB\_G0267448 Erg24-like in *Dictyostelium* cells the protein localizes at the ER and is absent from the nuclear envelope. An N-terminal GFP fusion of DDB\_G0284407 (Erg24) localizes to both the nuclear envelope and the ER. At the nuclear envelope GFP-Erg24 behaves differently from the immobile lamin NE81 in FRAP experiments. In contrast to NE81, GFP-Erg24 is

highly mobile, which makes an interaction with the nuclear lamin NE81 unlikely. In addition, immunogold transmission EM with GFP-Erg24 localizes this protein only at the ONM, which disqualifies this protein as an LBR homologue. Taken together, both Erg4/Erg24-family proteins are more likely to be involved in ergosterol biosynthesis than in nuclear envelope functions and the existence of a *bona fide* LBR orthologue in *Dictyostelium* is rather unlikely (Batsios, Gräf *et al.*, unpublished results).

**LINC complex**

One function for the nuclear envelope lies in coupling mechanical signals from the cytoplasm to the nucleus. In higher organisms the nuclear envelope is connected to cytoskeletal elements in the cytoplasm. This coupling is important for maintaining mechanical stability, cellular organization during movement, influencing gene expression, and arrangement of chromatin (Herrmann *et al.*, 2007). LINC complexes typically involve a diverse group of KASH-domain containing proteins that on one end, bind into the nucleoskeleton, and on the other end to cytoskeletal components such as actin filaments or microtubules. SUN-domain proteins are trimeric transmembrane proteins of the INM (Jahed *et al.*, 2018) which project through the inner nuclear envelope to interact with nuclear lamina or other nuclear components (Razafsky and Hodzic, 2009; Starr and Fridolfsson, 2010) and bind on short tail domains to KASH-domain proteins in the perinuclear lumen, that in turn connect the nuclear envelope to cytoskeletal elements. Defects in these linkages are impactful in multiple human diseases, such as muscular dystrophies, neurological defects (ataxia) or skin, and premature-aging disorders (Starr and Fridolfsson, 2010).

The *Dictyostelium* genome contains two SUN-domain proteins, Sun1, as described below (Schulz *et al.*, 2009a; Xiong *et al.*, 2008) and SunB which participates in a transcriptional signaling pathway with a key role during development (Shimada *et al.*, 2011); however, *bona fide* KASH-domain proteins could not be identified (discussed below). Endogenous Sun1 was shown to localize at the pericentrosomal region of the nuclear envelope and the centrosome itself during interphase (Fig. 4B) and mitosis (Schulz *et al.*, 2009a). By immunogold electron microscopy it was shown to reside on both the inner, as well as the outer, nuclear membrane. Sun1 dysfunction interferes with the nucleus-centrosome connection and partially causes aberrant centrosome numbers, similar to the described NE81 knockout phenotypes. This is in agreement with the interaction of both proteins in BioID experiments (see above).

FRAP analysis revealed two populations of Sun1: (1) the immobile fraction associated with the centrosome required for the maintenance of a stable coupling of the centrosome to the nucleus, and (2) a mobile fraction in the nuclear envelope (Schulz *et al.*, 2009a).

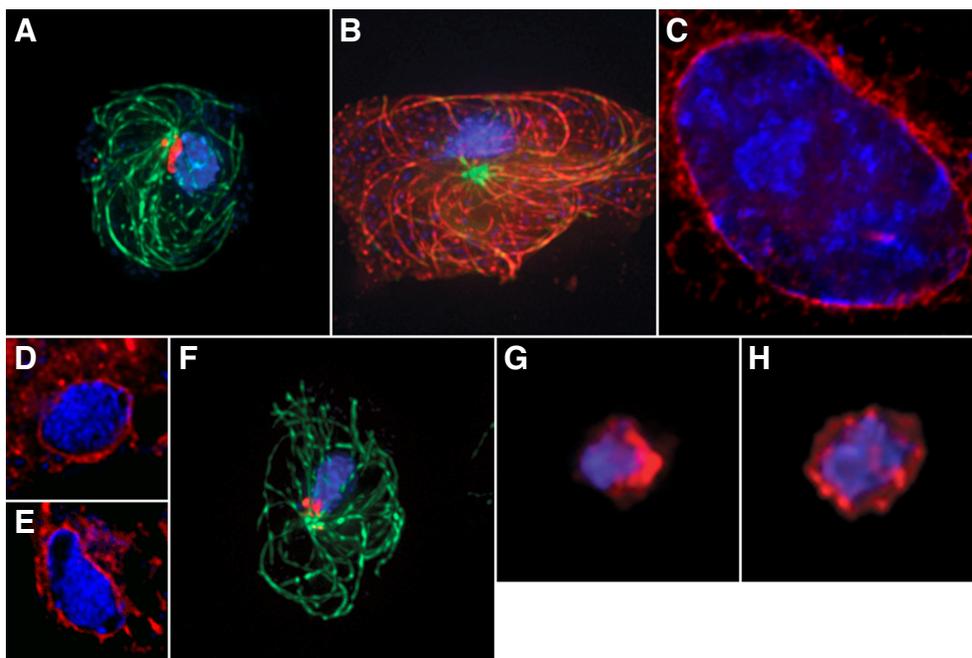
Although the *Dictyostelium* genome lacks readily identifiable KASH-domain containing proteins, two KASH-type activities have been reported at the ONM of this organism. Interaptin is an  $\alpha$ -actinin superfamily member with a carboxy-terminal transmembrane domain that shows weak homology to KASH proteins (Rivero *et al.*, 1998; Xiong *et al.*, 2008). Though it does not appear to directly bind Sun1, it does localize to the outer nuclear envelope and may mediate a nuclear linkage with actin filaments. The only other KASH-like activity so far reported in *Dictyostelium* is the kinesin Kif9 (Fig. 5) that provides a nucleus linkage to the centrosome (Tikhonenko *et al.*, 2013). This linkage is conspicuous and impactful in multiple respects.

### The centrosome/nucleus connection

One form of contact between the centrosome and nucleus likely occurs through a series of thin fibrils that are visible by electron microscopy (Omura and Fukui, 1985). The molecular nature of these fibrils is currently unknown, but they provide an adhesive-like activity to maintain close association of the interphase centrosome to the persisting nuclear envelope. This arrangement becomes abrogated at the onset of mitosis, when the centrosome inserts into the nuclear envelope, duplicates and organizes an intranuclear bipolar spindle to segregate chromosomes (Ueda *et al.*, 1999). Here, the fibrils may guide the insertion of the duplicating centrosome into the nuclear envelope. Although the molecular composition of these fibrils is unknown, a couple of proteins have been proven to play a role in the centrosome-nucleus linkage. *Dictyostelium* appears to have adapted a kinesin motor (Kif9) that is anchored

in the nuclear envelope and engages microtubules to draw the centrosome close to the nucleus (Tikhonenko *et al.*, 2013).

Kif9 is an internal motor kinesin (Kin-I) that does not readily group with the common kinesin family members. Similar to other Kin-Is (e.g. MCAK, Kinesin-13 family) Kif9 contains an amino terminal SxIP domain thought to regulate molecular interaction with microtubule tip binding proteins (Tikhonenko *et al.*, 2013). Unique to Kif9 is a carboxy-terminal ~23 residue transmembrane domain that targets the motor to membranes, and specifically the nuclear envelope. GFP tagging illustrates that Kif9 distribution is tightly restricted on the nuclear envelope, accumulating in a region underlying the centrosome (Fig. 5A). This distribution is preserved even in isolated nuclei. Removal of the carboxy-terminal 43 residues, which includes the transmembrane domain, results in redistribution of the motor throughout the cytoplasm (Fig. 5B). Expression of the carboxy-terminal domain alone targets GFP to the nuclear envelope, not only in *Dictyostelium* but also in mammalian cells (Fig. 5C). Interesting though, the distribution of the carboxy-terminal fragment is different in wild type and kif9 null backgrounds. In kif9 null cells (and in HeLa cells which lack a comparable kinesin isoform), the GFP tag is distributed evenly around the nuclear envelope (Fig. 5 D,E). However, in the presence of the wild type Kif9, the short tail domain is focused into the narrow region close to the centrosome (Fig. 5F). This is important for it implies that the tail domain alone interacts either directly with the full length Kif9 (unlikely) or with a nuclear envelope network component that is influenced by Kif9 activity. One such candidate component is the Sun1 protein. It has been hypothesized that Kif9 and Sun1 form the LINC complex in *Dictyostelium* (Tikhonenko *et al.*, 2013). The motor clearly binds microtubules and is anchored into the nuclear envelope. Kif9 and Sun1 show related asymmetric nuclear distributions and Kif9 removal results in redistribution of Sun1 across the nuclei (Fig. 5 G,H). In addition, dominant-negative expression of



**Fig. 5. Kif9 localizations.** (A) Full length GFP-Kif9 expression (in red) in *Dictyostelium* cells. Note the restricted distribution in the nuclear envelope region underlying the centrosome. The distribution is the same whether expressed in wild-type (shown) or null cells (not shown). Microtubules are in green, nuclei in blue. (B) GFP-Kif9 $\Delta$ 43 expression in *Dictyostelium* cells. In the absence of the carboxy-terminal 43 residue tail, the Kif9 motor shifts distribution into the cytoplasm and onto microtubules. (C) GFP-43aa carboxy-terminal tail expression in a HeLa cell. Note that the tail domain including the membrane anchor alone is sufficient to target GFP into the nuclear envelope, but it requires the presence of wild-type Kif9 to restrict its distribution. (D) and (E) Two examples of GFP-43 aa carboxy-terminal tail expression (red) in *Dictyostelium* kif9 null cells. (F) GFP-43aa carboxy-terminal tail expression (red) in a *Dictyostelium* control strain. (G). Sun1 (red) distribution on nuclei isolated from a *Dictyostelium* control strain. (H) Sun1 (red) distribution on nuclei isolated from *Dictyostelium* kif9 null cells. In the absence of Kif9, Sun1 becomes more evenly distributed on the nucleus. Images (G, H) adapted with permission from (Tikhonenko *et al.*, 2013). In all subfigures, blue color indicates DAPI staining and green color shows microtubules.

tail expression (red) in a *Dictyostelium* control strain. (G). Sun1 (red) distribution on nuclei isolated from a *Dictyostelium* control strain. (H) Sun1 (red) distribution on nuclei isolated from *Dictyostelium* kif9 null cells. In the absence of Kif9, Sun1 becomes more evenly distributed on the nucleus. Images (G, H) adapted with permission from (Tikhonenko *et al.*, 2013). In all subfigures, blue color indicates DAPI staining and green color shows microtubules.

Sun1 constructs disconnect centrosomes from nuclei and lead to enhanced aneuploidy (Schulz *et al.*, 2009a; Xiong *et al.*, 2008). Tikhonenko and co-workers reasoned that Kif9's motor domain engages microtubules and perhaps acting as a depolymerase, effects a directed pulling force on the centrosome.

This not only draws the centrosome toward the nuclear envelope, but would also move the kinesin laterally through the nuclear envelope to accumulate in the region underlying the centrosome. If the centrosome breaks free of its fibrous tethers, this Kif9-based mechanism could reel the centrosome back to a proximal nuclear location. This is in line with the observation that deletion of Kif9 results in centrosome displacement during interphase and leads to multiple mitotic perturbations that significantly impact cell growth (Tikhonenko *et al.*, 2013). In *C. elegans* dynein has a similar function for centrosome-nucleus linkage, as is predicted for Kif9. It is possible that in *Dictyostelium* both Kif9 and dynein (with its activator LIS1) pull the centrosome close to the nucleus. Another scenario could be that, similar to MCAK and dynein at kinetochores in mitosis (Maiato *et al.*, 2004), dynein pulls the microtubules close to the centrosome and Kif9 depolymerizes microtubules at the centrosome due to an inherent microtubule minus end depolymerisation activity. Such a role of dynein is supported by the observation that perturbation of the dynein regulator LIS1 does effect centrosome attachment in *Dictyostelium* (Rehberg *et al.*, 2005), albeit a dynein-mediated mechanism does not appear sufficient to compensate for the loss of Kif9.

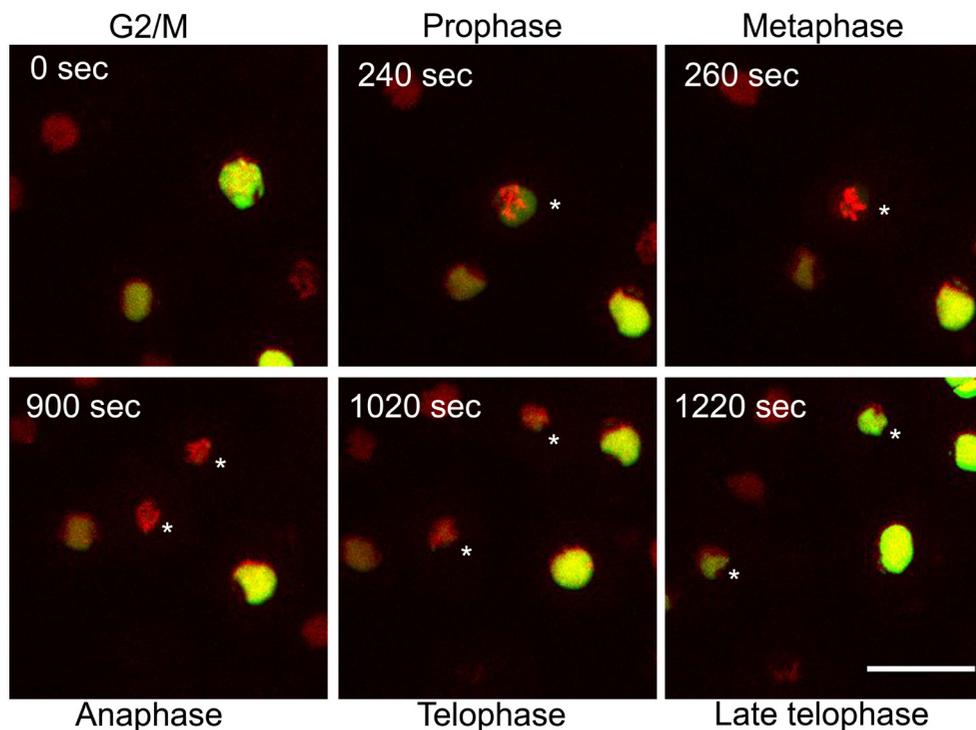
It is still unclear, if Kif9 connects directly to Sun1 or if it participates in a network that includes Sun1. The arrangement of Sun1 in the nuclear envelope is complex and multifaceted. Sun proteins are known to trimerize and/or assemble into higher order oligomers (Hennen *et al.*, 2018), as well as interact with other components (e.g. DdNKPAP, Burgute *et al.*, 2016, or NE81 Batsios *et al.*, 2016a). Connectivity to Sun1 or an underlying nuclear network may serve a couple of important functions. First, it would provide individual kinesin motors with a firmer and collective foothold to exert force on cytoplasmic microtubules than would be afforded by a single transmembrane domain alone. Second, Sun1 also appears to participate in the clustering of centromeres (Fig. 4A) (Eichinger *et al.*, 2005; Schulz *et al.*, 2009a), possibly in interaction with Ima1 (see above). In the absence of Kif9, their cluster position is random, without a bias toward the centrosome (Tikhonenko *et al.*, 2013). Positioning the centromeres close to the centrosome position would facilitate the connectivity of mitotic microtubules to kinetochores in the earliest stages of centrosome duplication and spindle assembly, and thus potentially enhance mitotic fidelity.

Multiple other components participate in the *Dictyostelium* centrosome–nuclear linkage. Some are integral to

the organization of the electron-dense corona that nucleates and anchors microtubules (e.g. DdCP250, CP148, CP55; Blau-Wasser *et al.*, 2009; Kuhnert *et al.*, 2012b; Kuhnert *et al.*, 2012a), others participate in multiple contexts to affect the linkage (e.g. LIS1, DdCenB; Mana-Capelli *et al.*, 2009; Rehberg *et al.*, 2005). As mentioned above, functional characterization of Ima1 in *Dictyostelium* is a key to elucidate the mechanism that connects the centrosome to the centromere cluster in *Dictyostelium*.

### The dynamics of the nuclear envelope during mitosis

The centrosome inserts into the nuclear envelope at the G2/M transition, duplicates and forms the two spindle poles. Failure of this insertion process leads to mitotic delays and supernumerary centrosome formation (Leo *et al.*, 2012; Meyer *et al.*, 2017). Centrosome insertion certainly requires close proximity to the nuclear envelope, but also deformation of the nuclear envelope in order to form a fenestra which ultimately harbours the duplicating centrosome. The mechanism how this fenestra forms is unknown. However, a recent study has revealed that the centrosomal protein CP75 is necessary for permeabilization of the nuclear envelope at the G2/M transition (Meyer *et al.*, 2017). Transmission EM observations further suggest that the nuclear envelope is a contiguous structure during nuclear division, without further fenestrations until late telophase, when the nuclear envelope bridge between the two separating daughter nuclei ruptures resulting in two distinct fenestrae, one at each daughter nucleus, that are penetrated by the central spindle (McIntosh *et al.*, 1985). Despite the general preservation of nuclear envelope integrity during this semi-closed mitosis, the nuclear envelope becomes permeable to larger pro-



**Fig. 6. GFP-Cenp68 diffuses into the cytosol upon permeabilization of the nucleus at the onset of mitosis.** Live cell imaging of cells expressing GFP-Cenp68 (green) and RFP-histone2B (red). Selected time points are shown. Mitotic nuclei are marked by asterisks. Scale bar 10  $\mu$ m.

TABLE 1

OVERVIEW OF *Dictyostelium* NUCLEAR ENVELOPE PROTEINS

Name	Gene ID	Localization	Information	Refs.
NE81	DDB_G0289429	Lamina	Lamin B type	1, 2
Nup43	DDB_G0277955	NPC	component of the nuclear pore complex	3
Nup62	DDB_G0274587	NPC	component of the nuclear pore complex	4
Sec13L/Seh1	DDB_G0277257	NPC	component of the nuclear pore complex	4
GLE2/Rae1	DDB_G0283835	NPC	component of the nuclear pore complex, mRNA export	4
FhkA	DDB_G0293656	Nucleolus	Putative protein kinase	5
Hsp32/HspC	DDB_G0272819	Nucleolus	Heat shock protein	5
Src1	DDB_G0293138	INM	LEM-domain protein	6
Sun1	DDB_G0272869	INM, ONM	LINC complex, centrosome-nucleus connection, centromere-centrosome connection	3, 7
Ima1	DDB_G0292450	unknown	centromere-centrosome connection	*
Cenp68	DDB_G0293620	Centromeres	Localizes to the clustered centromeres at the INM	8, 9
Kif9	DDB_G0274603	ONM	KASH type activity, centrosome-nucleus connection	10
Lis1	DDB_G0288375	ONM	Dynein-associated, microtubule-nucleus connection	11
Dynein heavy chain	DDB_G0276355	ONM	Dynein-associated, microtubule-nucleus connection	11
Interaptin/AbpD	DDB_G0287291	ONM	KASH type activity	3
CP75	DDB_G0283111	Centrosome	Centrosomal core, Mitotic centrosomal membrane insertion	12
TMEM33	DDB_G0286009	ONM, ER	Transmembrane protein 33	#
RTNLC	DDB_G0293088	ER	Membrane shaping	#
Nurim	DDB_G0288111	INM, ONM, ER	Transmembrane protein	#
Erg24	DDB_G0284407	ONM, ER	Ergosterol biosynthesis, no LBR activity	#
Erg4/Erg24	DDB_G0267448	ER	Ergosterol biosynthesis, no LBR activity	#

All molecular players at the nuclear envelope characterized so far are listed, also still unpublished ones, which are not further described in the text. Reference numbers refer to the bibliography except: <sup>1</sup>Batsios *et al.*, 2012; <sup>2</sup>Krüger *et al.*, 2012; <sup>3</sup>Xiong *et al.*, 2008; <sup>4</sup>Beck and Medalia, 2008; <sup>5</sup>Catalano and O'Day, 2015; <sup>6</sup>Batsios *et al.* 2016b; <sup>7</sup>Schulz *et al.* 2009a; <sup>8</sup>Kuhnert *et al.*, 2012b; <sup>9</sup>Samereier *et al.*, 2011; <sup>10</sup>Tikhonenko *et al.*, 2013; <sup>11</sup>Rehberg *et al.*, 2005; <sup>12</sup>Meyer *et al.*, 2017; \*unpublished Larochelle *et al.*; #unpublished Batsios, Gräf *et al.*

teins upon centrosome insertion. This is evident from studies with an NLS-tagged GFP. This label is restricted to the nucleoplasm during interphase but enters the cytoplasm at the onset of mitosis (Zang *et al.*, 1997). More recently, the soluble centromere protein GFP-Cenp68 (Samereier *et al.*, 2011) was also proven as a useful reporter for nuclear permeabilization. The size of the fusion protein (approx. 100 kDa) clearly demands Ran-dependent import. Upon high expression, excess GFP-Cenp68 that cannot bind to centromeres concentrates in the nuclear matrix and remains diffusible without any further mutant phenotype.

Fig. 6 shows that the nucleoplasmic GFP-Cenp68 rapidly diffuses out of the nucleus at mitotic onset. In late telophase high permeability ceases and GFP-Cenp68 becomes concentrated in the nucleoplasm again, indicating closure of the fenestrae at the centrosomes and the karyokinetic abscission sites. The simplest explanation for the fast redistribution of the protein from the nucleus into the cytoplasm in prophase is that it occurs through the above-mentioned fenestrae. An alternative mechanism has been shown in *Aspergillus nidulans* (De Souza *et al.*, 2004), where the NPCs partially disassemble at mitotic onset to allow proteins without an NLS, e.g. the tubulin, to diffuse through the pores into the nucleus independent of the Ran-driven transport. Current research indicates a similar mechanism in *Dictyostelium* that may complement fenestra-based permeabilization (Meyer *et al.*, unpublished results).

### **Dictyostelium nuclear envelope dynamics during development**

*Dictyostelium* development from an amoeboid state to a multicellular fruiting body offers an escape mechanism to ensure survival in harsh environmental conditions. It includes cell differentiation into stalk cells and durable spore cells, which also results in several morphogenetic changes of the nuclei. These changes are a con-

sequence of environmental changes and of adjustments in gene activity. Nuclei of aggregating cells are no longer spherical but more elongated. A further difference to the vegetative state is that the nucleoli fuse into one single patch upon aggregation (Sameshima, 1985). The single nucleolus locates mainly opposite to the centrosome and nuclear protrusions so-called “nuclear nozzles” appear at sites associated with nucleoli (Sameshima, 1985; Sameshima *et al.*, 1991). Interestingly, “nuclear nozzles” at the nucleoli can be induced also in vegetative cells by overexpression of Src1 (Batsios *et al.*, 2016b). As Src1 has a naturally increased expression level at the aggregation stage, the idea that nozzle formation during development is solely triggered by the increased expression of Src1 is not far-fetched. Yet, this idea remains to be proven in further experiments.

### **Outlook**

The nuclear envelope is not uniform, but it features several sub-regions, i.e. the ONM, the INM, the lamina, nucleolus-associated regions and the pericentrosomal region that are each characterized by a typical set of proteins (Fig. 1). Table 1 lists all known molecular players at the nuclear envelope of *Dictyostelium* and their localization. This list is certainly not complete and will grow with further studies of the interactome of the known players by BioID analyses. It will also be interesting to investigate how nuclear envelope-associated proteins cooperate in order to orchestrate the intriguing dynamic processes that happen during semi-closed mitosis. For example, despite the abovementioned involvement of the centrosomal core protein CP75 (see above) little is known regarding the process of fenestra formation during centrosome insertion into the nuclear envelope and, later in telophase, centrosome extrusion back into the cytoplasm. During this extrusion process the fenestrae of the nuclear envelope that embraced the mitotic centrosomes have

to be closed, together with the fenestrae at the abscission site, where the central microtubule spindle penetrates the separating daughter nuclei. According to the situation in animal cells during nuclear envelope re-formation (Sundquist and Ullman, 2015), the ESCRT machinery could be involved in this processes in *Dictyostelium* as well. The *Dictyostelium* genome contains homologs of the required ESCRT proteins, and future studies will disclose whether these membrane-shaping and repair processes are orchestrated through similar mechanisms as in mammalian cells. Future studies will also reveal how far the nuclear lamina is involved in chromatin organization, especially with regard to heterochromatin formation within so-called LADs (lamina-associated domains) (Van Bortle and Corces, 2013). Lamina dependent heterochromatin formation could also be deeply involved in cell differentiation as it has also been shown for developmental processes in animal cells such as retina development (Van Bortle and Corces, 2013).

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