Disentangling the molecular determinants for Cenp-F localization to nuclear pores and kinetochores

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Abstract

Cenp-F is a multifaceted protein implicated in cancer and developmental pathologies. The Cenp-F C-terminal region contains overlapping binding sites for numerous proteins that contribute to its functions throughout the cell cycle. Here, we focus on the nuclear pore protein Nup133 that interacts with Cenp-F both at nuclear pores in prophase and at kinetochores in mitosis, and on the kinase Bub1, known to contribute to Cenp-F targeting to kinetochores. By combining *in silico* structural modeling and yeast two-hybrid assays, we generate an interaction model between a conserved helix within the Nup133 β -propeller and a short leucine zipper-containing dimeric segment of Cenp-F. We thereby create mutants affecting the Nup133/Cenp-F interface and show that they prevent Cenp-F localization to the nuclear envelope, but not to kinetochores. Conversely, a point mutation within an adjacent leucine zipper affecting the kinetochore targeting of Cenp-F KT-core domain impairs its interaction with Bub1, but not with Nup133, identifying Bub1 as the direct KT-core binding partner of Cenp-F. Finally, we show that Cenp-E redundantly contributes together with Bub1 to the recruitment of Cenp-F to kinetochores.

Keywords Cenp-F; *in silico* modeling; kinetochores; mitosis; nuclear pore

Subject Categories Cell Cycle; Structural Biology

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Introduction

Cenp-F (centromere protein F, also known as mitotin in human and Lek1 in mouse) is a large protein that contributes to multiple cellular processes including cell division, gene expression, cell morphogenesis, vesicular transport, and ciliogenesis (reviewed in [1–3]; see also [4–6]). Overexpression of this multifaceted protein has been

linked to various cancers, whereas its inactivation or mutations lead to diverse developmentally related disorders (reviewed in [1,2]; see also [5–7]). Cenp-F has a complex and dynamic expression and localization pattern [8–10]: its expression peaks in G2 when it is mainly detected within the nuclear matrix, with a minor fraction detected at the centrosomes and mitochondria [11,12]. At the G2/M transition, Cenp-F relocates to the nuclear envelope (NE) where it contributes via NudE/EL to dynein/dynactin recruitment to the NE. This facilitates the positioning of the centrosomes close to the NE in prophase cells [13] and the migration of the nucleus toward the centrosomes prior to mitotic entry in rat neural stem cells [14,15]. In prophase, Cenp-F is recruited to the outer kinetochores (KT) where it persists until early anaphase when it localizes to the spindle midzone before being degraded [8–10]. Cenp-F depletion by RNA interference in human cell lines revealed its implication in kinetochore-microtubule attachment and its contribution to the sustained activation of the spindle assembly checkpoint [16–21]. Some of these phenotypes may reflect kinetochore-dependent functions of Cenp-F, involving interactions with microtubules or its other kinetochore partners such as NudE/EL or Cenp-E [20,22–25]. However, Cenp-F implication in mitosis remains controversial as no major viability or proliferation defect was observed upon its knock-out in mouse embryonic fibroblasts, or upon CRISPR/Cas9-induced depletion in HeLa cells [7,26].

The localization of Cenp-F at kinetochores involves multiple domains including a C-terminal KT-core, an internal repeated sequence, and a C-terminal CAAX farnesylation site [27,28] (see Fig 1A). In addition, RNA interference experiments have shown that kinetochore localization of Cenp-F is impaired upon depletion of various kinetochore proteins including the Bub1 kinase and the kinesin-like motor protein, Cenp-E ([29,30]; reviewed in [1]). However, its direct tether(s) at kinetochores is yet unknown. In contrast, Cenp-F recruitment to the NE in late G2 cells was shown to rely on its direct interaction with a nuclear pore constituent, Nup133 [13], but also implies its farnesylation [28] and its Cdk1-dependent nuclear exit [15]. Nup133 is a subunit of the Nup107-160

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Figure 1. Minimal domains required for the Y2H interaction between Nup133 and Cenp-F.

A Schematics of Cenp-F and its C-terminal domain based on data gathered for human and mouse Cenp-F. The 115-aa domain found in human but not mouse Cenp-F is indicated by a gray triangle (see the corresponding sequence alignment in Fig EV1 for details). Predicted leucine zippers (black diamonds), internal repeated sequence (arrows), binding sites for microtubules (MT-binding), Cenp-E, NudE/EL, Rb and Miro, the core region required for kinetochore binding (KT-core), nuclear localization signal (NLS), the conserved KEN7 degradation motif (KEN), and farnesylation site (F) are indicated (see text for references). Numbers refer to amino acid positions for human and mouse Cenp-F. The position of hCenp-F-Ct, mCenp-F-Ct1, and mCenp-F-Ct2 and of human and mouse Cenp-F-SID segments is also shown.

B Scheme of mNup133 protein organization, and structure of its N-terminal β -propeller. The dashed lines correspond to the unstructured N-terminal residues (aa 1–66) and the extremities of the ALPS loop (aa 251–268) not included in the model. The four helices or loop regions (α 1, L2, α 2/3, L4) protruding out of the modeled mNup133 β -propeller structure are colored in red.

C Interactions between the indicated mNup133 fragments cloned into pB27-LexA and mCenp-F C-terminal fragments cloned into pP6-Gal4-AD were assayed by Y2H based on growth on -LWH medium as described in Materials and Methods. Empty bait and prey vectors were used as negative controls (–). Western blots demonstrating the proper expression of the LexA and GAL4-AD fusions are presented in Appendix Fig S2A(i) and B(i).

complex (Y-complex), a key structural element of the nuclear pore complex (NPC). In interphase, repeated copies of the Y-complex assemble to form the cytoplasmic and nuclear rings of the NPCs [31,32] although a fraction of this complex can also be found inside the nucleoplasm [33]. In mitosis, a pool of the Y-complex is recruited to the kinetochores where it is required for proper chromosome segregation [34,35]. Noteworthy, kinetochore localization of

the Y-complex relies on both Cenp-F and the Ndc80 complex [34], showing a dual function of the Nup133/Cenp-F interaction at both nuclear pores and kinetochores. Our previous studies revealed that the N-terminal domain (NTD) of Nup133 interacts with a C-terminal region of Cenp-F (hCenp-F; [aa 2,644–3,065]; Fig 1A) [13,34], but how this interaction is regulated in time and space remains largely unknown. Moreover, this region of Cenp-F encompasses one of its

two microtubule binding domains [20] and binding sites to diverse partners including Rb [36], ATF4 [37], and Miro [12], but also its KT-core domain [27] (Fig 1A).

In view of the multiplicity of Cenp-F partners within its C-terminal region, and considering the interplay between Nup133 and Cenp-F at nuclear pores and kinetochores, functional studies of Cenp-F in mitosis critically require the generation of separation-of-function mutations that discriminate Cenp-F interaction with Nup133 from its other targeting determinants, notably at kinetochores.

By combining *in silico* modeling and yeast two-hybrid (Y2H) assays, we have now identified critical surfaces and specific amino acids required for Cenp-F/Nup133 interaction. We could thus define mutations that differentially impact Cenp-F localization at the NE in prophase and kinetochores in mitosis. Moreover, we show that Bub1 is the direct KT-core binding partner of Cenp-F that contributes, along with Cenp-E, to the recruitment of full-length Cenp-F to kinetochores.

Results and Discussion

In silico modeling of Cenp-F/Nup133 interaction

To specifically disrupt the Cenp-F/Nup133 interaction without affecting the other functions of these proteins, we aimed at refining their assembly mode and identifying critical amino acids specifically required for their interaction.

We used ULTImate Y2H screens (Hybrigenics) to precisely delineate the Nup133 binding domain on both human and mouse Cenp-F (see Materials and Methods). Pairwise protein sequence alignment revealed that the predicted minimal domain of Cenp-F interacting with Nup133 (selected interacting domain, SID[®] as defined by Hybrigenics) independently identified in these two species corresponded to nearly the same conserved domain within the Cenp-F C-terminal region (Figs 1A and EV1). We validated this predicted binding domain by demonstrating that a construct encompassing aa 2,655–2,723 of mCenp-F (subsequently named mCenp-F-SID) interacted with mNup133-N-terminal domain (NTD) in the Y2H assay as did the original fragments isolated in the screen (mCenp-F-Ct1 and mCenp-F-Ct2; Fig 1A and C).

We then sought to model the structure of the mCenp-F-SID segment. This region of mCenp-F has not been crystallized so far, but the C-terminal domain of Cenp-F features potential leucine zippers and was reported to homodimerize in a Y2H assay [9,10]. Bioinformatics analyses indeed predicted that most of this mCenp-F segment adopts coiled-coil conformations ([9]; Fig EV2A). We first confirmed the dimerization of the mCenp-F-Ct2 domain by *in vivo* crosslinking of HeLa cells transfected with HA-tagged-mCenp-F-Ct2 (Fig EV2C). Because the mCenp-F-Ct2 segment encompasses two predicted leucine zippers, we next characterized the oligomeric status of the most conserved region in Cenp-F-SID domain (Cenp-F-miniSID, aa 2,663–2,706 in mCenp-F; Fig EV2A and B) using SEC-MALS (size-exclusion chromatography–multi-angle light scattering; Fig EV2D). This approach revealed the propensity of this short wild-type (WT) peptide to dimerize, notably at higher salt concentration (Fig EV2D(ii)). This trend is consistent with the high isoelectric point of the studied peptide (pI = 9), whose numerous positively

charged residues induce repulsive electrostatic forces counteracting the stability of the coiled-coil.

We could therefore confidently model the Cenp-F-miniSID domain as a continuous parallel coiled-coil (see Materials and Methods; Fig EV2B). Consistent with this model, mutation of two buried leucines (d-position in the coiled-coil heptad; Fig EV2B) to glycine (mCenp-F^{L2668G/L2696G}) disrupted the stability of the coiled-coil as revealed both by *in vivo* crosslinking and by SEC-MALS assays. In contrast, the L2681E/L2683E mutant targeting surface-exposed residues did not affect the dimerization properties *in vivo*, and even stabilized the miniSID dimeric coiled-coil, likely by counteracting the repulsive electrostatic forces (Fig EV2C and D).

With regard to mNup133, we modeled the structure of its NTD based on the experimental structure of the hNup133 N-terminal β -propeller (PDB: 1XKS; see Materials and Methods). Two regions were not resolved in this structure and could therefore not be modeled [38]: (i) the first 66 residues predicted as disordered by MobiDB server [39], and (ii) a disordered loop (aa 244–266 of mNup133) encompassing an ALPS motif (Amphipathic Lipid Packing Sensors) [40] (Fig 1B). However, deletion of either of these domains preserved the interaction between mNup133-NTD and mCenp-F (Fig 1C and Appendix Fig S2).

Because individual reliable structural models could be generated for both the mNup133 β -propeller and mCenp-F C-terminal dimeric coiled-coil segment, we could then use protein–protein docking simulations to explore their possible binding modes. We therefore performed a free docking simulation based on the 3D structures modeled for both domains. We used the InterEvDock server [41], which is based on a pipeline that previously yielded the best predictions in the 6th community-wide CAPRI (Critical Assessment of PRediction of Interactions) evaluation meeting [42,43]. By default, the 10 most probable binding modes are proposed by InterEvDock. For each of these 10 models, the position of the center of mass of the Cenp-F dimer with respect to Nup133 β -propeller is illustrated by a colored sphere in Fig 2A(i) and (ii).

Challenging mNup133/mCenp-F docking models using Y2H assay

To evaluate whether any of the 10 models represented a relevant binding mode, we first focused on the importance of α -helices (α 1 and α 2/3; [38]) and loops (L2 and L4) decorating the Nup133 β -propeller (Figs 1B and EV3A). This choice was motivated by other examples of β -propellers within the NPC that interact with their partners via loop regions between consecutive blades [44–46]. We first individually replaced each of the four helices or loops within mNup133 β -propeller with glycine–serine-rich sequences (Appendix Table S1). Because replacement of helices α 1 and α 2/3 impaired the expression of the corresponding mNup133 mutant in yeast (Fig EV3B(ii)), we also generated mutants for superficial residues within these two loops. Replacing residues V89, M92, and T96, which form a conserved apolar patch within helix α 1 of mNup133, by aspartic acids (α 1-mut: mNup133^{V89D/M92D/T96D}; Fig 2A(iii)) abrogated interaction with mCenp-F (Figs 2B and EV3B). In contrast, replacing loops L2 and L4 by glycine–serine-rich sequences or mutating/deleting residues E317, D321, and Y330 Δ of helix α 2/3 (α 2/3-mut: mNup133^{E317R/D321R/Y330 Δ}) did not affect interaction with mCenp-F (Fig EV3). This indicated that the Nup133 α 1 helix is the major binding site for Cenp-F.

Figure 2. Modeling of mNup133 recognition by the mCenp-F and validation by Y2H of mutants designed to impair Cenp-F interaction with Nup133.

A Predicted Model 3, involving interaction of mCenp-F-SID (parallel coiled-coil, brown) with helix $\alpha 1$ (red) of mNup133 β -propeller (green). Two orthogonal views are presented in panels (i) and (ii). In (i), Cenp-F mutated residues impairing dimerization L2668G/L2696G are highlighted. (iii) presents a detailed view of the interface, highlighting the interaction between ^{mCenp-F}R2687 and ^{mNup133}E93. Mutations designed to test this model are indicated as sticks and labeled in panels (i) and (iii). The colored spheres in (i) and (ii) represent the center of mass of Cenp-F dimeric segments for the 10 most likely binding modes of Cenp-F to Nup133 as predicted by InterEvDock server (M1 to M10) [41].

B Y2H interactions between the indicated fragments of mNup133 and mCenp-F (Ct1, Ct2, or SID), either WT or bearing the indicated mutations, were assayed based on growth on -LWH medium. The corresponding Western blots are presented in Appendix Fig S2A(ii) and B(i).

Among the best 10 models proposed by the InterEvDock server, only Model 3 (evaluated as the second best model by the InterEvScore [47] and SOAP-PP [48] scoring methods) was consistent with a binding mode involving the $\alpha 1$ helix on mNup133 as the binding site for mCenp-F (Fig 2A). To further assess the relevance of this model, we next designed a set of mutants within either mCenp-F-SID or mNup133 β -propeller and analyzed them by Y2H (Fig 2B and Appendix Fig S2). Consistent with our model that predicted mCenp-F dimer formation to be the structural basis for interaction with mNup133, we observed a loss of mNup133 β -propeller interaction with the mCenp-F-SID L2668G/L2696G mutant that disrupts coiled-coil assembly (Figs 2B and EV2B–D). We then designed mutants targeting surface-exposed residues of mCenp-F and predicted to prevent interaction with Nup133 without impairing Cenp-F dimerization. We generated a double-point mutant (^{mCenp-F}L2681E/L2683E) affecting amino acids predicted to interact with Nup133 residues ^{mNup133}V89/M92/T96, as well as two single-point mutants, ^{mCenp-F}K2678E and ^{mCenp-F}R2687E (Fig 2A). As predicted by the InterEvScore model, the three mutants impaired interaction with mNup133-NTD [aa 1–513] and [aa 67–513] (Fig 2B). Note however that a likely non-specific interaction was observed when the ^{mCenp-F}R2687E mutant was assayed against full-length mNup133 (Fig EV4). To further confirm that our selected InterEvScore model represents an accurate picture of mNup133/mCenp-F interaction,

we finally designed the ^{mNup133}E93R mutation that affects one of the most conserved residues in the mNup133 N-terminal domain (Fig EV3A; Appendix Fig S1). Importantly, this mutation abolished the interaction with wild-type mCenp-F-SID but specifically compensated the R2687E mutation on Cenp-F-SID (Fig 2B). This compensation points to a direct and fundamental contact between ^{mCenp-F}R2687 and ^{mNup133}E93, fully supporting our predicted docking model (Fig 2A(iii) and B).

Together, these data validated our *in silico* structural model, in which the conserved $\alpha 1$ helix protruding out of the mNup133 β -propeller (Fig EV3A) binds to a parallel coiled-coil segment of mCenp-F, centered around a set of conserved apolar residues exposed to the surface of both partners. This analysis further provided us with specific Cenp-F C-terminal mutants affecting its interaction with Nup133.

Recruitment of Cenp-F C-terminal fragments to intranuclear GLFG bodies underlines spatiotemporal regulations of Nup133/Cenp-F interaction

To validate the impact of the characterized Cenp-F mutants in a cellular context, we next transfected HeLa cells with GFP-mCenp-F-Ct1 and GFP-mCenp-F-Ct2 constructs. Both fusions lack the KEN box that leads to Cenp-F degradation after anaphase onset ([49];

Fig 1A) and are thus stably expressed throughout the cell cycle. In interphase cells, both fusions localize in the nucleus (Figs 3A and EV5A). In mitotic cells, a faint albeit clear enrichment at kinetochores is observed in cells expressing the GFP-mCenp-F-Ct2 fusion that encompasses the previously described KT-core domain of Cenp-F (Figs 3B and EV5B(ii); [27]). In contrast, we never detected the GFP-mCenp-F-Ct1 fusion at kinetochores, a result consistent with the fact that it lacks both the NLS and the second leucine zipper present in the KT-core region ([27]; our unpublished data). Finally, unlike endogenous Cenp-F, neither GFP-mCenp-F-Ct1 nor GFP-mCenp-F-Ct2 was detected at the NE in late G2/prophase cells, possibly because they lack the CAAX farnesylation site [28] and/or determinants required for their nuclear export at mitotic onset [15].

Noteworthy, a fraction of both GFP-mCenp-F-Ct1 and GFP-mCenp-F-Ct2 was detected along with Nup133 within discrete intranuclear structures, stained by the GLFG repeat-containing Nup98 nucleoporin and thus corresponding to the GLFG bodies present in HeLa-E cells [33,50] (Figs 3A and EV5A). In contrast, GFP-mCenp-F-Ct2 was not recruited to the Nup98-positive but Nup133-negative GLFG bodies present in HEp-2 cells [51] (Fig EV5A). This indicates that the recruitment of GFP-mCenp-F-Ct2 to the GLFG bodies present in HeLa-E cells relies on the Y-complex, via Nup133. The observation that these C-terminal fragments of

Cenp-F can interact with Nup133 in GLFG bodies throughout the cell cycle demonstrates that cell-cycle-dependent posttranslational modifications are not strictly required for Nup133/Cenp-F interaction. Conversely, unlike GFP-mCenp-F-Ct1 and GFP-mCenp-F-Ct2, full-length Cenp-F is not recruited to Nup133-containing GLFG bodies (Fig EV5B and C) most likely because retention of full-length Cenp-F at other intranuclear sites may restrict its diffusion within the nucleoplasm (as also revealed by its exclusion from the nucleoli). These data, along with previous studies revealing the implication of Cdk1-dependent nuclear exit of Cenp-F for its targeting to the NE [15,52], point to a crucial role of compartmentalization in the regulation of Cenp-F/Nup133 interaction.

Mutations within Cenp-F C-terminal domain discriminate NPC/nuclear bodies from kinetochore targeting

We next used the dual localization of the GFP-mCenp-F-Ct2 domain at both intranuclear bodies in interphase and kinetochores in mitosis as readout to analyze in human cells the effect of the mutations impairing mCenp-F-SID interaction with Nup133. Indeed, since these mutations were clustered around the previously described KT-core domain of Cenp-F [27,37] (Figs 1A and EV1), it was critical to determine whether they impaired KT-targeting of Cenp-F. Upon transient transfection in HeLa-E cells, all the GFP-mCenp-F-

Figure 3. Mutations within GFP-mCenp-F-Ct2 can differentially affect its targeting to intranuclear GLFG bodies and to kinetochores.

A, B HeLa-E cells transiently transfected with GFP-mCenp-F-Ct2, either WT or bearing the indicated mutations, were fixed 2 days after transfection and stained with (A) an antibody directed against Elys, a Y-complex partner, that labels the NPCs and the GLFG bodies in interphase cells or (B) an anti-Hec1 antibody that stains kinetochores in mitotic cells. Cells were also stained with DAPI. For each transfected construct, a representative interphase cell (3D reconstruction covering 1.6–2 μ m, A) and a mitotic cell (single focal plane, B) are presented. The corresponding Western blots and larger fields of transfected cells are presented in Appendix Fig S3. Scale bars, 10 μ m.

Ct2-derived mutants assayed were similarly expressed (Appendix Fig S3A) and accumulated in the nucleus and nucleolus as the wild-type construct (Fig 3A). The GFP-mCenp-F-Ct2 L2668G/L2696G fusion, bearing mutations predicted to prevent dimerization, localized neither to the GLFG bodies nor to kinetochores, pointing that *in vivo* the coiled-coil folding and dimerization are prerequisite for the correct localization of this Cenp-F domain at both locations (Fig 3A and B). In agreement with our Y2H data, the GFP-mCenp-F-Ct2 L2681E/L2683E and K2678E mutants that displayed impaired interaction with Nup133 did not localize to the GLFG bodies in HeLa-E cells (Fig 3A). Importantly, these mutants were targeted to kinetochores, indicating that they are properly folded and able to dimerize (Fig 3B).

Because the C-terminal fragments of Cenp-F are not targeted to the NE in prophase, we next introduced mutations impairing

Nup133 interaction in the context of full-length GFP-hCenp-F (L2797E/I2799E in human Cenp-F, corresponding to L2681E/L2683E in mCenp-F; Fig EV1). Consistent with a previous study [49], the WT form of GFP-hCenp-F mimicked the localization of endogenous Cenp-F (Figs EV5C and 4A). In particular, a clear NE staining was observed in nearly all ($n = 23$ out of 27 cells) prophase cells in which GFP-hCenp-F is already localized at kinetochores (Fig 4A). In contrast, the L2797E/I2799E mutant form of GFP-hCenp-F, while properly targeted to kinetochores, was in most cases either not detectable or barely enriched at the NE in late G2/prophase cells ($n = 18$ and 12, respectively, out of 32 cells), even in cells displaying a clear kinetochore staining (Fig 4B and C). Together, these functional data thus validated *in vivo* the mutants generated based on our *in silico* modeling of Nup133/Cenp-F interaction.

Figure 4. L2797E/I2799E mutations prevent GFP-hCenp-F targeting to the NE in late G2/prophase cells.

A–C HeLa-E cells transiently transfected with GFP-hCenp-F-WT (A) or GFP-hCenp-F-L2797E/I2799E (B, C) were fixed 1 day after transfection and immunolabeled with the indicated antibodies. A single plane from widefield acquisitions of prophase cells (identified based on phospho-histone H3 staining and persistence of a NE) is shown. Scale bars, 10 μ m. Arrows point to kinetochores labeled by GFP-hCenp-F. Line scans (yellow lines on images, plotted from the cytoplasm toward the nucleoplasm, distances in pixels) measuring the intensity of GFP-hCenp-F (green lines) and Nup133 (red lines) signals in late G2/prophase cells reveal the peak of GFP-hCenp-F-WT that co-localizes with Nup133 at the NE (A). In contrast, either no enrichment (B) or a very minor accumulation (C) is detectable in GFP-hCenp-F-L2797E/I2799E-transfected cells.

Figure 5. Cenp-F direct interaction with Bub1 is inhibited by mutation of a conserved cysteine and is involved, together with Cenp-E, in its recruitment to kinetochores.

A Y2H interactions between full-length mNup133 or hBub1 and mCenp-F (-Ct1 or -Ct2), either WT or bearing the indicated mutations, were assayed based on growth on -LWH medium supplemented with 5 mM 3AT. The corresponding Western blots are presented in Appendix Fig S2A(iii) and B(ii).

B (i) HeLa-E cells were transfected with siRNA duplexes targeting Cenp-F (-) or both Cenp-F and Cenp-E (+siCenp-E) along with a plasmid directing the expression of a Cenp-F-siRNA-resistant form of GFP-hCenp-F (GFP-hCenp-F^{siR}), either WT or bearing the C2865S mutation (CS). Two days after transfection, cells were treated with Nocodazole (20 μ M for 4 h), fixed, and immunolabeled with the indicated antibodies and DAPI. A projection of 11 z confocal sections of the individual stainings and the overlay between the GFP signal and Hec1 staining (used to identify and quantify kinetochores) are presented. Scale bar, 10 μ m. **(ii)** The relative enrichment of GFP-Cenp-F and Cenp-E at kinetochores was quantified as described in Materials and Methods for *n* cells from two independent experiments. Box plots were generated using KaleidaGraph (Synergy Software): Each box encloses 50% of the normalized values obtained, centered on the median value. The bars extending from the top and bottom of each box mark the minimum and maximum values within the dataset falling within an acceptable range. Values falling outside of this range are displayed as an individual point. Statistical analyses were performed using Wilcoxon–Mann–Whitney rank-sum test. Standard conventions for symbols indicating statistical significance are used: ns, not significant: $P > 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Bub1 is the direct KT-core binding site for Cenp-F that contributes, along with Cenp-E, to its recruitment to kinetochores

A previous study had identified a conserved cysteine essential for the kinetochore targeting function of Cenp-F KT-core region (C2865 in hCenp-F, corresponding to C2748 in mCenp-F; [27]; Fig EV1). To evaluate a possible contribution of Nup133 (possibly via a distinct domain) to the kinetochore targeting of Cenp-F, we mutated this cysteine in the mCenp-F-Ct2 segment and tested its interaction with Nup133. In the Y2H assay, the C2748S mutation did not prevent the interaction of mCenp-F-Ct2 with full-length mNup133 (Fig 5A). Consistently, the GFP-mCenp-F-Ct2 C2748S mutant still co-localized with Nup133 within the GLFG bodies in HeLa-E cells (Fig 3A), while as anticipated [27], it was not detectable at kinetochores (Fig 3B).

We thus aimed to determine which other kinetochore partner of Cenp-F would rely on this conserved cysteine. Among the constituents whose depletion affects the targeting of Cenp-F to kinetochores, Bub1 kinase was reported to interact with Cenp-F in a Y2H assay (unpublished results from T.J. Yen cited in [22]). This prompted us to assess whether Bub1 could specifically interact with the KT-core fragment of Cenp-F. Y2H analysis revealed that unlike Nup133, hBub1 interacts with mCenp-F-Ct2 but not with mCenp-F-Ct1 that lacks the second leucine zipper motif present in Cenp-F KT-core domain (Figs 1A and 5A). Moreover, the C2748S mutation strongly inhibits Cenp-F interaction with Bub1 (Fig 5A). These data indicate that Bub1 represents the main KT-core-dependent tether for Cenp-F to kinetochores. Note that a direct interaction between the Bub1 kinase domain and a dimeric coiled-coil in Cenp-F C-terminal domain has been meanwhile demonstrated through biochemical reconstitution [preprint: 53]. Importantly, when introduced within mCenp-F-Ct2, none of the mutations impairing Cenp-F association with Nup133 affected its Y2H interaction with Bub1 (Fig 5A). This demonstrates that the lack of interaction with Nup133 or Bub1 is not merely a consequence of global misfolding of the corresponding mutants and that Cenp-F features two distinct binding sites for Nup133 and Bub1 located on two consecutive leucine zipper-containing segments.

To evaluate the functional implication of the Bub1/Cenp-F interaction, we next generated siRNA-resistant forms of full-length GFP-hCenp-F and cotransfected HeLa cells with the resulting GFP-hCenp-F^{siR}-WT or -C2865S plasmids along with Cenp-F siRNA duplexes. To increase the percentage of mitotic cells and prevent a potential contribution of the MT-binding sites of Cenp-F to its kinetochore localization, cells were treated with Nocodazole prior to immunolabeling. Both qualitative and quantitative fluorescence image analyses revealed that even in the absence of endogenous Cenp-F (that may have contributed to the GFP-transgene localization via heterodimerization), GFP-hCenp-F^{siR}-C2865S was largely retained at kinetochores (Fig 5B). This differed from the behavior of the corresponding GFP-mCenp-F-Ct2 C2748S mutant that is not detectable at kinetochores (Fig 3). This result was also unexpected since depletion of Bub1 or the lack of its C-terminal tail was reported to cause an efficient mislocalization of Cenp-F from kinetochores [30, 54, preprint: 53]. Together, these data pointed to the existence of redundant targeting determinants of Cenp-F, involving Bub1-mediated interactions outside of the Cenp-F-Ct2 domain. A

potential candidate was the kinesin-like motor protein, Cenp-E. Indeed, Cenp-E and Cenp-F directly interact with each other [22] (see Fig 1A) and were reported to stabilize each other at kinetochores [30]. Moreover, Cenp-E targeting to kinetochores also indirectly relies on Bub1 [30]. We thus analyzed the localization of GFP-hCenp-F^{siR}-WT or -C2865S in HeLa cells co-depleted for endogenous Cenp-F and Cenp-E. Under our experimental conditions, the kinetochore targeting of GFP-hCenp-F^{siR}-WT was not significantly affected by Cenp-F/Cenp-E co-depletion. In contrast, this RNAi treatment nearly entirely abolished the kinetochore localization of GFP-Cenp-F^{siR}-C2865S (Fig 5B). Our data thus demonstrate that Bub1 is the direct KT-core binding partner of Cenp-F that contributes, along with Cenp-E, to the localization of full-length Cenp-F to kinetochores.

In conclusion, our study provides a successful example of a hybrid analysis using Y2H interaction studies and *in silico* structural modeling. With this approach, we could elaborate an interaction model between the N-terminal domain of Nup133 and a short dimeric coiled-coil segment of Cenp-F, which was validated in an *in vivo* context upon expression of either short fragments or the full-length Cenp-F protein. This robust experimental pipeline demonstrates that despite being located within a very short domain, the determinants required for the interaction of Cenp-F with Nup133 and Bub1 can be functionally separated. At kinetochores, Cenp-F may thus simultaneously interact with both Bub1 and Nup133. However, our localization studies demonstrate that affecting Cenp-F/Nup133 interaction specifically impairs its prophase NE localization. In contrast, Bub1 appears as the major KT-core tether for Cenp-F that operates in a redundant manner with Cenp-E to localize full-length Cenp-F to kinetochores. While previous functional studies were solely based on Cenp-F depletion, our separation-of-function mutations will be instrumental in the future to determine the extent to which the kinetochore and/or NE localization of Cenp-F contribute to its multiple functions in physiological and pathological situations.

Materials and Methods

Structural models of Nup133 and Cenp-F domains

Pairwise sequence alignment of human/mouse Cenp-F proteins was performed using EMBOSS Matcher (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/).

Coiled-coil conformation predictions on mCenp-F C-terminal domain were performed using COILS/PCOILS on the Bioinformatics Toolkit server [55]. Because the structural deformation induced by kinks in phase-shifting regions is difficult to predict, a model for the entire segment spanning mCenp-F-Ct1 and mCenp-F-Ct2 could not be generated with high confidence. In contrast, most of mCenp-F-SID segment was predicted as a canonical, continuous, and conserved coiled-coil region (miniSID, gray box in Fig EV2A). This stretch consists of 44 residues [aa 2,663–2,706] containing six heptads in which half of the (a/d) coiled-coil positions are leucine residues. This is consistent with previous predictions [10], which suggested that this coiled-coil region is a leucine zipper. Since the vast majority of leucine zippers dimerize in a parallel mode [56], we used a canonical parallel coiled-coil

template to model residues [2,663–2,706] using CCBUILDER server [57] with default options.

mNup133 was modeled by a conservative template-based modeling method using the SwissModel server [58] based on the structure of the human ortholog (PDB: 1xks, exhibiting 81% sequence identity to mNup133).

Docking simulation for the Nup133 and Cenp-F domains

The rigid-body docking step was performed using the InterEvDock server that takes into account both the physicochemical nature of protein surfaces [48,59] and the co-evolutionary information between two binding partners [41,47] (<http://bioserv.rpbs.univ-paris-diderot.fr/services/InterEvDock/>). We took as input the structural models of Nup133 and Cenp-F and used the advanced options allowing to specify the multiple sequence co-alignments. Indeed, Cenp-F is a homodimer and its multiple sequence alignment had to be adapted to contain a copy of every sequence accounting for the dimeric structure. Besides, both monomers in the Cenp-F PDB file were changed to the same chain label. The structures of each of the 10 consensus rigid-body models recovered from the server were relaxed to remove steric clashes and optimize side-chain contacts using Rosetta following the protocol described in [41]. The 3D coordinates of the structural model shown in Fig 2A are provided in Appendix Fig S6.

Size-exclusion chromatography with multi-angle laser light scattering (SEC-MALS)

N-ter acetylated and C-ter amidated peptides were purchased from custom synthesis service (ProteoGenix; > 95% purity) and verified by MALDI. Peptide solutions at a concentration of 5 mg/ml were injected on Superdex 75 10/300 GL (GE Healthcare) equilibrated in 50 mM Tris pH 7.4 with four different salt conditions, 0.15, 0.5, 1, or 2 M NaCl, at a flow rate of 0.5 ml/min (20°C). Separation and ultraviolet detection were performed using Shimadzu HPLC system, light scattering was monitored using mini DAWN TREOS system (Wyatt Technology), and concentration was measured by Optilab T-rex differential refractometer (Wyatt Technology). Molar masses of proteins were calculated using ASTRA 6.1 software (Wyatt Technology).

Plasmids

Plasmids used in this study are listed in Appendix Table S1. They were either previously published or generated using standard molecular cloning techniques including PCR amplification using proofreading DNA polymerases (Phusion HF, NEB) and Clontech In-Fusion HD Cloning Kit or NEBuilder HiFi DNA Assembly Cloning Kits. For all constructs, PCR-amplified fragments and junctions were checked by sequencing. Plasmid maps are available upon request.

Yeast two-hybrid

The minimal Nup133-binding site of human Cenp-F (defined as SID[®], Selected Interaction Domain; Hybrigenics) was identified by reanalyzing the outcome of a previous Y2H screen performed using

hNup133-NTD [aa 1–500] as bait to screen a human breast tumor epithelial cells RP1 cDNA library [13].

The pB27-LexA-mNup133 [aa 1–1,156] plasmid was used to screen a random primed mouse Embryonic Stem Cell_RP1 cDNA library cloned into the pP6-Gal4-AD plasmid using a high-throughput proprietary yeast two-hybrid-based technology (ULTIMATE Y2H Screen; Hybrigenics). To investigate pairwise interactions between mNup133 and mCenp-F by yeast two-hybrid, the wild-type or mutant mNup133 and mCenp-F fragments were cloned in the pB27-LexA and pP6-Gal4-AD plasmid, respectively. pB27-LexA-derived plasmids were transformed in L40ΔGal4 strain, while pP6-Gal4-AD-based plasmids were transformed into the Y187 strain as described previously [31]. Bait and prey strains were mated in rich medium, and diploids were grown for 2–3 days on minimum medium lacking leucine and tryptophan (–LW, as control to ensure mating efficiency) or lacking leucine, tryptophan, and histidine (–LWH) supplemented, when indicated, with 1 or 5 mM 3-Aminotriazole (3AT) a competitive inhibitor of the *HIS3* gene product that increases the stringency of the Y2H assay.

To analyze the expression of the yeast two-hybrid constructs, total proteins were extracted from yeast cells using the NaOH–TCA lysis method essentially as described [60]. Briefly, three OD₆₀₀ values of exponentially growing yeast cells were collected by centrifugation, washed once in water, resuspended in 550 µl of 0.17 M NaOH, and incubated for 10 min on ice. 50 µl of 50% TCA was then added, and the samples were incubated again for 10 min on ice. After a 2-min centrifugation at 12,000 g, pellets were resuspended in 60 µl of Laemmli 2×, 15 µl 1 M Tris, and 2% β-mercaptoethanol, denatured 5 min at 95°C, and analyzed by Western blot (Appendix Fig S2).

Cell culture, plasmid and siRNA transfections, crosslinking, and whole-cell extracts

HeLa-E and HEp-2 are subclones of HeLa cells initially received from F. Perez (Institut Curie, Paris, France) and Luc Mouthon (Institut Cochin, Paris, France), respectively. These cell lines were grown at 37°C in DMEM (Life Technologies) supplemented with 10% fetal calf serum, 1% L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. For plasmid and siRNA transfections, ~0.4–0.5 million cells plated on 35-mm dishes were treated with 10 µl Lipofectamine 2000 (Invitrogen) and (i) 4 µg of HA-mCenp-F-Ct2, GFP-mCenp-F-Ct1, or GFP-mCenp-F-Ct2 WT or mutant plasmids, (ii) 2 µg of the pUG6SP-tTA activation plasmid (gift from M.N. Boddy; Addgene plasmid #41029; [61]) and 2 µg of GFP-hCenp-F full-length WT or L2797E/L2799E plasmids (for Fig 4), or (iii) 4 µg of GFP-mCenp-F^{siR}-WT or GFP-mCenp-F^{siR}-C2865S plasmid DNA and 240 µg of Cenp-F siRNA duplex (5'-CAGAATCTTAGTAGTCAAGTA-3'; [13]) either alone or combined with 240 µg of Cenp-E siRNA duplex (5'-CCACUAGAGUUGAAA GAUAAU-3'; [62]; for Fig 5B). The transfection medium was removed after 4 h, and the cells were analyzed by Western blot or immunofluorescence 24 or 48 h after transfection.

For *in vivo* crosslinking, HeLa-E cells transfected with HA-mCenp-F-Ct2 WT or mutant plasmids were treated 48 h after transfection with 0.5 mM disuccinimidyl suberate (DSS, a non-cleavable and membrane permeable homobifunctional NHS-ester

crosslinker; Thermo Scientific) in PBS for 5 min and then quenched with 10 mM Tris pH 8.6 for an additional 15 min prior to cell lysis.

To prepare whole-cell lysates, transfected human cells were lysed in 2× Laemmli lysis buffer [150-mM Tris-HCl (pH 6.8), 5% (wt/vol) SDS, 25% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue]. Lysates were incubated for 3 min at 95°C, clarified by sonication [Bioruptor (Diagenode): 3 cycles of 30 s on/off, high power], and denatured again for 3 min at 95°C. Except for crosslinking experiments, protein concentration was then determined using a BCA assay kit (Thermo Scientific). Total protein extracts supplemented with β-mercaptoethanol (750 mM final) were analyzed by Western blot (~15 µg per lane).

Western blot analyses

For Western blot analysis, yeast or human cell lysates were separated on 8% Tris-glycine SDS-PAGE gels or on NuPAGE™ 4–12% Bis-Tris gels (using MOPS as running buffer) and transferred to nitrocellulose membranes. The resulting blots were stained using Ponceau, saturated with TBS, 0.1% Tween, and 5% dried milk, and probed with affinity-purified monoclonal mouse anti-LexA (Santa Cruz SC-7544; 1:5,000), polyclonal rabbit anti-Gal4AD (SIGMA G9293; 1:2,000), mouse monoclonal antibody HA.11 (clone 16B12; Eurogentec #MMS-101R; 1:2,000), or mouse monoclonal anti-GFP antibody (Roche Diagnostic #11814460001; 1:1,000). Incubations of the membrane with primary and HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were done in TBS buffer (0.1% Tween, 5% dried milk), and signals were detected by enhanced chemiluminescence (SuperSignal® Pico or Femto; Thermo Scientific).

Immunofluorescence microscopy

Antibodies used in this study were as follows: affinity-purified rabbit polyclonal anti-human Nup133 (#756-77; 1:100; [31]; used in Fig EV5A and B); rat monoclonal anti-mouse Nup133 (#74; clone 9C2; 1:100; used in Figs 4 and EV5C) generated against recombinant histidine-tagged mouse Nup133 [aa 66–512] and purified by ProteoGenix (validation of this antibody for IF experiments is provided in Appendix Fig S4); rat monoclonal anti-Nup98-Nter (clone 2H10, Abcam ab50610, 1:500); mouse monoclonal anti-Cenp-F (clone 11/Mitosin; BD Biosciences #610768; 1:400); rabbit polyclonal anti-Cenp-F (Abcam ab5, 1:400); rabbit polyclonal anti-Elys/Mel28 antibody (FID1 serum, directed against histidine-tagged human Mel28 protein, aa 1,208–1,800; generously provided by R. Walczak and I. Mattaj, EMBL, Heidelberg, Germany; 1:1,000; [63]); mouse monoclonal anti-HEC1 (clone 9G3; Abcam ab3613; 1:500); rabbit anti-Cenp-E antibody (generously provided by T.J. Yen, Philadelphia, USA; 1:1,000; [22]); autoimmune CREST serum (Antibodies Incorporated 15-235; 1:500); and mouse monoclonal anti-phospho-H3 Ser10 (Abcam ab14955; 1:4,000). Secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (cy3, cy5) and were generally used at 1:500 dilution (except at 1:2,000 dilution when anti-mouse cy3 was used in combination with anti-phospho-H3).

Fluorescence signals within the intranuclear bodies and at kinetochores were best detected upon pre-extraction of cells, followed by a fixation-extraction procedure as previously described [33].

Briefly, cells grown on coverslips were quickly pre-extracted for 15 s in PHEM buffer (20 mM K–1,4-piperazinediethanesulfonic acid, 10 mM K–ethylene glycol tetraacetic acid, 1 mM MgCl₂, pH 6.8) containing 0.2% Triton and then fixed for 10 min in the same buffer further containing 4% paraformaldehyde supplemented with 0.85% of 1 N NaOH. For Fig 5B, the transfected cells were not pre-extracted but directly fixed for 20 min with 3% paraformaldehyde in PBS supplemented with 0.85% of 1 N NaOH and then permeabilized with 0.5% Triton X-100 in PBS for 8 min. In both cases, following a 15- to 30-min saturation in PBS containing 1% BSA, coverslips were then successively incubated for 1 h with primary and secondary antibodies diluted in PBS + 1% BSA. Cells were then stained with 0.1 µg/ml of DAPI and were mounted in Movioli medium.

Image acquisition and analysis

Widefield fluorescence images were acquired using a microscope (DM6000B; Leica) with a 100×, numerical aperture 1.4 (HCX Plan-Apo) oil immersion objective and a charge-coupled device (CCD) camera (CoolSNAP HQ; Photometrics). A piezoelectric motor mounted underneath the objective lens enabled rapid and precise Z-positioning. Spinning disk images were acquired on an inverted microscope (DMI8; Leica) with a CSU-W1 spinning disk head (Yokogawa) and a sCMOS ORCA-Flash 4 V2+ camera (Hamamatsu) using 100×/1.4 oil objective. Image stacks were acquired without camera binning, with a plane spacing of 0.2 µm. Either a unique plane or 3D maximum-intensity projections performed using MetaMorph (Universal Imaging Corp.) or ImageJ/Fiji software are presented as indicated on the figures.

Line scan analyses were performed using ImageJ. A line of 10 pixels width was drawn on the focal plane of the NE (defined based on Nup133 staining), and the average intensity along this line was plotted for each channel using Microsoft Excel. Quantifications of fluorescence intensities at kinetochores were carried out using a Fiji macro (provided in Appendix Fig S5) on 3D projections covering the entire mitotic cells (raw data are included in Appendix Table S2). Briefly, kinetochores were identified based on Hec1 staining. Fluorescence intensities for each kinetochore were then calculated and normalized to the average intensity measured on the entire cell. For each cell, all kinetochores were independently quantified and their intensity was averaged. Box plots were generated using KaleidaGraph (Synergy Software). Statistical analyses were performed using Wilcoxon–Mann–Whitney rank-sum test provided by the KaleidaGraph software.

Expanded View for this article is available online.

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Author contributions

AB, RG, FO, JD, and VD conceived and designed the experiments; AB, SM-B, FO, and CB performed the wet experiments; JY and RG performed the modeling simulations and designed the mutants; AB, SM-B, RG, JY, FO, RV, and VD analyzed the data; and VD, AB, and RG wrote the manuscript with contribution from all co-authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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