The Ndc80 Loop Region Facilitates Formation of Kinetochore Attachment to the Dynamic Microtubule Plus End

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Summary

Proper chromosome segregation in mitosis relies on correct kinetochore-microtubule (KT-MT) interactions. The KT initially interacts with the lateral surface of a single MT (lateral attachment) extending from a spindle pole and is subsequently anchored at the plus end of the MT (end-on attachment) [1]. The conversion from lateral to end-on attachment is crucial because end-on attachment is more robust [2–4] and thought to be necessary to sustain KT-MT attachment when tension is applied across sister KTs upon their biorientation [1]. The mechanism for this conversion is still elusive. The Ndc80 complex is an essential component of the KT-MT interface [1,5], and here we studied a role of the Ndc80 loop region, a distinct motif looping out from the coiled-coil shaft of the complex [6], in Saccharomyces cerevisiae. With deletions or mutations of the loop region, the lateral KT-MT attachment occurred normally; however, subsequent conversion to end-on attachment was defective, leading to failure in sister KT biorientation. The Ndc80 loop region was required for Ndc80-Dam1 interaction and KT loading of the Dam1 complex, which in turn supported KT tethering to the dynamic MT plus end [3,7]. The Ndc80 loop region, therefore, has an important role in the conversion from lateral to end-on attachment, a crucial maturation step of KT-MT interaction.

Results and Discussion

KT-MT interaction develops in a step-wise manner [1]. The KT initially interacts with the MT lateral surface (lateral attachment) and slides along the MT towards a spindle pole (Figure 1A, i, ii). Then, the KT is tethered at the end of the MT (end-on attachment) and transported further as the MT shrinks (end-on pulling) (Figure 1A, iii). Subsequently both sister KTs interact with MTs, and aberrant KT-MT interactions are removed by error correction (Figure 1A, iv, v) until sister KT biorientation (i.e., sister KTs attaching to MTs from opposite spindle poles) is established (Figure 1A, vi).

The Ndc80 complex is an outer (i.e., closer to the MT) KT component, composed of four proteins (Figure 1B), and has a central role in comprising the KT-MT interface [1,5]. The Ndc80 complex binds directly to the MT lateral surface in vitro, at the calponin-homology (CH) domain within Ndc80 protein (also called Hec1) [8–10], and the complex is indeed required for the lateral KT-MT attachment in vivo [11]. Presumably the Ndc80 complex is also involved in the end-on KT-MT attachment. Consistent with this, an injection of an antibody against the Ndc80 CH domain changed the dynamics of KT-MT interactions in metaphase [12]. Moreover, the Ndc80 complex can couple a microsphere at the end of a dynamic MT in an in vitro reconstituted system [13]. Thus, it is likely that the Ndc80 complex is directly involved in both the lateral and end-on KT-MT attachment. Given this, the Ndc80 complex may play a key role in the conversion from the lateral to end-on attachment.

Mutations within the Ndc80 Loop Region Lead to Cell Lethality or Temperature-Sensitive Cell Growth

The Ndc80 complex forms a long rod-shape structure with two globular domains at each end [8–10] (Figure 1B). While one globular domain interacts with a MT, the other binds the Mtw1 complex (Mis12 complex in metazoa), a relatively inner KT component, i.e., closer to the centromere. These two globular domains are connected by long coiled-coil motifs. Peculiarly, this coiled-coil shaft is interrupted in the middle of Ndc80 protein [6] by a region of 50–60 amino acid residues that does not conform to the coiled-coil structure [10] (Figure 1C), thus presumably looping out from the coiled-coil shaft and hence called the loop region (Figure 1B). Indeed, electron microscopy revealed that the coiled-coil shaft of the Ndc80 complex showed a kink or flexible bend at the position of the loop region [14]. Intriguingly, the loop region contains several evolutionarily conserved amino acid residues (Figure 1D) and probably forms a β-sheet structure that may be involved in protein-protein interaction [14].

To address the role of the Ndc80 loop region, we constructed yeast strains whose only ndc80 harbors a deletion of 20–40 amino acid residues within the loop region, i.e., ndc80Δ480-520, ndc80Δ480-510, and ndc80Δ490-520, and ndc80Δ490-510 (Figure 1C). Deletions ndc80Δ480-520, ndc80Δ480-510, and ndc80Δ490-520 could not support cell viability at any temperature tried (data not shown), whereas ndc80Δ490-510 cells showed growth at 25 °C but not at 35 °C (Figure 1E). We also constructed strains whose only ndc80 had substitution of alanines for seven conserved amino acid residues within the loop region (and thus called ndc80Δ-7A; Figure 1D). The ndc80Δ-7A mutant cells showed growth at 25 °C but not at 35 °C (Figure 1E). Such temperature-sensitive growth of ndc80Δ490-510 and ndc80Δ-7A cells was not due to reduced expression of mutant Ndc80 proteins or a defect in interaction with Nuf2, another component of the Ndc80 complex (Figure 1F).

Mutations in the Ndc80 Loop Region Support Initial KT-MT Interaction Normally but Sister KT Biorientation Is Defective

To address possible roles of the Ndc80 loop region in KT-MT interactions, we visualized MTs and a selected centromere (CEN5) by live-cell imaging and compared their behavior in wild-type, ndc80Δ490-510, and ndc80Δ-7A mutant cells at 35 °C. spc24-1 mutants are defective in KT-MT attachment...
In *ndc80* mutants, sister *CEN5* were on the spindle but their separation was delayed ([Figure 2B](#)), and in most *spc24-1* cells, sister *CEN5* remained unseparated and did not localize on the spindle ([Figure 2B](#) ii). Thus, in mutants of the Ndc80 loop region, the establishment of sister KT biorientation is defective although KTs are caught on the spindle.

Meanwhile, the *ndc80* mutants also showed failure to satisfy the spindle-assembly checkpoint [19] ([Figure S2C](#)). We also compared the nature of the biorientation defect in these mutants with that found in *ipl1* mutants with that found in *C. elegans* (Figure S1A) [11]. This procedure prevented *CEN3* from localizing on the mitotic spindle. While cells were arrested in metaphase, we reactivated *CEN3*, which led to KT reassembly and interaction with MTs extending from
a spindle pole (spindle-pole MTs). This assay allowed observation of the individual KT-MT interaction with high spatial resolution because CEN3 moved away from the spindle prior to its reactivation [11].

In agreement with the results in Figure 2, in ndc80Δ490-510 and ndc80-7A mutant cells, CEN3 was captured by the lateral surface of a spindle-pole MT at 35°C with similar kinetics as wild-type cells; by contrast, subsequent sister CEN3 separation on the spindle proceeded more slowly compared with wild-type cells, indicative of a delay in sister CEN3 biorientation (Figure 3B). On the other hand, in spc24-1 cells, the initial CEN3 capture by MTs was defective [11].

By using live-cell imaging, we investigated CEN3-MT interaction in further detail. In wild-type cells, after the initial CEN3-MT interaction, CEN3 moved by sliding along a MT lateral surface toward a spindle pole [11]. While CEN3 was on the MT lateral surface, this MT often underwent depolymerization at its plus end and shrank until its plus end caught up with CEN3 (Figure 3C, i). When this happened, either of the following two events occurred in wild-type cells [3]: (1) CEN3 was tethered at the MT end (end-on attachment) and pulled toward a spindle pole as the MT shrank further (end-on pulling) (40% of cases) or (2) the MT showed regrowth (MT rescue at CEN3) (60% of cases) (Figure 3C, i).

In ndc80Δ490-510 and ndc80-7A mutant cells, CEN3 sliding occurred almost normally, except for a small number (~5%) of ndc80Δ490-510 cells showing CEN3 pausing on a MT during an extended period (data not shown). Remarkably, in both ndc80Δ490-510 and ndc80-7A mutants, the end-on attachment was rarely established at 35°C (Figure 3C, ii), thus making subsequent end-on pulling infrequent (Figure 3C, ii), compared with wild-type cells. Thus, Ndc80 loop region is required for the efficient conversion from lateral to end-on KT-MT attachment.

Notably, defects in end-on attachment correlate well with defects in sister KT biorientation. For example, the ndc80-7A mutant showed milder defects in both end-on attachment and biorientation, compared with ndc80Δ490-510 mutant cells, which showed separated and un-separated sister CEN5s, respectively. (ii) The percentage of cells showing separation of sister CEN5s on the bipolar spindle for at least two consecutive time points, until indicated time points (0 min: establishment of bipolar spindle). Sister CEN5s were scored as “separated” when two signals were discernible. See also Figure S2.
The Dam1 complex has the ability to track the plus end of a shrinking MT [3, 7] and, once loaded on the KT, it mediates the end-on pulling of the KT by a shrinking MT [3]. During this process, the Dam1 complexes form oligomers and/or a ring structure encircling a MT [21]. Thus, the Ndc80 loop and the Dam1 complex may work together to support end-on KT-MT attachment. In this regard, it is intriguing that the Ndc80 and Dam1 complexes showed a physical interaction [22–25]. It was difficult to detect this interaction conclusively via coimmunoprecipitation or a protein pull-down (data not shown), but it could be detected with a yeast two-hybrid assay [23].

We therefore addressed whether the interaction between Ndc80 and Dam1 was dependent on the Ndc80 loop region by using a yeast two-hybrid assay. We first confirmed that all the wild-type Ndc80 and its mutants Ndc80Δ490-510 and Ndc80-7A showed interaction with Nuf2 (Figure 4A, right), consistent with the result in Figure 1F. We also found that wild-type Ndc80 showed a positive interaction with Dam1, as reported previously [23]. However, Ndc80Δ490-510 and Ndc80-7A mutants showed very little interaction with Dam1 (Figure 4A, left). Thus the loop region indeed facilitates interaction between Ndc80 and Dam1.

What is the functional consequence of the Ndc80-Dam1 interaction? The Ndc80 complex is required for loading of the Dam1 complex on the KT [22, 26] and an Ndc80-Dam1 interaction may facilitate this process. If so, the Ndc80 loop region might be required for Dam1 complex loading on
the KT. We tested this possibility by using chromatin immuno-
precipitation. In wild-type cells, centromere DNA (CEN3) was
clearly precipitated with the Dam1 protein and also with the
NuF2 protein (Figure 4B, i, ii). Remarkably, in ndc80 Δ490-510 and
ndc80-7A mutants, CEN3 precipitation with Dam1 was
considerably reduced (Figure 4B, i, ii), although CEN3 precip-
itation with NuF2 was similar between the mutants and wild-
type. This result suggests a defect in Dam1 loading on KTs
in these mutants.

We also compared the localization pattern of Dam1 and
Mtw1 in metaphase (Figure 4C; Figure S4A). Mtw1 is a compo-
nent of the KT [20] and should represent the position of KTs.
Dam1 and Mtw1 showed almost perfect colocalization in
wild-type cells. In ndc80 Δ490-510 cells, the total amount of
Mtw1 and Dam1 on the spindle was not altered (Figure S4A),
but Dam1 signals were often present between two globular
Mtw1 signals (Figure 4C; Figure S4A). Results in Figures 4B
and 4C suggest requirement of the Ndc80 loop region for
Dam1 loading on the KT.

The Ndc80 Loop Region Facilitates Interaction
with the Dam1 Complex to Anchor the KT at the Dynamic
MT Plus End
Our study has revealed that the Ndc80 loop region mediates
the interaction with the Dam1 complex to ensure proper
KT-MT attachment (Figure 4D). With Ndc80 loop-region
mutants, the lateral KT-MT attachment is still largely normal;
consistently, this process does not require the Dam1
complex [11]. On the other hand, the Dam1 complex has an
important role in the end-on KT-MT attachment and subse-
quent end-on pulling of the KT by a MT [3, 7]. With Ndc80
loop region mutants, the Ndc80 and Dam1 complexes cannot
interact properly, leading to the failure in the end-on
attachment.
It was recently demonstrated that the Dam1 complex is able to enhance MT binding of the Ndc80 complex (e.g., its cosedimentation with MTs) in vitro [24, 25]. Given this, by using a condition reported in [24], we evaluated MT cosedimentation of the purified Ndc80 complex with loop mutants; its enhancement by the Dam1 complex was similar to that of the wild-type Ndc80 complex (Figure S4B). We reason that the loop-dependent Ndc80-Dam1 interaction in vivo was not recapitulated in this particular condition in vitro. Alternatively, an additional factor, which is missing in the in vitro reaction, may be necessary for the interaction between Dam1 and the Ndc80 loop region.

In this regard it is intriguing that, in fission yeast, Dis1 (an ortholog of Stu2 in budding yeast and XMAP215/chTOG in vertebrates) showed interaction with the Ndc80 loop region [27]. However, in contrast to fission yeast, Ndc80 and Stu2 showed no interaction in budding yeast (Figure S4C) and Ndc80 loop mutants did not alter Stu2 localization at KTs (Figure S4D). Nonetheless, Stu2 shows interaction with Dam1-complex components in a two-hybrid assay [23]; data not shown). Thus we cannot exclude the possibility that Stu2 (possibly at the end of a shrinking MT) is involved in the Ndc80-Dam1 interaction.

Our finding that the Ndc80 loop region mediates the interaction with the Dam1 complex is consistent with nanometer-scale mapping of KT components in metaphase [28]. The Ndc80 complex bridges between the inner KT and a MT, and its Ndc80/Nuf2 globular head locates further outside (away from the inner KT) of the Dam1 complexes (see Figure 4D). In this configuration, the location of the Ndc80 loop region along the KT-MT axis approximately corresponds to that of the Dam1 complex [28]. The KT-MT attachment has advantages for the initial KT-MT interaction because the MT lateral surface provides a large contact surface, whereas the end-on attachment ensures more robust KT-MT interaction [2–4], which is presumably required for sister KT biorientation. Thus the conversion from lateral to end-on attachment is an inevitable vital step in developing a proper KT-MT interaction. The Ndc80 and Dam1 complexes play central roles in comprising this particular condition in vitro. Alternatively, an additional factor, which is missing in the in vitro reaction, may be necessary for the interaction between Dam1 and the Ndc80 loop region.

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