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COMMENTARY

LINCing the eukaryotic tree of life – towards a broad evolutionary comparison of nucleocytoplasmic bridging complexes

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ABSTRACT

The nuclear envelope is much more than a simple barrier between nucleoplasm and cytoplasm. Nuclear envelope bridging complexes are protein complexes spanning both the inner and outer nuclear envelope membranes, thus directly connecting the cytoplasm with the nucleoplasm. In metazoans, they are involved in connecting the cytoskeleton with the nucleoskeleton, and act as anchoring platforms at the nuclear envelope for the positioning and moving of both nuclei and chromosomes. Recently, nucleocytoplasmic bridging complexes have also been identified in more evolutionarily diverse organisms, including land plants. Here, I discuss similarities and differences among and between eukaryotic supergroups, specifically of the proteins forming the cytoplasmic surface of these complexes. I am proposing a structure and function for a hypothetical ancestral nucleocytoplasmic bridging complex in the last eukaryotic common ancestor, with the goal to stimulate research in more diverse emerging model organisms.

KEY WORDS: KASH protein, SUN protein, Opisthokonta, Archeaplastida, Evolution, Coiled-coil domain, LINC complex

Introduction

The nucleus of eukaryotic cells is enveloped by two layers of membrane, the inner nuclear membrane (INM) and the outer nuclear membrane (ONM), together called the nuclear envelope. Although the perforation of the nuclear envelope by the nuclear pore complexes (NPC) has long been known, only fairly recently, another direct connection between the cytoplasm and the nucleoplasm has been discovered, the linker of nucleoskeleton and cytoskeleton (LINC) complexes, also referred to as nucleoplasmic bridging complexes (for a review, see Rothballer and Kutay, 2013).

The core components of LINC complexes are the INM SUN (Sad1, UNC84 homology) domain proteins and ONM KASH (Klarsicht, ANC-1, Syne homology) domain proteins, hereafter called SUN and KASH proteins. Both types of proteins contain transmembrane domains (TMDs), and the actual SUN and KASH domains interact with each other in the space between the ONM and INM, the nuclear envelope lumen or perinuclear space (PNS) of the nucleus. The KASH domain is a stretch of 50 to 60 amino acids at the very C-terminus of KASH proteins, consisting of a single TMD and the 'KASH tail', a sequence of variable amino acids that resides in the PNS, and typically ends with the highly conserved motif PPPx, frequently PPPT (reviewed in Starr and Fridolfsson, 2010).

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The structure of a human SUN–KASH complex has been resolved; it shows a SUN domain trimer – facilitated by an adjacent trimeric coiled-coil domain also located in the PNS – binding to three copies of the KASH tail (Sosa et al., 2012). The KASH tail is necessary for targeting KASH proteins to the ONM, and this depends on the presence of the SUN proteins (reviewed in Starr and Fridolfsson, 2010).

LINC complexes were first discovered by forward-genetic screens in Drosophila and *Caenorhabditis elegans* for mutants with defects in nuclear positioning and/or nuclear migration. These LINC complexes were shown to connect nuclei to elements of the cytoskeleton, with the cytoplasmic domains of KASH proteins binding to actin, a microtubule motor or intermediate filaments. On the nucleoplasmic side, SUN proteins bind to lamins, thus connecting the cytoskeleton to the nucleoskeleton (for recent reviews, see Tapley and Starr, 2013; Luxton and Starr, 2014; Kim et al., 2015; Bone and Starr, 2016).

The founding members of KASH proteins are of the nesprin class, so called after both their nuclear location and their similarity to spectrins (and also called Synes in several publications). Their cytoplasmic domains consist of a variable length of spectrin-repeat domains, with some KASH proteins being very large, such as the giant isoforms of human nesprin-1 (Syne-1) with over 8000 amino acids. LINC complex functions in nuclear movement and positioning include pronuclear migration in fertilized eggs, cell-cycle-dependent nuclear oscillations during neuronal differentiation, nuclear repositioning during fibroblast migration, nuclear positioning during *C. elegans* embryo development, nuclear migration during *Drosophila* eye disk development, as well as meiotic chromosome movement to facilitate recombination (reviewed in Burke and Roux, 2009; Razafsky and Hodzic, 2009; Starr and Fridolfsson, 2010; Bone and Starr, 2016). There is also growing evidence that LINC complexes are connected to human diseases, such as Emery–Dreifuss muscular dystrophy, cerebellar ataxia, arthrogyrposis and hereditary hearing loss, and that they are involved in mechanical signal transduction (Lombardi and Lammerding, 2010; Banerjee et al., 2014; Guilluy et al., 2014).

The current eukaryotic evolutionary tree places all organisms between yeast and man into the supergroup Opisthokonta (Adl et al., 2012; Field et al., 2012; Gräf et al., 2015). Almost all information available on LINC complexes is derived from research on model organisms within this supergroup, with the 'canonical' LINC complexes being those that connect the lamina to the three types of cytoskeletal filaments in the cytoplasm. However, even within this supergroup, LINC complexes with different structures and functions are becoming increasingly known (see below).

Recently, the first LINC complexes in the Archeaplastida, the supergroup containing the land plants, have been reported, and they are both similar and divergent from their opisthokont counterparts in important ways (Graumann et al., 2010; Zhou et al., 2012, 2015c; Tatout et al., 2014). At first glance, it appears that SUN proteins are

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well conserved across this evolutionary distance of ~1 billion years. In contrast, the plant SUN-binding ONM proteins have no sequence similarity with opisthokont KASH proteins. Nevertheless, they interact with plant SUN domains in the same way as described above – through binding of short tail domains to the conserved SUN domains in the PNS – and they have the same overall structure of an N-terminal cytoplasmic domain, a single C-terminal TMD, and a short tail, ending in a highly conserved, but different, short amino acid sequence. In other words, during evolution, plants and opisthokonts seem to have recruited a different set of proteins into becoming the ONM-binding partners of SUN proteins (Zhou et al., 2012, 2014, 2015c).

If this were indeed the case, it would beg for the interesting question as to whether SUN proteins are evolutionarily older than KASH proteins, and what their possible ancestral function in the absence of their KASH partners could have been. Here, I will rather argue a slightly different case, namely that ancestral 'SUN–KASH' complexes did exist, but that their closest extant relatives cannot be found among the metazoan nesprin types, but among another, more broadly conserved KASH protein structure. I will first review 'non-canonical' LINC complexes in unikonts (Adl et al., 2012), then discuss what can be learned from the characterization of the first bikont (Adl et al., 2012) LINC complexes – those in higher plants – and finally discuss what this comparison might tell us about the structure and function of ancestral SUN-interacting proteins.

Non-canonical LINC complexes and emerging LINC complex functions in unikonts

Although the prime focus of LINC complex studies has long been the cytoskeletal connection and the nuclear movement and positioning mechanisms (Luxton and Starr, 2014; Tapley and Starr, 2013; Bone and Starr, 2016), several additional roles for opisthokont LINC complexes have been identified, and several 'unusual' KASH proteins with more variable cytoplasmic domains and KASH tails have been added to the list. In the mouse, tethering of telomeres during meiosis requires SUN1 and the meiosis-specific KASH protein KASH5 (also known as CCDC155) (Horn et al., 2013). KASH5 is a dynein-binding outer nuclear envelope protein that contains a C-terminal TMD, an N-terminal EF-hand domain and a centrally located coiled-coil domain (Figs 1 and 2). Is has sequence similarity to zebrafish LRMP, a protein with similarity to KASH proteins that is involved in pro-nuclear fusion in the zygote. LRMP is located at the nuclear envelope but no SUN-binding partner has been identified, and it is not known whether LRMP is part of a zebrafish LINC complex. Similar to KASH5, zebrafish LRMP contains several coiled-coil domains instead of spectrin repeats (Figs 1 and 2) (Lindeman and Pelegri, 2012; Horn et al., 2013). A second unusual mammalian KASH protein is mammalian LRMP (also known as Jaw1), which is also related to zebrafish LRMP, if by similarity of different domains (Shindo et al., 2010; Horn et al., 2013). It was originally described as an endoplasmic reticulum (ER) protein in lymphoid tissue (Behrens et al., 1994, 1996) and also interacts with the type III inositol 1,4,5-triphosphate receptor in taste buds (Shindo et al., 2010). Like for zebrafish LRMP, no SUN interactor is currently known.

C. elegans LINC complexes were among the first identified in the context of nuclear positioning and movement (Wilhelmsen et al., 2006; Razafsky and Hodzic, 2009; Razafsky and Hodzic, 2015), but an unusual KASH protein was found here, too. ZYG-12 has a less-conserved KASH domain termed the KASH mini domain, and a coiled-coil region in the cytoplasmic portion of the protein (Figs 1 and 2). Mutations in ZYG-12 perturb the centrosome–nucleus attachment, ultimately leading to segregation defects and lethality. A ZYG-12 isoform that lacks its KASH domain (ZYG12a) also exists and the model is that the two proteins form a dimer, with ZYG12 binding to the nuclear envelope and ZYG12a to the centrosome (Malone et al., 2003) (Fig. 2). ZYG-12 interacts in the nuclear envelope lumen with SUN1 (also called matefin, MTF), requiring the ZYG-12 KASH mini domain but not the SUN domain, which suggests an unusual SUN–KASH interaction (Malone et al., 2003; Minn et al., 2009; McGee et al., 2009).

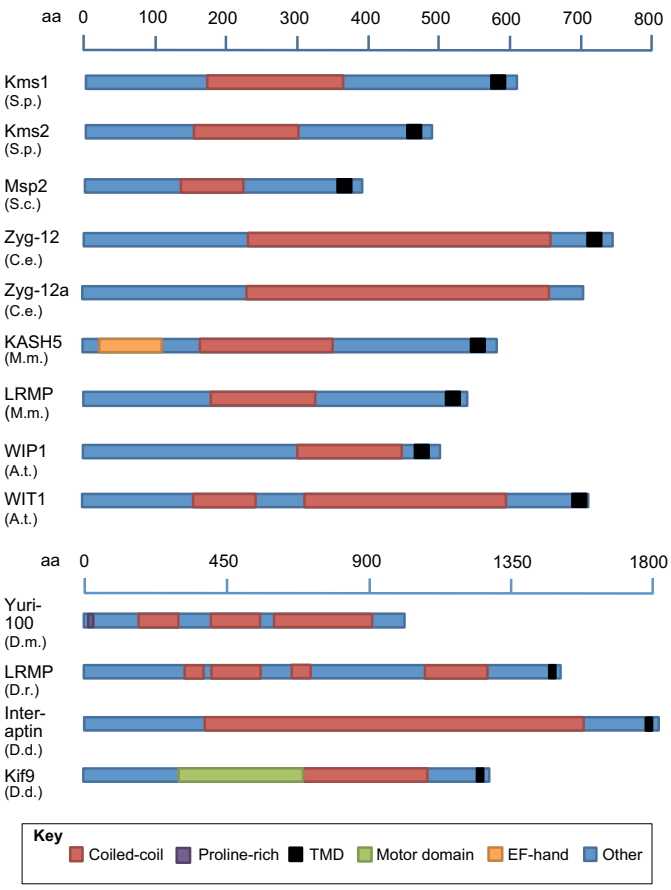

Fig. 1. Domain organization of coiled-coil KASH proteins and coiled-coil proteins involved in KASH-like functions. Proteins are drawn to two scales for better visibility of domain organization. Domain color code is explained in the key. Zyg-12a, Yuri-100, and WIT1 are not KASH proteins, but are included because they might fulfill similar functional roles or are part of a KASH complex. Species abbreviations from top to bottom are: S.p., *Schizosaccharomyces pombe*; S.c., *Saccharomyces cerevisiae*; C.e., *Caenorhabditis elegans*; M.s. *Mus musculus*; A.t., *Arabidopsis thaliana*; D.m., *Drosophila melanogaster*; D.n., *Danio rerio*; D.s., *Dictyostelium discoideum*. Yuri-100 is the longest isoform of Yuri Gagarin (Texada et al., 2008).

Besides the classical LINC complex proteins Klaroid (SUN) and Klarsicht and MSP-300 (KASH) (Luxton and Starr, 2014), *Drosophila* also has the SUN protein Spag4 (also called Giacomo) and the cytoplasmic long coiled-coil protein Yuri Gagarin (Figs 1 and 2). Yuri Gagarin has neither a TMD nor similarity to the KASH tail, but functionally interacts with Spag4 in spermatogenesis and nucleus–centrosome attachment through dynein and dynactin. Yuri Gagarin is specifically expressed in the male reproductive organ and co-localizes at the nuclear envelope with Spag4 and dynein and dynactin. In contrast, Klarsicht and MSP-300 are dispensable for fly spermatogenesis (Texada et al., 2008; Kracklauer et al., 2010).

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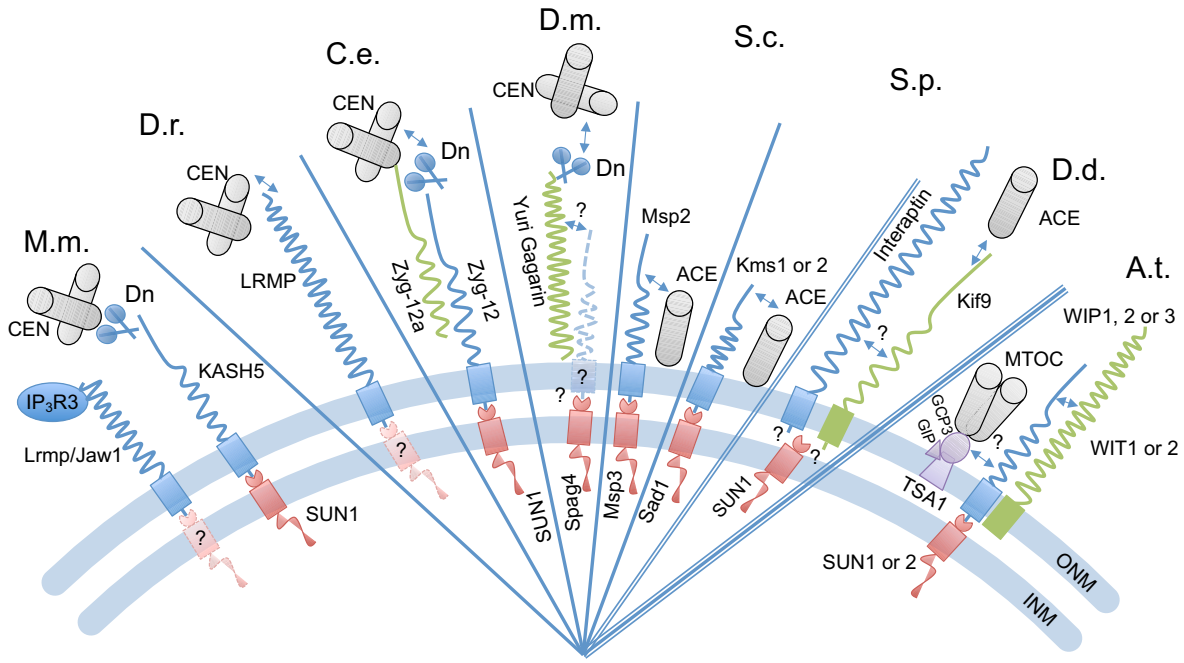

Fig. 2. Functional interaction between coiled-coil-type KASH and KASH-like proteins with MTOCs is broadly conserved in unikonts and possibly beyond. Coiled-coil KASH proteins and KASH-related proteins are depicted that either have a role in the association of MTOCs with the nuclear envelope, or that are good candidates for such a role based on similarity to other systems. MTOCs are represented by symbols for centriole-containing centrosomes (CEN), acentriolar centrosomes (ACE), and a generic, nuclear-envelope-associated MTOC in plants. Proteins with dotted outlines and question marks have not been identified, but are predicted here to exist. Green proteins are cytoplasmic coiled-coil proteins without a KASH domain. Double-pointed arrows show known interactions. Question marks indicate interactions that have not been shown, but would be interesting to investigate. Single radial lines separate opisthokont organisms, the double radial line separates the Opisthokonta from the Amoebozoa, and the triple radial line separates the Archaeplastida from the unikonts (see also Fig. 4). Species are ordered (from left to right) by distance from *Homo sapiens*: vertebrates, invertebrates, fungi, amoebae, and plants. Species name abbreviations are: M.s., *Mus musculus*; D.r., *Danio rerio*; C.e., *Caenorhabditis elegans*; D.m., *Drosophila melanogaster*; S.c., *Saccharomyces cerevisiae*; S.p., *Schizosaccharomyces pombe*; D.s., *Dictyostelium discoideum*; A.t., *Arabidopsis thaliana*. IP_3R, type III inositol 1,4,5-triphosphate receptor; Dn, dynein.

In budding yeast, the interaction between the SUN protein Msp3 and the KASH protein Msp2 leads to the recruitment of the yeast acentriolar centrosome, the spindle pole body (SPB), into the nuclear envelope (Friederichs et al., 2011). Msp2 is a 387-amino-acid protein with a C-terminal TMD and a coiled-coil domain of ∼100 amino acids in the cytoplasmic portion of the protein (Figs 1 and 2). In fission yeast, the SUN protein Sad1 and the KASH proteins Kms1 and Kms2 are involved in SPB insertion (King et al., 2008). In Kms2 mutants, mitotic entry is delayed, and the bipolar spindle is disrupted, in addition to aberrant SPB insertion phenotypes. Kms1 and Kms2 contain centrally located coiled-coil domains of ∼150 amino acids (Kim et al., 2015) (Figs 1 and 2).

Dictyostelium discoideum is of great interest in a broad comparison of LINC complexes, because it is a member of the sistergroup of the opisthokonts, the Amoebozoa (which together make up the unikonts) (Adl et al., 2012). Thus, it would be expected that *Dictyostelium* LINC complexes share similarities with ancestral opisthokont LINC complexes. *Dictyostelium* goes through a form of closed mitosis similar to *Aspergillus* (De Souza et al., 2004) and has acentriolar centrosomes like yeast (Gräf et al., 2015). Two SUN proteins and two potential candidates for KASH proteins are currently known. Sun-1 is associated with the INM and plays a role in centrosome–nucleus attachment (Xiong et al., 2008; Schulz et al., 2009). SunB is a mid-SUN protein, with the SUN domain located between two transmembrane domains (Shimada et al., 2010). There are two KASH protein candidates, Interaptin and Kif9. Interaptin is an ONM protein of the α-actinin superfamily (Rivero et al., 1998) with a long central coiled-coil domain and a C-terminal domain consisting of a TMD and a short tail ending in -PT, thus similar to the C-terminal PPPT motif of KASH proteins (Figs 1 and 2). The C-terminal domain consisting of the TMD and the KASH-like tail are sufficient for nuclear envelope association (Xiong et al., 2008; Rivero et al., 1998). Interaptin overexpression displaces Sun-1 from the nuclear envelope, which the authors interpret as an interaction that is somewhat different from that of the canonical SUN–KASH complexes.

Kif9 is an unusual kinesin that comprises a motor domain, a coiled-coil domain and a C-terminal TMD but no discernible KASH-like tail (Tikhonenko et al., 2013). Kif9 is associated with the nuclear envelope and accumulates in the vicinity of the centrosomes; this localization overlaps with that of Sun-1. Sun-1 depletion leads to a shift of Kif9 localization to a more random distribution on the nuclear envelope, suggesting a functional interaction.

LINC complexes in bikonts

Excavata

Trypanosoma brucei belongs to the supergroup Excavata, and is thus as distantly related to the opisthokonts as the Archaeplastida (Field et al., 2012). The nuclear pore complex and the karyopherins are conserved, but there is no evidence for LINC complexes. There are, however, SUN-like proteins encoded in other Excavata lineages, such as diplomonads and heterolobosids, and it is thus possible that the trypanosomatids have selectively lost LINC complexes (Field et al., 2012). Identifying Excavata KASH-like proteins would be extremely helpful in broadening the current data set on possibly ancestral LINC complexes.

Archaeplastida

Plant SUN proteins

The first SUN protein homologs in plants were reported as *Arabidopsis thaliana* (At)Sad1a and AtSad1b (based on the similarity to *S. pombe* Sad1), hereafter AtSUN1 and AtSUN2, respectively, in the context of a large-scale screen for cell-cycle-associated proteins (van Damme et al., 2004). Two studies recognized AtSUN1 and AtSUN2 as bona fide homologs of mammalian SUN1 and showed that they are associated with the nuclear envelope, have a classic SUN domain, and can form homo- and hetero-complexes (Graumann et al.; 2010; Oda and Fukuda, 2011). AtSUN1 and AtSUN2 are shorter than mammalian SUN proteins and more similar to yeast Msp3 and *S. pombe* Sad1 (Evans et al., 2014). They share with non-plant SUN proteins an N-terminal domain with a nuclear localization signal, a transmembrane domain, a coiled-coil domain and the SUN domain. Homologs of AtSUN1 and AtSUN2 were found throughout the land plants, including Lycophytes and moss, indicating that they are broadly conserved and evolutionary old (Zhou and Meier, 2013).

Amino acid sequence comparisons of plant homologs of AtSUN1 and AtSUN2 with that of human SUN2 showed that plant SUN proteins have an N-terminally expanded conserved SUN domain, but the function of this extension is not known (Zhou and Meier, 2013). Nevertheless, the SUN domain of AtSUN1 can be modeled onto the structure of human SUN2. Although there is very little sequence conservation in the region that comprises the 'KASH lid' in human SUN2, the computed KASH-binding surface of AtSUN1 was successfully used for site-directed mutagenesis to produce plant SUN mutants that are disrupted in KASH-domain binding, suggesting that the domain is functionally conserved (Zhou and Meier, 2013; Zhou et al., 2014).

In addition to AtSUN1 and AtSUN2, there is a group of atypical SUN proteins in plants with a SUN domain in the central region of the polypeptide, flanked by transmembrane domains (denoted mid-SUNs). Three mid-SUN proteins have been described both in *Arabidopsis* (AtSUN3, AtSUN4 and AtSUN5) and in maize [*Zea mays* (Zm)SUN3, ZmSUN4 and ZmSUN5] (Murphy et al., 2010; Murphy and Bass, 2012; Graumann et al., 2014). Mid-SUN proteins have also been found in mice (Sohaskey et al., 2010), budding yeast (Friederichs et al., 2011) and *Dictyostelium* (Shimada et al., 2010). Like opisthokont mid-SUN proteins, plant mid-SUN proteins are present at the ER in addition to the nuclear envelope, suggesting a possible sub-functionalization. However, *Arabidopsis* mid-SUN proteins bind to the KASH proteins AtWIP1 and AtTIC (see below), indicating that they have the potential to be part of nuclear-envelope-bridging complexes (Graumann et al., 2014).

Plant KASH proteins

Although the sequences and the KASH-binding properties of opisthokont SUN proteins are conserved in plants, plant genomes do not code for homologs of known opisthokont KASH proteins (Zhou and Meier, 2013). WPP-domain-interacting proteins (WIPs) are a class of outer nuclear-envelope-associated plant-specific proteins that are involved in anchoring plant RanGAP to the nuclear envelope. They consist of a cytoplasmic coiled-coil domain, a TMD and a short tail domain terminating in the highly conserved tripeptide -VPT (Zhou et al., 2012). RanGAP binds through its N-terminal WPP domain to the coiled-coil domain of AtWIP1, AtWIP2 and AtWIP3. AtWIP1 has been shown to localize at the ONM by immunogold labeling (Xu et al., 2007). Because of the overall domain similarity and location, AtWIP1, AtWIP2 and AtWIP3 were tested for SUN binding in a combination of co-immunoprecipitation and yeast-two-hybrid experiments, and found to indeed bind to AtSUN1 and AtSUN2 (Zhou et al., 2012). Here, the 9-amino-acid tail located C-terminal of the transmembrane domain is required both for binding to SUN proteins and nuclear envelope localization, and binding depends on the domain of AtSUN1 and AtSUN2 that is equivalent to the KASH-binding lid in opisthokont SUN proteins (Zhou and Meier, 2013). The *Arabidopsis* WIP proteins also showed reduced nuclear envelope association in a SUN-mutant background and can therefore be considered plant analogs of KASH proteins, despite the lack of sequence similarity. WIT1 and WIT2 are two coiled-coil nuclear-envelope-associated proteins that bind to WIP1, WIP2 and WIP3 (Zhao et al., 2008) (Figs 1 and 2). They have a C-terminal TMD but no tail domain. The coiled-coil domains of WIP and WIT family members are required for their interaction, with heterodimers of higher-order hetero-complexes of WIP and WIT proteins likely being associated with the ONM (Zhao et al., 2008).

AtTIK was identified through a split-ubiquitin yeast two-hybrid screen with AtSUN2 (Graumann et al., 2014). It has a C-terminus that is more similar to mammalian KASH proteins than that of WIP1, WIP2 and WIP3, with a longer linker between TMD and C-terminus and a terminal -PPPS motif, which is required for SUN protein interaction. The cytoplasmic domain of AtTIK contains an interleukin 1-receptor/Toll-like receptor (TIR) domain of unknown function. AtTIK has biological roles in root development and root nuclear size, but the mechanism by which the protein operates and its cytoplasmic binding partners are elusive.

The broadly conserved C-terminal tail domain of the WIP family was used to computationally identify more plant KASH candidates. These new plant KASH proteins share with the WIP family a C-terminal -VPT motif that is separated from a TMD by a short linker. Termed SUN-interacting nuclear envelope proteins (SINEs), they all bind to AtSUN1 and AtSUN2 through an interaction of their VPT tail with the KASH-binding lid of the SUN proteins, and depletion of AtSUN1 and AtSUN2 leads to a reduced association with the nuclear envelope (Zhou et al., 2014) (Fig. 3). SINE1 and SINE2 are related proteins that both have an N-terminal domain with similarity to armadillo repeat domains. By contrast, SINE3 and SINE4 are short proteins with N-terminal domains that show no similarities (Fig. 3). In addition, SINE5, a protein that is apparently restricted to *Medicago* species, has been identified that also fulfills the criteria described above (Zhou et al., 2014).

Functions of plant LINC complexes

Targeting RanGAP to the nuclear envelope

The first bona fide function for an identified plant LINC complex was the anchoring of RanGAP1 to the nuclear envelope (Xu et al., 2007). Indeed AtWIP1 was identified as the interaction partner of the *Arabidopsis* RanGAP1 N-terminal nuclear-envelope-targeting domain. This mechanism differs from that in metazoans, where RanGAP is associated with the nuclear pore through an interaction of its C-terminal, SUMOylated domain with the nucleoporin RanBP2 (Matunis et al., 1998). The N-terminal RanGAP-anchoring WPP domain is unique to plants, and so is the WIP protein family (Meier, 2000; Rose and Meier, 2001; Xu et al., 2007). Aside from RanGAPs from different plant species, only a group of small, fairly uncharacterized plant proteins, named the WPP proteins, share sequence similarity with this domain (Meier, 2000; Patel et al., 2004). The WPP domain binds to the coiled-coil domain of both WIP and WIT family members, with the latter also being involved in anchoring RanGAP to the nuclear envelope (Xu et al., 2007; Zhao et al., 2008) (Fig. 1). A SUN2–WIP1–RanGAP1 complex was

Journal of Cell Science (2016) 129, 3523-3531 doi:10.1242/jcs.186700

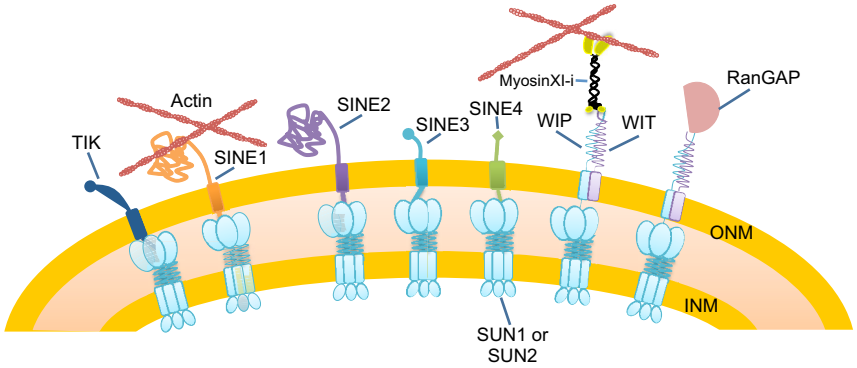

Fig. 3. Arabidopsis LINC complexes and known cytoplasmic interactors. Arabidopsis SUN1 and SUN2 are shown as a hypothetical trimer, based on the structure of human SUN. Arabidopsis KASH proteins that are known to bind SUN1 and/or SUN2 through their tail domains are depicted. SINE1 either directly or indirectly interacts with F-actin. The WIP–WIT complex interacts with myosin XI-i, which in turn binds to F-actin. The WIP–WIT complex interacts with *Arabidopsis* RanGAP, but it has not been shown whether myosin XI-i and RanGAP can bind simultaneously to the complex. WIP is used for either WIP1, WIP2 and/or WIP3, and WIT for WIT1 and/or WIT2. Except for TIK, all other KASH proteins have been shown to depend on an interaction with a SUN protein for their ONM association.

shown to exist by co-immunoprecipitation, and depletion of SUN1 and SUN2 disrupts the association of RanGAP1 with the nuclear envelope (Zhou et al., 2012).

Nuclear shape

Although embryonic *Arabidopsis* nuclei are near spherical, nuclei adopt a wide variety of shapes and sizes in mature tissues (plant nuclear morphology and its molecular players have recently been reviewed by Meier et al., 2016). Elongated and super-elongated nuclei dominate in root epidermal and root hair cells, whereas leaf epidermal and meristem cell nuclei are spindle-shaped, and the vegetative nucleus in pollen grains can be highly lobulated. Extensive grooves and invaginations of nuclear envelope membranes have also been documented in cultures of plant cells (Collings et al., 2000). In both WIP and SUN loss-of-function mutants, the nuclei of differentiated cells are returned to an embryonic spherical shape, with the exception of the lobulated pollen nucleus, which is not affected (Oda and Fukuda, 2011; Zhou et al., 2012, 2014). Similar spherical nuclei have been observed in WIT double mutants and in mutants of *KAKU1*, which encodes myosin XI-i (Tamura et al., 2013). KAKU1 binds to WIT1, and it has been suggested that a complex formed between KAKU1, WIT1, a WIP and SUN1 or SUN2 transmits cytoskeletal forces to the nuclear envelope. In dissecting this complex further, it was shown that SUN1, but not SUN2, and WIT2 but not WIT1, play a predominant role in determining nuclear shape. The spherical nuclei in *SUN* or *WIT* mutants have invaginated nuclear envelopes, suggesting a disruption in the homeostasis between nuclear membrane surface area and nuclear volume (Zhou et al., 2015a). In AtTIK-knockout seedlings, root nuclear size is reduced; however, it is currently not known how this relates to the *SUN*, *WIP*, *WIT* and *KAKU* phenotypes described above (Graumann et al., 2014).

Nuclear movement and nuclear anchoring

Nuclear movement is integral to several processes throughout plant development, including root hair growth, trichome (leaf hair) development, the initiation of both agonistic and antagonistic plant–microbe interactions and high-light avoidance in leaves, as well as the response to mechanical stimulation. Some of these movements have been associated with the actin cytoskeleton. The most striking among these is likely the journey of the pollen vegetative nucleus down the pollen tube towards the female reproductive apparatus (for a recent review of nuclear movement in plants, see e.g. Griffis et al., 2014). It has been shown that KAKU1, in conjunction with the SUN–WIP–WIT complex is involved in nuclear movement in fully developed root hairs, as well as in the shade-recovery aspect of the high-light avoidance response in leaves (Tamura et al., 2013). In addition to nuclear movement, nuclear positioning has been found to be relevant for developmental and signaling processes, such as in *C. elegans* embryo development (Tapley and Starr, 2013; Bone and Starr, 2016). In plants, SINE1 is involved in the symmetric central positioning of the *Arabidopsis* guard cell nuclei into close association with the guard cell plasma membranes that are adjacent to the stomatal pore (Zhou et al., 2014). This positioning requires F-actin, and the armadillo-repeat-like N-terminus of SINE1 has been shown to associate *in vivo* with the actin filaments of guard cells. However, the physiological role of this type of nuclear positioning is currently not known. Interestingly, mutants in SINE2, a close homolog of SINE1, which is expressed in leaf epidermal and mesophyll cells, are more susceptible to an oomycete pathogen (Zhou et al., 2014). Although the detection of this pathogen by the plant indeed involves nuclear re-positioning (Caillaud et al., 2012), a role of SINE2 in this event has not yet been demonstrated. The functions of SINE3, SINE4 and SINE5 are currently not known.

Pollen tube termination

During fertilization, a SUN–WIP–WIT LINC complex is required for the nuclear migration of vegetative pollen and for successful pollen tube termination (Zhou and Meier, 2014; Zhou et al., 2015b). In *Arabidopsis*, a mature pollen grain contains the nucleus of the vegetative cell and the two engulfed sperm cells with their own, highly condensed nuclei, which are attached to the vegetative nucleus by membrane bridges (McCue et al., 2011). Upon pollen germination, the vegetative nucleus typically emerges first from the pollen grain and ‘leads’ the sperm cells down the pollen tube to the ovule, the female reproductive apparatus. In *Arabidopsis* lines that combine null mutations in WIP1, WIP2 and WIP3, or WIT1 and WIT2, as well as a combination of all five mutant alleles, this process is disrupted and the vegetative nucleus frequently becomes misplaced during pollen tube growth, whereas the sperm cells still reach the female apparatus. However, the final stage of pollen tube development, its burst – or termination – is impaired in the mutants, leading to a drastic reduction in seed set. Both WIP1 and WIT1 have been shown to be located at the nuclear envelope of the vegetative

nucleus. Thus, the following model has been proposed: (1) a LINC complex at the vegetative nucleus is required to establish cytoplasmic forces that transport the vegetative nucleus along the pollen tube, and (2) the presence of the vegetative nucleus at the tip of the pollen tube is required for a process that is crucial to pollen tube termination (Zhou and Meier, 2014; Zhou et al., 2015b).

Mitosis and meiosis

In higher plants, like in metazoans, but unlike in budding yeast, *Aspergillus* or *Dictyostelium*, the nuclear envelope breaks down at the onset of mitosis. During open mitosis in *Arabidopsis*, AtSUN1 and AtSUN2 associate with the bulk mitotic membrane faction, which in plants, unlike metazoans, traverses the spindle apparatus (Graumann and Evans, 2011). Upon nuclear envelope re-formation, both AtSUN1 and AtSUN2 accumulate first at the side of the chromosomes that is closest to the spindle poles, before they subsequently fill in the entire nuclear rim (Oda and Fukuda, 2011; Graumann and Evans, 2011). This pattern of reassociation with the re-forming nuclear envelope was also seen for WIP1 (Xu et al., 2007), but its functional significance is not known.

During meiotic prophase I, the meiotic telomeres are associated with the nuclear envelope in mammals and worms, and are connected to cytoplasmic motors through LINC complexes (Sato et al., 2009; Kracklauer et al., 2013; Yamamoto, 2014; Link et al., 2015). Cytoplasmic forces lead to the well-described phenomenon of telomere clustering, or bouquet formation, which is important for the pairing of homologous chromosomes and for meiotic recombination. Both in maize and in *Arabidopsis*, SUN proteins have been found at specific locations during meiosis, such as at a nuclear envelope belt in maize and at punctate structures in the meiotic nucleus in *Arabidopsis* (Murphy et al., 2014; Varas et al., 2015). Deficiency in AtSUN1 and AtSUN2 leads to defects in the distribution of meiotic crossover sites, delayed mitotic progression, incomplete synapsis and unresolved interlocks (Duroc et al., 2014; Varas et al., 2015); these phenotypes are also found in the Arabidopsis mutants of the kinesin AtKin-1 (also called AtPSS1). AtKin-1 has been shown to interact with WIP1 and WIP2 in yeast two-hybrid assays, suggesting that WIPs are also involved in the meiotic functions of AtSUN1 and AtSUN2 through a complex they form with AtKin-1 (Duroc et al., 2014). Thus, plant SUN proteins and possibly their associated KASH proteins also have a function during meiosis.

In summary, although plant KASH proteins are structurally similar to opisthokont KASH proteins, they are not homologous based on sequence similarity. Plant KASH proteins are involved in a variety of biological processes that might, but do not always have to, involve interactions with the cytoskeleton. On the nuclear side, the protein interaction partners of plant SUNs are still largely unknown. An exception is their interaction with one of the long coiled-coil CRWN proteins, which have been suggested to fulfill a lamin-like role in plants (Graumann, 2014).

Are the most ancestral SUN-interacting proteins coiled-coil-type centrosome adaptors?

According to the most current phylogenetic tree, extant eucaryotes are grouped into five supergroups, with the Amoebozoa and the Opisthokonta together forming the unikonts (one flagellum) and the Archaeplastida, SAR-CCTH (Stramenopile, Alveolata, Rhizaria and Cryptophyta, Centrohelida, Telonemia and Haplophyta) and the Excavata forming the bikonts (two flagella) (Adl et al., 2012; Field et al., 2012; Gräf et al., 2015) (Fig. 4). Plants are currently the only

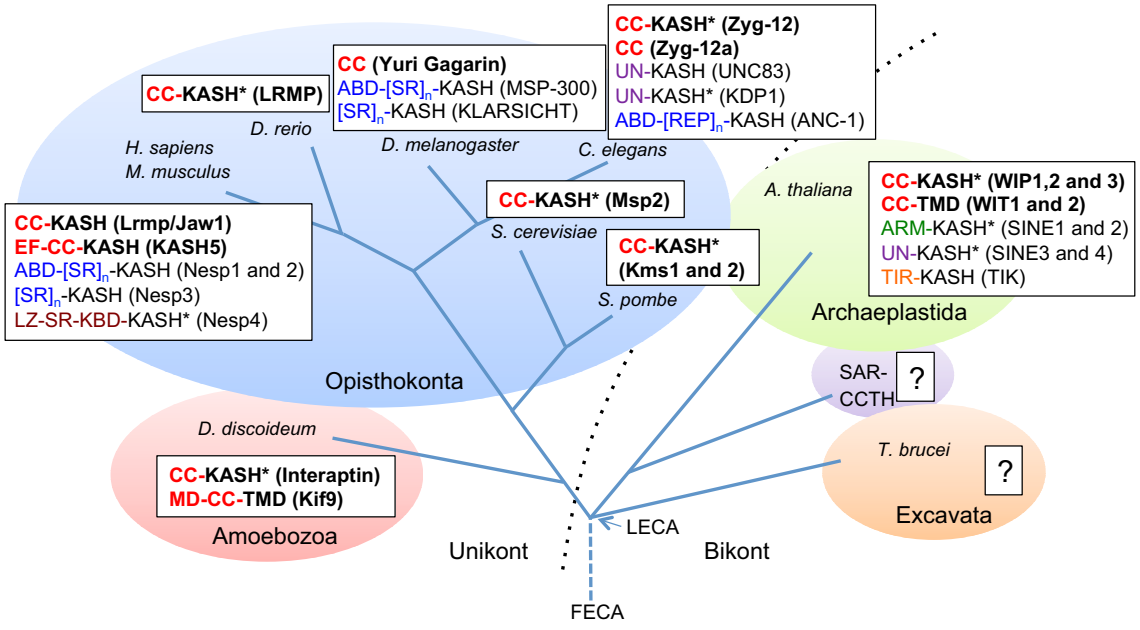

Fig. 4. Conservation of coiled-coil domain KASH proteins across different supergroups. KASH proteins are organized based on their types of cytoplasmic domains and presence in organismal groups. Abbreviations for domains are: CC, coiled-coil domain; EF, EF-hand domain; $[SR]_n$, spectrin-repeat domain of variable length; ABD, actin-binding domain; MD, kinesin motor domain; LZ-SR-KBD, leucin-zipper–spectrin-repeat–kinesin-binding region domain combination; ARM, armadillo-repeat domain; TIR, interleukin 1 receptor/Toll-like receptor domain; UN, domain of unknown structure; KASH, KASH domain; KASH*, short or unusual KASH domain; TMD, C-terminal transmembrane domain with no discernible KASH tail. Protein organization is written as domain abbreviations connected by dashes and to be read left to right as N-terminal to C-terminal. For example ABD-$[SR]_n$ -KASH stands for an N-terminal actin-binding domain, followed by a stretch of several spectrin repeats, followed by a C-terminal KASH domain. Cytoplasmic domains are color coded by similarity, for example all coiled-coil domains are in red. Coiled-coil KASH proteins are in bold. Protein names are given in parentheses after each structure. FECA, first common eukaryotic ancestor; LECA, last common eukaryotic ancestor. Question marks for SAR-CCTH and Excavata indicate that currently no KASH or KASH-like proteins are known.

bikont group members for which we have any information regarding SUN and KASH protein structure and function. Neither for the Excavata nor for SAR-CCTH have any SUN or KASH protein homologs been described.

Fig. 4 summarizes the domain organization of known KASH and KASH-like proteins across the current eukaryotic tree of life. Although among the opisthokont LINC complexes, the primary focus has long been on those that contain a spectrin-like KASH protein and their connection to the cytoskeleton, in all investigated model organisms, at least one nucleoplasmic-bridging complex exists that contains a KASH or KASH-related protein with a cytoplasmic coiled-coil domain (indicated in bold letters in Fig. 4).

In *Drosophila*, no coiled-coil-KASH protein is known, but it is possible based on the known biology that Yuri Gagarin interacts indirectly with Spag4 and is thus anchored to the nuclear envelope. If such a nucleoplasmic-bridging complex exists in *Drosophila* (as conceptualized in Fig. 2) it would be similar to the *C. elegans* Zyg-12a, Zyg-12 and SUN1 connection. In *Drosophila*, it would comprise a coiled-coil heterocomplex of two likely unrelated proteins in the cytoplasm. This, in turn, is very similar to the WIT–WIP–SUN complex in plants, where SUN-bound WIP interacts with cytoplasmic WIT to form a heterocomplex, and WIT is the connector to kinesin. It has been shown that the C-terminal predicted TMD of WIT1 is not required for its association at the nuclear envelope in the presence of WIP1 (Zhao et al., 2008), suggesting that the coiled-coil domains of WIP and WIT are the primary nuclear-envelope-anchoring mechanism for the complex. Similarly, Kif9, which is also involved in the centrosome–nucleus interaction, might not directly interact with SUN1 in *Dictyostelium*, but through an unidentified bridging protein. It should be noted that Interaptin is not a likely candidate for this, because the mutant has no defect in the centrosome-nucleus association (Xiong et al., 2008).

Interestingly, in unikonts, the described coiled-coil KASH or KASH-related proteins all have some role in associating a spindle-forming MTOC with the nuclear envelope (Fig. 2). In several metazoans, they have specific functions during spermatogenesis or meiosis, such as has been shown for KASH5 in mammals, Zyg-12 in worms, and Yuri Gagarin and Spag4 in flies. If such a function for a similar KASH–SUN complex also existed in the bikonts, it would suggest that the most ancestral SUN-binding proteins in eukaryotes might have been coiled-coil-domain-containing proteins that are involved in connecting the nuclear envelope with a microtubule-organizing center (MTOC).

Unfortunately, the nature of the plant spindle-forming MTOC is poorly understood. However, it is known that γ-tubulin complex (γ-TuC) components are associated with the nuclear envelope and that microtubule growth can initiate on the nuclear surface (Batzenschlager et al., 2013). Proteins involved are γ-TuC protein 3 (GCP3) and GCP3-interacting protein (GIP) (Janski et al., 2012; Batzenschlager et al., 2015). GCP3 interacts with GIP, and GIP in turn interacts with TonSoku-associating protein 1 (TSA1), a TMD protein associated with the nuclear envelope (Batzenschlager et al., 2013). It could therefore be tested whether members of the WIP and/or WIT families interact with any components of the plant γ-TuC that are associated with the nuclear envelope, such as GIP or GCP3 (Fig. 2). If this was indeed the case, it would lend great support to the idea that a truly ancestral LINC complex consisted of a SUN protein similar to extant variants and one or more coiled-coil proteins located at the ONM, which either directly or indirectly interact with the SUN protein and an MTOC.

It has been proposed that the last eukaryotic common ancestor (LECA) had a precursor centrosome, which consisted of a membrane and chromatin-associated MTOC (Gräf et al., 2015). What precise function the earliest nucleocytoplasmic bridging complex could have had in its attachment to the nuclear envelope is hard to predict, and would among other things depend on whether we assume that the LECA underwent open or closed mitosis. During open mitosis, the INM-associated proteins might have had a role in attaching and detaching membranes from chromatin during the cell cycle. In any case, a double-membrane-spanning complex is a great candidate for coordinating an MTOC on the cytoplasmic side with a chromosome-organizing function on the nucleoplasmic side of the nuclear envelope, especially if such a complex is already implicated in telomere clustering (Yamamoto, 2014; Link et al., 2015; Ebrahimi and Cooper, 2016).

Conclusions and outlook

To follow up on this hypothesis, further knowledge about LINC complexes from additional bikont groups is required. It is interesting to note that the coiled-coil type of KASH proteins in plants is deeply conserved within the Archaeaplastida, unlike other, likely more specialized plant KASH proteins, such as TIK, SINE3 or SINE4 (Fig. 3; Zhou et al., 2014). In this regard, however, more sequences from green algae are required, especially from those freshwater algae that have been proposed to be ancestral to land plants. Furthermore, if *T. brucei* has indeed lost its LINC complexes, mining sequences from other Excavata would be a powerful approach to gain additional bikont information.

In summary, it is worthwhile considering that the first LINC complexes that had been discovered might be a special case that has evolved in the metazoans and that has been extended along with increasing functions in nuclear anchoring and movement, possibly in the context of evolving hematopoietic, neuronal and muscular cell types. To further understand ancestral LINC complexes, it will be important to probe into their additional, diverse functions and to investigate both the more specialized and the more broadly conserved proteins that form the cytoplasmic interaction surface of the nucleoplasmic bridging complexes.

Acknowledgements

I would like to thank an anonymous reviewer for providing valuable additional information about the *Dictyostelium* work.

Competing interests

I declare no competing or financial interests.

Funding

Research in this area in my laboratory is funded by grants from the National Science Foundation [grant numbers MCB-1243844, MCB-1613501].

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