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COMMENTARY

The dynamic protein Knl1 – a kinetochore rendezvous

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ABSTRACT

Knl1 (also known as CASC5, UniProt Q8NG31) is an evolutionarily conserved scaffolding protein that is required for proper kinetochore assembly, spindle assembly checkpoint (SAC) function and chromosome congression. A number of recent reports have confirmed the prominence of Knl1 in these processes and provided molecular details and structural features that dictate Knl1 functions in higher organisms. Knl1 recruits SAC components to the kinetochore and is the substrate of certain protein kinases and phosphatases, the interplay of which ensures the exquisite regulation of the aforementioned processes. In this Commentary, we discuss the overall domain organization of Knl1 and the roles of this protein as a versatile docking platform. We present emerging roles of the protein interaction motifs present in Knl1, including the RVSF, SILK, MELT and KI motifs, and their role in the recruitment and regulation of the SAC proteins Bub1, BubR1, Bub3 and Aurora B. Finally, we explore how the regions of low structural complexity that characterize Knl1 are implicated in the cooperative interactions that mediate binding partner recognition and scaffolding activity by Knl1.

KEY WORDS: BubR1, Knl1, MELT motif, Kinetochore, Mitosis, Mitotic checkpoint

Introduction

Knl1, also known as Spc105 in budding yeast, Spc7 in fission yeast, and in humans as CASC5, AF15q14 and Blinkin, is a central component of the Knl1–Mis12–Ndc80 (KMN) network, a ten-subunit macromolecular assembly that also comprises the Mis12 complex (Mis12, Dsn1, Nsl1 and Nnf1), and the Ndc80 complex [Hec1 (also known as Ndc80), Nuf2, Spc24 and Spc25] (DeLuca and Musacchio, 2012; Tooley and Stukenberg, 2011; Varma and Salmon, 2012). Knl1 functions as a signaling hub during early mitosis and contributes to the formation of kinetochore–microtubule (MT) attachments (Przewloka and Glover, 2009; Santaguida and Musacchio, 2009). Knl1, the largest subunit of the KMN network, is required for accurate chromosome segregation during mitosis (Desai et al., 2003), and for both activating and inactivating the spindle assembly checkpoint (SAC), a conserved signaling cascade that delays anaphase onset in the presence of unattached or improperly attached chromosomes (Kiyomitsu et al., 2007; Meadows et al., 2011; Rosenberg et al., 2011; Espeut et al., 2012). Defects in Knl1 function have been implicated in genome instability, leukemia, microcephaly and neurological disorders (Kiyomitsu et al., 2007; Kiyomitsu et al., 2011; Yang et al., 2014; Genin et al., 2012). Interestingly, the phenotype observed in cells in which Knl1 is depleted is similar to that associated with the suppressed expression of the mitotic checkpoint proteins Bub1 and BubR1 (Kiyomitsu et al., 2007; Kiyomitsu et al., 2011; Cheeseman et al., 2006; Cheeseman et al., 2008), suggesting that a major function of Knl1 is to coordinate Bub1 and BubR1 signaling, a notion that has seen significant support from a number of recent studies in yeasts, *Caenorhabditis elegans*, *Drosophila melanogaster* and cultured human cells (Desai et al., 2003; Cheeseman et al., 2008; Schittenhelm et al., 2009; Venkei et al., 2012; Varma et al., 2013; reviewed by Caldas and DeLuca, 2013). Knl1 has also emerged as the central 'switchboard' for Aurora B activity. Aurora B itself is thought to govern the attachments between MTs and the kinetochore (Box 1). Knl1 is required for Aurora-B-mediated phosphorylation of outer kinetochore proteins, including Hec1, the primary kinetochore–MT attachment protein (Cheeseman et al., 2006; Alushin et al., 2010), and Dsn1, a protein that contributes to the stabilization of kinetochore–MT attachments (Kline et al., 2006; Welburn et al., 2010; Caldas and DeLuca, 2013).

Knl1 orthologs show low amino acid sequence similarity and are characterized by an abundance of regions of low structural complexity and the presence of a C-terminal globular domain. Knl1 binds to MTs (Welburn et al., 2010; Espeut et al., 2012) and has key roles in the kinetochore recruitment of the SAC kinase Bub1 and pseudokinase BubR1 (Kiyomitsu et al., 2007), as well as of the protein phosphatases PP1 (directly) and PP2A (indirectly through BubR1) (Liu et al., 2010; Suijkerbuijk et al., 2012; Kruse et al., 2013). A number of recent reports have provided important insight into how Knl1 functions as a docking platform that integrates a number of SAC kinase and phosphatase activities. Clues as to how Knl1 coordinates these activities have emerged owing to the recent identification and characterization of functional regions in Knl1, including the protein–protein interaction motifs SILK, RVSF, MELT and KI, and a C-terminal domain that adopts the RWD fold (Fig. 1). In this Commentary, we discuss our current understanding of the different functions of Knl1 during mitosis and advance a mechanistic view of the interactions that are mediated by this protein to ensure proper chromosome segregation. We emphasize, in particular, recent insights into the function and regulation of the protein-interacting motifs of Knl1.

Knl1 – a major kinetochore scaffolding protein

As a central component of the kinetochore scaffolding machinery, Knl1 is important for the recruitment of a number of kinetochore proteins, both within the KMN network itself, as well as proteins implicated in the SAC and chromosome congression machinery (Cheeseman et al., 2008; Kiyomitsu et al., 2007; Pagliuca et al., 2009; reviewed in Caldas and DeLuca, 2013). A variety of functional motifs and protein partners, together with its overall low structural complexity, define Knl1 as a multifactorial docking platform. The C-terminal region of Knl1 is responsible for its

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Box 1. Aurora B regulation of kinetochore–MT attachments

The regulation of kinetochore–MT attachments by Aurora B is complex. The current model in the literature, the so-called displacement model, posits that Aurora B at the centromere generates an outwardly radiating activity gradient that causes the phosphorylation of outer-kinetochore proteins that are brought into close proximity to the centromere as a result of incorrect kinetochore–MT attachments. Phosphorylation then results in the decreased stability of these kinetochore–MT interactions, thus allowing the spindle and kinetochore machinery to repair the inappropriate attachments. Upon the application of tension or the kinetochore stretch that occurs as a consequence of spindle biorientation, the outer kinetochore targets of Aurora B are displaced from the centromere; they become less accessible to the 'sphere of influence' of Aurora B and are thus no longer efficiently phosphorylated (for excellent recent reviews see Funabiki and Wynne, 2013; Tanaka, 2013; Lampson and Cheeseman, 2011).

KMN network proteins (although not Knl1 itself) have also been shown to directly interact with the spindle and kinetochore-associated (SKA) protein complex formed by SKA1, SKA2 and SKA3, which has been strongly implicated in maintaining stable kinetochore–MT interactions (Hanisch et al., 2006; Gaitanos et al., 2009; Theis et al., 2009; Daum et al., 2009; Raaijmakers et al., 2009). The association between the KMN and SKA networks is negatively regulated by Aurora B activity (Chan et al., 2012). Whether phosphatases that are recruited by Knl1 regulate interactions between KMN and SKA is an open question. SKA3 has also been identified as a binding partner of B55-β and B55-δ, regulatory subunits of protein phosphatase PP2A (Glatter et al., 2009), although the significance of the interaction remains unclear (Foley et al., 2011).

kinetochore localization and is required for its interaction with Nsl1, a component of the Mis12 complex (Cheeseman et al., 2006; Kiyomitsu et al., 2007; Petrovic et al., 2010; Petrovic et al., 2014) (Fig. 1). The C-terminal region of Knl1 (residues 1904–2316 in humans) also mediates its binding to Zwint (Petrovic et al., 2010) a kinetochore protein that is required for binding the Rod–Zwilch–Zw10 (RZZ) complex in prometaphase (Kiyomitsu et al., 2011; Kops et al., 2005; Wang et al., 2004). Because the kinetochore localization of Knl1 depends on Mis12 and Zwint (Varma et al., 2013), it is possible that the two complexes act cooperatively to recruit Knl1 to the kinetochore. In human Knl1, a fragment encompassing the amino acid residues R_{2096}–D_{2311} directly interact with Nsl1 (Petrovic et al., 2014). Knl1 orthologs are also able to directly bind to MTs through an N-terminal MT-binding region that includes a highly conserved basic patch (Welburn et al., 2010). In the context of a reconstituted KMN network from *C. elegans*, these MT-binding activities have been shown to act cooperatively (Cheeseman et al., 2006; Wei et al., 2007), although evidence for a stable protein complex between Hec1 and Knl1 has not been reported.

Knl1 protein-interacting regions

SILK and RVSF motifs

The SILK [consensus sequence (SG)ILK] and RVSF (consensus RVxF) motifs are located at the far N-terminus of Knl1 and mediate the direct binding of protein phosphatase PP1 (Liu et al., 2010; Rosenberg et al., 2011), an enzyme that counteracts the kinase activity of Aurora B and that mediates SAC silencing (Lesage et al., 2011; Espeut et al., 2012). Through a feedback mechanism, Aurora B inhibits the interaction between PP1 and Knl1 through phosphorylation of the RVSF motif (Liu et al., 2010). In *C. elegans*, PP1 binding to Knl1 plays a role in SAC silencing, which might ensure the timely removal of Bub1 and BubR1 from the kinetochore (Espeut et al., 2012). In agreement with this, the Nilsson laboratory recently reported that deletion of the first 150 or 300 amino acids of Knl1, or the mutation of the N-terminal Knl1 PP1-binding site, results in increased levels of Bub1 and BubR1 at the kinetochore compared with that in cells expressing wild-type Knl1, suggesting that PP1 negatively regulates the recruitment of Bub1 and BubR1 (Zhang et al., 2014; London et al., 2012; Meadows et al., 2011; Rosenberg et al., 2011). In addition to PP1, Knl1 indirectly mediates the recruitment of PP2A through the recruitment of BubR1, which binds directly to the B56 family of PP2A regulatory subunits (Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013). PP2A is important for stabilizing kinetochore–MT attachments through dephosphorylation of outer kinetochore substrates in late mitosis (Foley et al., 2011; Liu et al., 2010; Suijkerbuijk et al., 2012; Kruse et al., 2013). Although Knl1 mediates the kinetochore recruitment of PP1 and PP2A, these two enzymes potentially act upon different substrates. The further elucidation of how the kinetochore localization and substrate selectivity of these phosphatases is achieved will provide important insights into the mechanism underlying kinetochore regulation.

A major function of Knl1-bound PP1 is to regulate the activity of Aurora B. Deletion of a Knl1 N-terminal region or depletion of Knl1 abolishes Aurora-B-mediated phosphorylation of outer kinetochore proteins, including Hec1 and Dsn1, thus resulting in defects in kinetochore–MT attachment (Caldas et al., 2013). The observation that Aurora B activity is diminished is somewhat surprising, considering that the loss of binding of PP1 to the N-terminal region of Knl1 is expected to enhance phosphorylation of Aurora B substrates (Liu et al., 2010). Indeed, Caldas and collaborators have shown that, in contrast to previous observations (Liu et al., 2010), mutation of the PP1-binding site of Knl1 (RVSF to AAAA) does not significantly affect Aurora B autophosphorylation (Caldas et al., 2013). The reason for the disparity between these studies is not clear, but might be due to different sensitivities of the readouts that were used for Aurora B activity [namely, the use of phosphospecific antibodies versus targeted Förster resonance energy transfer (FRET) sensors]. A second facet of Aurora B regulation by Knl1 occurs indirectly, through Knl1-mediated recruitment of Bub1 (see below). Work from the DeLuca laboratory has demonstrated that the N-terminus of Knl1 facilitates the phosphorylation of the Bub1 substrate histone H2A at T120, an event which in turn promotes the centromere targeting of Aurora B (Caldas et al., 2013; Yamagishi et al., 2012). Interestingly, rescue of Aurora B localization with an N-terminal Knl1 fragment does not result in an equivalent rescue of Aurora B activity, suggesting that other regions of Knl1 might also promote Aurora B kinase activity (Caldas et al., 2013).

MELT motifs

A number of copies of the MELT motif with a sequence consensus of (M/I/L/V)-(E/D)-(L/M/I/V)-(T/S) are found in the N-terminal and the middle regions of Knl1 orthologs (Figs 1, 3). Despite the presence of multiple units of the MELT motif in Knl1/Spc105 being an evolutionary conserved feature (Cheeseman et al., 2004; Vleugel et al., 2012; Vleugel et al.,

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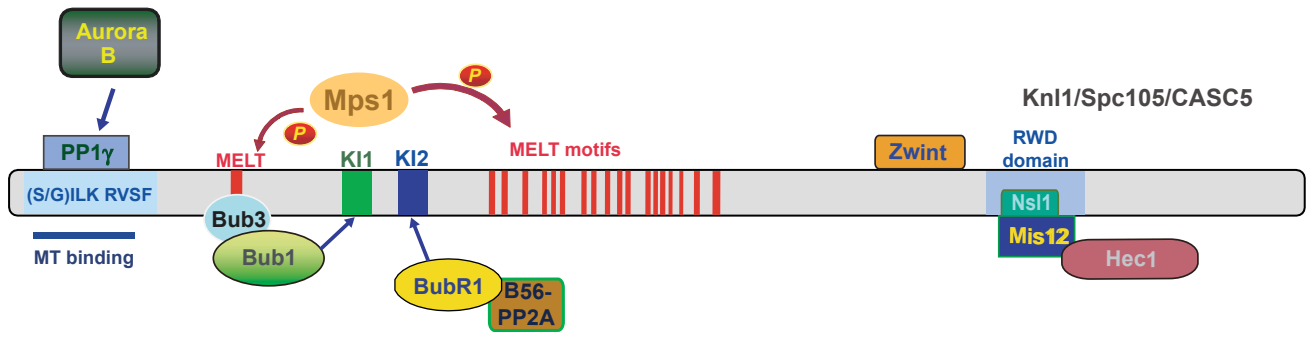

Fig. 1. Knl1 is a kinetochore scaffold. Schematic representation of the regions of Knl1 that mediate its binding to diverse protein partners. The balance between kinase and phosphatase activities on the N-terminal of Knl1 is likely to be a key factor in the regulation of the interactions. For instance, Bub3 recruitment to the kinetochore requires phosphorylation of the MELT motif repeats of Knl1 by Mps1, an important aspect of SAC signaling that is described in more detail in Fig. 3. In addition, the ability of Knl1 to recruit PP1γ is negatively regulated by Aurora B, whereas Aurora B recruitment to the kinetochore is enhanced by Bub1.

2013), the exact number of MELT or MELT-like motifs shows a large variation across species and thus suggests that Knl1 is a rapidly evolving protein (Vleugel et al., 2012; Vleugel et al., 2013).

It is now established that phosphorylation of the threonine residue in position 4 of the MELT motifs – or at least a subset thereof – is the target of the mitotic checkpoint kinase Mps1, and that this posttranslational modification of Knl1 is required for the recruitment of Bub1 and Bub3 to the kinetochore (London et al., 2012; Primorac et al., 2013; Shepperd et al., 2012; Yamagishi et al., 2012). Accordingly, preventing the phosphorylation of Knl1 by Mps1, results in attenuated binding of the BUB proteins to Knl1, chromosome congression defects, and failure to mount an appropriate checkpoint response (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012).

The question of how MELT sequences recruit BUB proteins has been investigated in more recent studies. Structural and biochemical evidence has demonstrated that phosphorylated MELT motifs bind directly and with high affinity to Bub3 (Primorac et al., 2013; Zhang et al., 2014). The crystal structure of Bub3 from budding yeast in complex with a synthetic MELT phosphopeptide and the Bub3-binding region of Bub1 (a protein motif commonly referred to as the GLEBS motif) identified a well-conserved region on the side of the β-propeller structure that is formed by the Bub3 WD40 repeats (Box 2) as the site that binds to the phosphorylated MELT motif (Primorac et al., 2013; Fig. 2A,B). In the crystalline state, few Bub1 residues appear to be implicated in the interaction of Bub3 with MELT motifs, whereas residues that flank the phosphothreonine of the phosphorylated MELT motif do not engage directly in the interaction with Bub3, thus suggesting the interaction between phosphorylated MELT and Bub3 has a moderate affinity, a notion that is supported by isothermal titration calorimetry (ITC) experiments of Bub3 with synthetic phosphopeptides that mimic MELT (Primorac et al., 2013). The structure of the ternary complex shows that a few Bub1 residues that are located at the N-terminal end define a β-hairpin (β1–β2; residues K_{307}–I_{309} and E_{315}–I_{317}, respectively). Amino acid residues of this Bub1 β-hairpin physically interact with the Bub3 residues that define two loop regions; namely, βA6 and βD5 (residues R_{242}–N_{244} and N_{249}–A_{251}, respectively). The interaction between Bub1 β1–β2 and Bub3 βA6–βD5 gives rise to the formation of one four-stranded β-sheet located above the phosphorylated MELT residues that are engaged in the interaction with Bub3 (Primorac et al., 2013). Further evidence of the importance of Bub1 for the Bub3–phosphorylated-MELT interaction was derived from quantitative binding studies of pure Bub1 and Bub3 proteins with peptides mimicking the phosphorylated MELT sequence as measured by ITC. The experiments revealed a 10-fold decrease in the affinity of the interaction between the phosphorylated MELT peptide and Bub3 (i.e. an approximate K_d of 2 μM) when Bub1 was absent (Primorac et al., 2013).

The stoichiometric relationship between phosphorylated MELT and Bub3 has led to the suggestion that each MELT repeat constitutes a functional binding site that is ‘read’ by a Bub3 molecule (Primorac et al., 2013; Zhang et al., 2014; Vleugel et al., 2013). This is supported by the observation that a drop in the amount of BUBs that are recruited is proportional to the number of MELT repeats that have been removed, and that increasing the number of MELT arrays proportionally augments the amount of Bub1 recruited (Zhang et al., 2014; Vleugel et al., 2013). The exact number and combination of MELT motifs required for efficient mitotic progression, however, remains unclear. One study has shown that any array of functional MELT motifs that could recruit sufficient amounts of Bub1 to the kinetochore could ensure proper chromosome segregation, suggesting that different MELT motifs have redundant functions (Vleugel et al., 2013). The same study also demonstrated, however, that not all MELT motifs are equally efficient at Bub1 recruitment; six repeats of MELT2 did not restore BUB levels, and consequently did not support chromosome congression nor SAC activity, whereas six repeats of MELT17 were able to do so (Vleugel et al., 2013). Whether MELT2 is a poor Mps1 substrate or a poor acceptor of Bub3 when it is phosphorylated remains to be clarified, and answering this question might shed some insight as to why – at least in human Knl1 – so many MELT motifs evolved when efficient BUB protein recruitment can be accomplished by only a subset thereof. Indeed, expression of a Knl1 construct that lacks all but the five C-terminal MELT repeats is able to recruit sufficient amounts of Bub1 and BubR1 to support normal chromosome congression (Zhang et al., 2014), although a considerable delay in the duration of mitosis was observed, perhaps due to the loss of the PP1-binding site (see above). Further removal of MELT motifs in the context of a C-terminal Knl1 fragment decreases the kinetochore level of Bub1 and BubR1 and does not support proper SAC function or chromosome alignment (Zhang et al., 2014). Remarkably, the 260 N-terminal residues, which contain

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Box 2. General features of protein domains that are known to interact with Knl1

TPR motif

The tetratricopeptide repeat (TPR) motif is a protein motif defined a consensus of 34 amino acids that are organized in a helix-loop-helix. TPR motif repeats define a right-handed super-helical twist of the entire structure that results from the packing of α-helices. The TPR motif is widely distributed in proteins from animals and plants that mediate a diversity of functions. Although the number of TPR units varies greatly between TPR-containing proteins, from a tandem of two repeats to more than ten, it generally functions as a versatile organizing module for the assembly of multiprotein complexes. Mitotic checkpoint proteins that contain tandem arrangements of this motif are Bub1, BubR1 and Mps1.

WD40 repeat

The WD40 repeat fold is typically composed of several WD repeats that form a four-stranded anti-parallel β-sheet or blade. Each WD repeat consists of a short structural motif of ~40 amino acid residues, often terminating in the pair tryptophan-aspartic acid (WD), hence the name. The blades fold together to adopt a circular solenoid architecture called the WD40 domain, with a seven-bladed β-propeller being the most common type. The blades interlock so that the last β-strand of one repeat interacts with the first three of the next repeat to form the 3D blade structure. WD40-repeat proteins are usually involved in the assembly of multi-protein complexes, where the WD40 repeat units serve as a rigid docking platform for the binding of protein ligands. Examples of mitotic checkpoint proteins that contain the WD40 fold are Bub3 and Cdc20.

only the first MELT motif, together with the KI1 and KI2 motifs, are able to recruit Bub1 proteins to ectopic chromosomal regions during mitosis. However, this Knl1 region is not strictly required for the SAC or congression, providing the remaining MELT motifs of Knl1 are present (Vleugel et al., 2013). When localized to the kinetochore, this N-terminal Knl1 construct supports the SAC but not chromosome congression, suggesting that alignment of sister chromatid pairs might require higher levels of BUB activity (Vleugel et al., 2013; Krenn et al., 2014). A systematic analysis of MELT motif phosphorylation and their capacity to support Bub3 binding, chromosome congression and the SAC will be needed to determine the functionality of individual MELT motifs.

In addition to variation in number, sequence diversity of the core MELT motif is also apparent across evolution. A recent bioinformatics analysis of human Knl1 revealed the presence of 19 repeating modules that resemble the MELT motif. The methionine and threonine residues of the MELT motif are highly conserved, whereas the intervening residues are often negatively charged, although species-specific variances do exist (Vleugel et al., 2013). The most divergent of the MELT-like sequences have been identified in drosophilids, where the threonine phosphoacceptor of the MELT repeat has been replaced by the acidic residues aspartate or glutamate, suggesting that Mps1 phosphorylation of the MELT sequence might not play an important role in BUB recruitment in flies (Vleugel et al., 2013). Unlike the situation in yeasts and mammalian cells, in *D. melanogaster*, these repeats are apparently dispensable for Spc105/Knl1 function (Shittenhelm et al., 2009). Further sequence analysis also showed that repeating MELT motifs are often flanked on the N-terminal side by the sequence T$\Phi\Phi$-(F/Y)-(ST)-(DE), where Φ denotes a hydrophobic residue, and the sequence SHT at the C-terminal end. Interestingly, the two flanking sequences appear to be essential for recruitment of Bub1 to the kinetochore in human cells (Vleugel et al., 2013). However, these features are not conserved in all species, and how they coordinate with phosphorylated MELT sequences to promote BUB recruitment awaits further clarification (Vleugel et al., 2013). Regardless of the exact number and sequence of MELT motifs, these sequences might serve as a 'rheostat' that allows the cell to fine-tune the SAC response and the chromosome congression machinery as needed. In this way, permutations and combinations of functional BUB-binding sites at any given moment are determined quantitatively by the number of MELT motifs that are phosphorylated and qualitatively by the affinity of Bub3 to the individual motif.

Although these recent studies all recognize the significance of phosphorylated MELT motifs for the recruitment of Bub1 and BubR1, many questions still remain. Importantly, Bub3–BubR1 can associate with a phosphorylated-MELT-containing peptide, but it is not clear whether this interaction is direct (Zhang et al., 2014). Moreover, unlike Bub1, BubR1 requires the context of kinetochores in order to bind to Knl1. In agreement with this, BubR1 recruitment to kinetochores is strictly dependent on Bub1 and Bub3, whereas that of Bub1 is independent of BubR1 (Millband and Hardwick, 2002; Millband et al., 2002; Chen, 2002). A related question is whether the occupancy of MELT motifs by Bub1 and BubR1 obey a 1:1 stochiometry and, if so, how the binding of these checkpoint proteins is regulated. Future efforts will need to focus on answering precisely how BubR1 is recruited to the kinetochore and whether this is achieved through a direct interaction with Bub1, in order to fully understand its mechanism of kinetochore docking.

Moreover, Bub1 kinase activity is required for chromosome biorientation; this raises the question of whether there is a feedback mechanism between Bub1 and Knl1. Can Bub1 phosphorylate Knl1 at its MT-binding domain and thus regulate its MT-binding activity? In addition, the threonine residue of the MELT motifs has emerged as a crucial Mps1 substrate for SAC functionality and chromosome alignment. However, are all MELT sequences equally well suited as substrates for Mps1, or is there a differential affinity of Mps1 for different MELT motifs that could constitute an additional layer of Mps1 regulation? Are the flanking sequences of the MELT motif, for example, TΦ-(F/Y)-(ST)-(DE), phosphorylated and does this have an effect on BUB recruitment? In human Knl1, the threonine residue of the T$\Phi\Phi$-(F/Y)-(ST)-(DE) sequence loosely resembles a Plk1 and/or Mps1 phosphorylation site in most MELT modules. In addition, the Knl1 region encompassing the TΦ-(F/Y)-(ST)-(DE) sequence is predicted to be of low structure complexity, suggesting that an incorporation of phosphate groups might be tolerated. The identification of the MELT motif and the characterization of Mps1 as the principal kinase that regulates this motif are important discoveries, and biochemical and structural studies of how the extended MELT motif coordinates BUB recruitment will shed more light onto the molecular interactions underpinning SAC regulation.

KI motifs

Two KI (lysine-isoleucine) motifs, defined by the consensus sequence KI(D/N)FxxF(L/I)xRL but named after the first two residues of the sequence present in human Knl1, are located near

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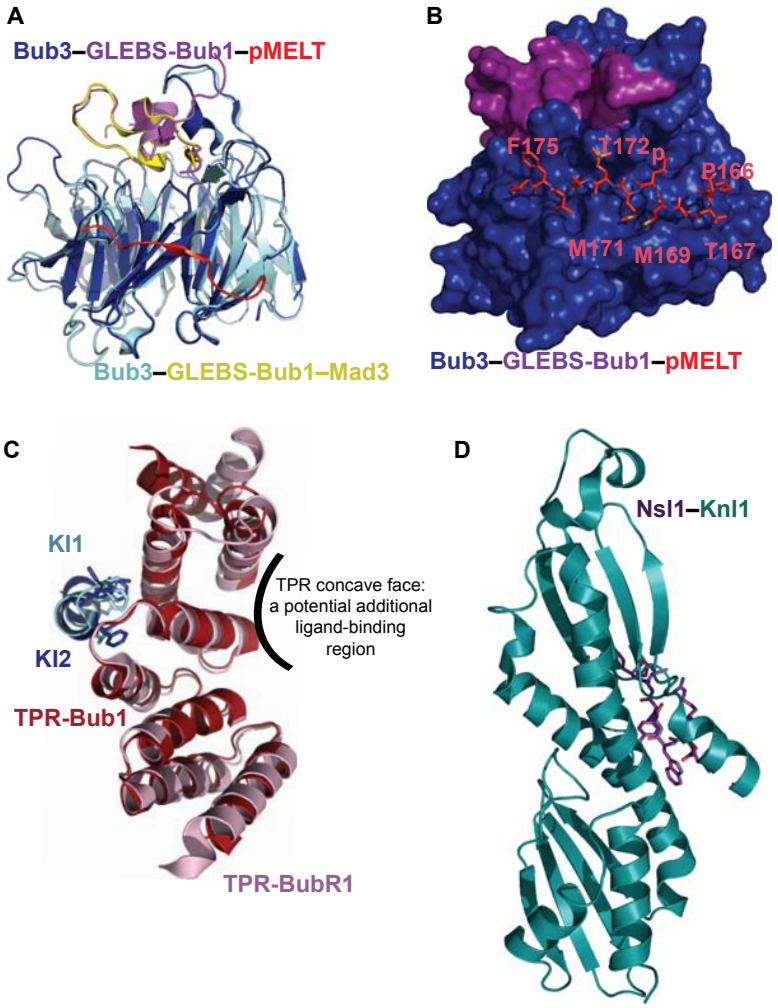

Fig. 2. Structures of SAC and kinetochore complex components. (A) Two protein-binding motifs have been identified in Bub3. Structure superposition of the Bub3–Bub1-GLEBS motif binary complex (PDB ID 2I3S) and the Bub3–Bub1-GLEBS–pMELT motif ternary complex (where pMELT is a peptide mimic of the phosphorylated MELT motif) (PDB ID 4BL0), showing that only minor conformational changes occur in Bub3 upon binding of the phosphorylated MELT motif. In contrast, upon complex formation the pMELT mimic peptide is likely to undergo important conformational changes from a disordered to an ordered state. (B) The structure of the ternary complex shows that few residues of the phosphorylated MELT motif contribute to the interaction and that Bub1 N-terminal residues define a 'roof' on the phosphorylated MELT. (C) Structure superposition of the binary complexes of the TPR from Bub1 and the Knl1 KI1 motif (PDB ID 4A1G), and the TPR from BubR1 and the Knl1 KI2 motif (PDB ID 3SI5), showing that both TPRs undergo little conformational changes upon Knl1 binding. (D) The C-terminal region of Knl1 is organized as double tandem arrangement of the RWD motif and mediates Knl1 binding to Nsl1 (PDB ID 4NF9).

the N-terminus of Knl1, downstream of the first MELT motif (Fig. 1; Fig. 3). Each KI motif shows unique functional features; the first one (KI1) mediates the recruitment of the Bub3–Bub1 complex and the second (KI2) that of Bub3–BubR1 (Kiyomitsu et al., 2007; Kiyomitsu et al., 2011). The binding of Bub1 and BubR1 to KI1 and KI2, respectively, is mediated by a tandem arrangement of the tetratricopeptide repeat (TPR) motif (Box 2), thus defining a TPR domain (Fig. 2C,D). In the case of Bub1, conserved residues (I_{177}, T_{179}, F_{182} and L_{186}) of KI (I_{177}xTxxFLxxL_{186}) define an extensive hydrophobic interface (Krenn et al., 2012). The interaction of the TPR domain of BubR1 with KI2 shows a similar extensive complementary hydrophobic interface that involves residues I_{213}, F_{215}, F_{218}, I_{219} and L_{222} of Knl1 (Bolanos-Garcia et al., 2011). Remarkably, neither the KI1 nor the KI2 motif can substitute for another, suggesting that the TPR domains of Bub1 and BubR1 are not interchangeable and have likely evolved specificity for their respective KI motifs and potentially other binding partners. Indeed, considering that the concave face that is defined by the tandem arrangement of the TPR motif conforms to the mode of ligand binding observed in several TPR–ligand complexes [such as protein phosphatase 5 (PP5) in complex with a heat shock protein 90 (HSP90) (PDB ID 2BUG); heat shock organizing protein (HOP, also known as STIP1) in complex with a heat shock 70 (HSC70) and HSP90 mimic peptides (PDB IDs 1ELW and 1ELR, respectively); and PEX5 in complex with a peroxisomal targeting signal-1 (PTS1) cognate peptide (PDB ID 1FCH) (revised in Bolanos-Garcia and Blundell, 2011)], and that Knl1 binding to Bub1 and BubR1 occurs principally at the non-canonical convex interface of the TPR fold (Bolanos-Garcia et al., 2011; Krenn et al., 2012), it is possible that the TPR domains of Bub1 and BubR1 recruit additional binding partners through alternative interfaces (Fig. 2C).

The presence of KI1 and KI2 has only been identified with confidence in vertebrate Knl1 orthologs (Kiyomitsu et al., 2011; Bolanos-Garcia et al., 2011; Krenn et al., 2012; Vleugel et al., 2012), suggesting that the KI motif has evolved recently (Vleugel et al., 2012; Vleugel et al., 2013). However, in the context of the full-length Knl1 molecule, the interaction of KI1 with the TPR domain of Bub1 is not sufficient for the recruitment of Bub1 to kinetochores (Krenn et al., 2012; Yamagishi et al., 2012). In addition, neither the presence of KI1 nor KI2 in Knl1 is an absolute requirement for SAC activity or chromosome alignment (Krenn et al., 2012; Yamagishi et al., 2012). Moreover, although the Bub1 TPR domain has been reported to be necessary for optimal Bub1 kinase activity (Krenn et al., 2012; Ricke et al., 2012), a Bub1 TPR mutant that is unable to interact with KI1 does not exhibit altered kinase activity or kinetochore localization of Bub1 (Krenn et al., 2012; Yamagishi et al., 2012), suggesting that it is not the interaction with KI1 per se that promotes catalytic activity. Similarly, KI2 is not necessary for the robust kinetochore recruitment of BubR1 (Yamagishi et al., 2012). Nevertheless, although it appears that the KI motifs are

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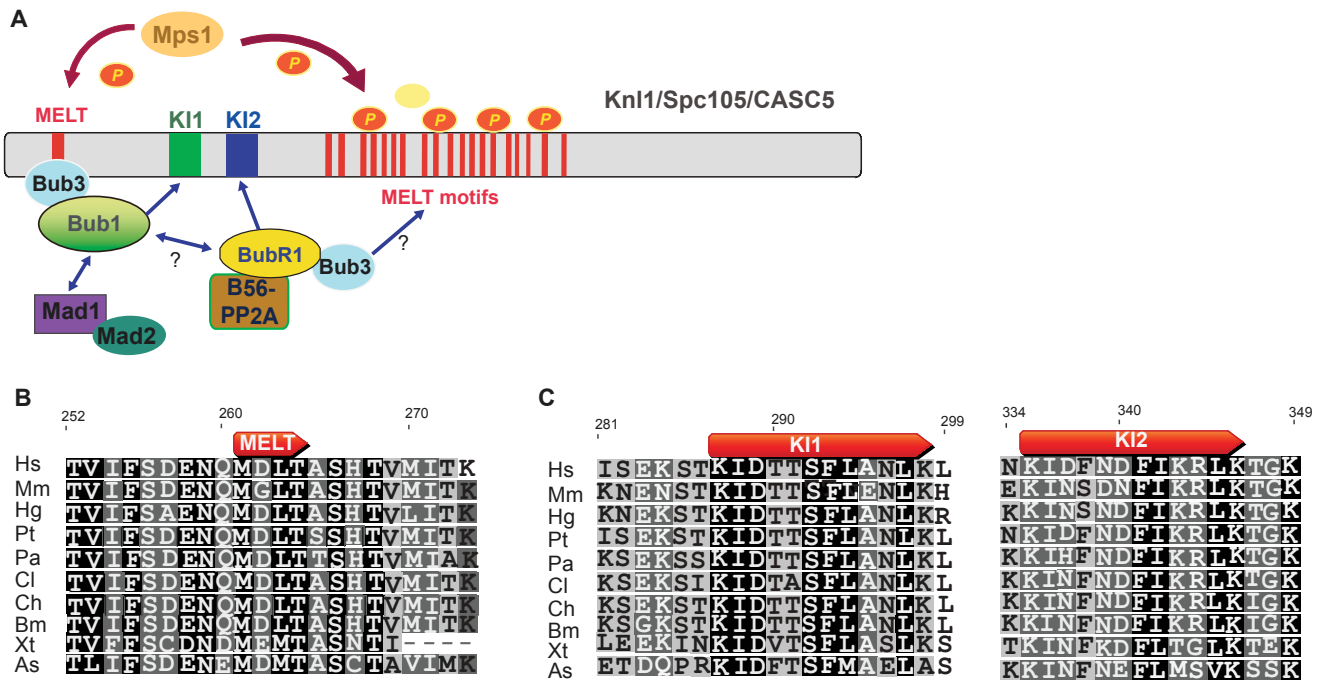

Fig. 3. Function of MELT motif repeats. (A) The threonine residues of the MELT repeats are the target of the mitotic checkpoint kinase Mps1. The KI1 and KI2 motifs of KNL1 can cooperate with Msp1-phosphorylated MELT motifs to promote and sustain binding to Bub1, BubR1 and Bub3. For simplicity, the cartoon shows the phosphorylation of only a few putative MELT repeats in human KNL1 that are located downstream of the KI motifs. The interactions with Bub1, BubR1 and Bub3 induce important local conformational changes in KNL1. Whether such structural rearrangements in KNL1 can stimulate the catalytic kinase domain of Bub1, contribute to the recruitment of BubR1 to the kinetochore, and mediate the direct interaction between Bub1 and BubR1 and/or the binding of Bub3 to additional KNL1 sites, are important aspects that require further investigations. Furthermore, other SAC components, such as Mad1 and Mad2, might also be involved. For example, work from the Biggins laboratory has shown that, in yeast, Mad1 recruitment to the kinetochore involves the binding of Mad1 to the middle region of Bub1 and that the process requires the phosphorylation of the latter protein by Mps1 (London and Biggins, 2014). (B,C) Amino acid sequences alignments of the MELT, KI1 and KI2 motifs, respectively, from diverse organisms. Hs, *Homo sapiens*; Mm, *Mus musculus*; Hg, *Hetecephalus glaber*; Pt, *Pan troglodytes*; Pa, *Pteropus alecto*; Cl, *Canis lupus*; Bm, *Bos mutus*; Ch, *Capra hircus*; Xt, *Xenopus tropicalis*; As, *Alligator sinensis*. The sequence alignments indicate that these functional motifs are highly conserved in these organisms.

dispensable for the overall presence of BUB proteins at the kinetochore, they might still contribute to the efficiency of chromosome congression (Krenn et al., 2014; Vleugel et al., 2013). In support of this notion, deletion of Knl1 residues 150–300 (encompassing MELT1, KI1, K2 and MELT2) results in a 5- to 10-minute delay in metaphase alignment compared to when full-length Knl1 is present, despite recruiting similar amounts of Bub1 to the kinetochore in both cases (Vleugel et al., 2013).

Studies of Knl1 truncations have come to similar conclusions: N-terminal Knl1 fragments that encompass only the first MELT motif and KI1 and KI2 have shown that both KI motifs cooperate strongly with the adjacent single MELT repeat to promote the recruitment of mitotic checkpoint proteins to the kinetochore (Krenn et al., 2014; Vleugel et al., 2013). Interestingly, one study reported that removal of the BubR1-specific KI2 motif in the context of a N-terminal Knl1 fragment results in reduced Bub1 recruitment, prompting the idea that interactions between BubR1 and KI2 stabilize Bub1–KI1 interactions (Krenn et al., 2012). Moreover, the concerted binding of Bub3 and Bub1 to a phosphopeptide that mimics the phosphorylated MELT1–KI region of Knl1 occurs in a cooperative manner, which is in agreement with their mutual dependency for kinetochore loading (Primorac et al., 2013). Strikingly, inclusion of KI1 and KI2 sequences downstream of a single functional MELT repeat from the central region of Knl1 provides this otherwise non-functional engineered fragment, with the capability of enhanced Bub1 recruitment and SAC function. These results collectively support the view that the KI1 and KI2 motifs act as enhancers of binding. Details of how exactly this is achieved is not readily apparent, although some clues have emerged from structural studies.

Superposition of the 3D structures of TPR Bub1 and TPR BubR1 domains with the structures of the Bub1–Knl1 and BubR1–Knl1 complexes indicate that the two TPR domains undergo only a little conformational change upon Knl1 binding, although we have reported a disorder-to-order transition of a Knl1 KI2 mimic peptide upon BubR1 binding (Bolanos-Garcia et al., 2011). Such a transition might be important in presenting unbound flexible regions of Knl1 to specific kinases and/or phosphatases, and it will be important to investigate whether Bub1 binding to Knl1 primes the adjacent MELT motif for recognition by Mps1 and binding to Bub3. Recruitment of the TPR domain of Bub1 to KI1 in Knl1 might also contribute to BubR1 recruitment to the kinetochore (Johnson et al., 2004; Klebig et al., 2009). Undoubtedly, future structural studies of the N-terminal phosphorylated MELT, KI1 and KI2 complex in association with Bub3 and a Bub1 fragment that includes both the TPR domain, as well as the Bub3-binding region, will go a long way to solving this issue.

RWD domain

In metazoans, the C-terminal region of Knl1 shows a high degree of amino acid residue conservation. This region is organized as a

compact globular domain that is characterized by the presence of the RWD domain and a coiled coil region (Petrovic et al., 2010; Petrovic et al., 2014; Fig. 2D). The RWD domain physically interacts with the Mis12 complex component Nsl1 (Petrovic et al., 2014), whereas the Knl1 coiled coil domain mediates the binding of Knl1 to Zwint (Fig. 1). Both the Mis12 complex and Zwint are required for proper kinetochore assembly (Caldas and DeLuca, 2013). The RWD domain, which is named after three major RWD-containing proteins: RING finger-containing proteins, WD-repeat-containing proteins and yeast DEAD (DEXD)-like helicases, is typically organized as α+β sandwich fold with an α-β-β-β-α-α topology. One structural feature of RWD domains is the presence of the YPxxxP motif (residues L_{231}PSPYP$_{236}$ in Ctf9 from *Kluyveromyces lactis*). Often, the YPxxxP motif forms a stable loop that includes three consecutive β-turns that overlap with each other by two residues (triple β-turn), as seen in the 3D structures of the RWD domains of GCN2 (also known as EIF2AK4), ubiquitin-conjugating enzymes (E2s) and the kinetochore proteins Spc24 and Spc25, to name a few (Nameki et al., 2004; Schmitzberger and Harrison, 2012). Mutation of residues within the YPxxxP motif often impairs protein stability (Nameki et al., 2004; Schmitzberger and Harrison, 2012). The recently reported crystal structures of the Knl1 C-terminal domain alone and in complex with Nsl1 (PDB 4NFA and 4NF9, respectively) confirm that the region adopts a RWD-fold that shares similar structural features to Csm1, Spc24, Spc25, Ctf19 and Mcm21 (Nishino et al., 2013; Petrovic et al., 2014), as well as the mitotic checkpoint protein Mad1 (Kim et al., 2012). Interestingly, in human Knl1, the RWD contains a non-canonical YPxxxP motif (YPxxP) that is defined by the residues Y_{2262}PSVP$_{2266}$ (supplementary material Fig. S1). Such a YPxxP motif is located far away from the Nsl1-binding region (by ~19 Å). However, given the important local rearrangements of the RWD domain upon Nsl1 binding, it would be important to define whether this motif contributes to Knl1 function(s), including its binding to the Mis12 complex. The overall structural architecture of RWD and E2s motifs, including the triple β-turn, is fundamentally conserved across species, suggesting that the RWD motif is a recurrent structural module of kinetochore architecture. Considering that tandem arrangements of repeat motifs are a common feature of the mitotic checkpoint kinases Bub1, BubR1 and Mps1 that regulate chromosome segregation, it is tempting to speculate that modular functional motifs of central components of the SAC and the KMN network have co-evolved.

A pattern of disorder-to-order transitions in SAC signaling is emerging from the structures of diverse complexes, including that of the TPR domains of Bub1 and BubR1 in complex with the KI motifs of Knl1, Bub3 bound to Knl1 MELT motifs and more recently, the Knl1 RWD domain in complex with a Nls1 mimic peptide (residues Q_{266}–P_{274}) (Bolanos-Garcia et al., 2011; Krenn et al., 2012; Primorac et al., 2013; Petrovic et al., 2014). In the latter case, Nsl1 appears to undergo a disorder-to-order transition that results in the formation of a tight helical turn upon formation of the complex (Petrovic et al., 2014). Another common feature that emerges from the analysis of these complexes is the predominance of cooperative hydrophobic interactions that stabilize them. At the same time, important differences can be noted. For example, important local conformational changes in the Knl1 RWD region surrounding residue Y_{2125} take place upon Nls1 binding, whereas in the complexes between BUB and the Knl1 KI motif or BUB and the MELT motif only small conformational changes are observed after formation of the complex. It will be important to establish to what extent other interactions of the KMN network that involve RWD-containing proteins are mediated by the RWD domain. Similarly, it is unclear whether similar important local structure rearrangements of the RWD domain underlie the interactions mediated by the C-terminal region of Knl1 and how such conformational changes can contribute to modulate SAC signaling.

Low structural complexity of Knl1 – implications for cell signaling

With the exception of the globular RWD domain, Knl1 shows multiple regions of low structure complexity that are distributed throughout its entire polypeptide chain. A high frequency of regions of low structure complexity is a feature of hub proteins that are found in interactome networks (Babu et al., 2012; Kim et al., 2006; Dosztányi et al., 2006; Dunker et al., 2005; Dunker et al., 2008; Haynes et al., 2006). Although it is not surprising that suppression of the expression or impairment of the stability of hub proteins can have a profound effect on the function of an entire interaction network (Albert, 2005; Albert et al., 2000), it would be interesting to study in greater detail the consequences that the suppression of Knl1 expression has on SAC signaling. The establishment of large and highly flexible surfaces that mediate productive intermolecular interactions is a crucial requirement for the proper assembly of a number of macromolecular complexes, such as ribosome proteins (Peng et al., 2014) and those defining the Wnt pathway (Xue et al., 2012; an excellent review of the role of regions of low structure complexity for the formation and regulation of macromolecular assemblies is Nishi et al., 2013). This might well be the case for MELT motif phosphorylation by Mps1, which could contribute to the presentation of the Knl1 KI motif to the TPR domain of Bub1, and of KI2 to the TPR domain of BubR1, thereby enhancing their binding to Knl1. Whether the binding of Bub1 and BubR1 to their specific KI motifs follows a concerted or sequential mechanism *in vivo* remains to be established. Moreover, the disorder-to-order transition of Knl1 upon binding of Bub1 or BubR1 might facilitate the exposure of flexible and unbound regions of Knl1 to specific kinases and/or phosphatases, a process that is important for its function in the SAC. The fact that the recognition sites for PP1 and Aurora B that have been mapped to the N-terminal region of Knl1 are in close proximity to KI1 and KI2 appears to support a mode of concerted interactions that underlie local disorder-to-order transitions (Liu et al., 2010; Rosenberg et al., 2011). In such a coordinated and possibly cooperative mode of interaction, protein colocalization and concentration to a defined region should provide a structural framework to mount an effective SAC response.

The association of proteins through the interaction of regions of low structure complexity in the crowded environment of the cell might influence the kinetics of association of protein complexes, as well as their stability and remodeling (Banks and Fradin, 2005; Cino et al., 2012; McGuffee and Elcock, 2010; Miermont et al., 2013; Dyson and Wright, 2005), including processes that underlie mechanotransduction events (Pan et al., 2012). It is tempting to speculate that these types of interactions have an important role in the communication between the KMN and the SAC. The levels of SAC proteins at kinetochores are dynamically regulated during the attachment of kinetochores to microtubules, with maximal levels being reached at unattached kinetochores (Lara-Gonzalez et al., 2012; Foley and Kapoor, 2013). It is likely that activation and inactivation of checkpoint proteins at kinetochores in response to

the status of kinetochore–MT attachment through phosphorylation and dephosphorylation events will prove to be crucial for the regulation of this essential, intricate and highly dynamic cellular process. Furthermore, binding of SAC proteins to the docking platform that is presented by Knl1 might induce allosteric conformational changes that coordinate multiple activities at the kinetochore. This is an interesting possibility that deserves further investigation.

Conclusions and perspectives

Intrinsically disordered proteins frequently associate with binding partners through low affinity, but highly specific, interactions to mediate an effective response in cell cycle regulation. Examples of these classes of interactions are $p21^{WAF1}$ (encoded by *CDKN1A*) binding to casein kinase II, and $p27^{KIP1}$ (encoded by *CDKN1B*) to Cdk2–cyclin-A. This often involves multiple linear motifs that mediate the interaction with one or more ligands, thus providing a layer of regulation of the cellular response. In addition to the interactions that are mediated by the Knl1 RWD domain, the intrinsic structural flexibility of Knl1 is likely to be crucial for the establishment of the numerous, productive and specific interactions that it undergoes in the crowded environment of the cell. Furthermore, regions of low structure complexity in other SAC proteins and KMN components might have essential roles in the control of chromosome segregation, as greater selectivity can be gained by the involvement of multiple components.

Most Knl1 homologs contain an array of repeating modules that are unique to this protein, although, as discussed above, the number and sequence of those modules varies considerably across species. It will be important to define more precisely the requirement of species-specific motifs that account for Knl1 partner recognition in different organisms. An understanding of the Knl1 interactions triggered in response to unattached kinetochores and how these are organized in space and time should provide fundamental insights into the molecular mechanism(s) that regulate the early events of SAC signaling. The molecular understanding of Knl1 functions in mitotic checkpoint signaling thus remains an important and challenging task that will require intensive – and indeed inventive – research efforts.

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Competing interests

The authors declare no competing interests.

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Supplementary material

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