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Nucleosomal regulation of chromatin composition and nuclear assembly revealed by histone depletion

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Abstract

Nucleosomes are the fundamental unit of chromatin, but the analysis of transcription-independent nucleosome functions has been thwarted by the confounding gene expression changes resultant of histone manipulation. Here we solve this dilemma by developing *Xenopus laevis* egg extracts deficient for nucleosome formation, and analyze the proteomic landscape and behavior of nucleosomal chromatin and nucleosome-free DNA. We show that while nucleosome-free DNA can recruit nuclear envelope membranes, nucleosomes are required for spindle assembly, lamina and nuclear pore complex (NPC) formation. In addition to RCC1, we reveal that ELYS, the initiator of NPC formation, fails to associate with naked DNA, but directly binds histones H2A–H2B and nucleosomes. Tethering ELYS and RCC1 to DNA bypassed the requirement for nucleosomes in NPC formation in a synergistic manner. Thus, the minimal essential function of nucleosomes in NPC formation is to recruit RCC1 and ELYS.

Eukaryotic nuclear DNA is not only the template for gene transcription but also the substrate and platform for many biological processes. DNA needs to be structurally organized, repaired if damaged, faithfully replicated, segregated during cell division, and separated from the rest of the cytoplasm by the nuclear envelope (NE) during interphase. However, these processes do not function on naked DNA but within a proteinaceous environment termed chromatin, whose main components are the core histone proteins, H2A, H2B, H3 and H4. These organize DNA into repeats of nucleosomes each of which containing ~146bp of DNA wrapped around a histone octamer, composed of two copies of each of the core histones¹. Nucleosomes are thus at the heart of all DNA-based processes and are generally thought to be major regulators of these, both by occluding DNA from interaction with DNA binding proteins, and by specifically recruiting other proteins. It is generally accepted that many of these functions are regulated by post-translational modifications of histones, which

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specifically determine interaction partners or affect chromatin structure more directly (for example by affecting chromatin structure)². However, the analysis of these functions is complicated by the fundamental roles that nucleosomes play in regulating transcription, as histone manipulations *in vivo* alter gene expression profiles³, which may indirectly affect a process of interest. Furthermore, vertebrate genomes harbor large copy numbers of histone genes⁴ and a variety of histone variants⁵, making it difficult to manipulate them. As a consequence, establishing functions of the nucleosome has not been straightforward, and the development of new model systems is required to address many fundamental functions of chromatin.

To investigate non-transcriptional histone functions, we employed *Xenopus laevis* egg extracts, which faithfully recapitulate chromatin functions in a manner identical to intact cellular physiology, but independently of transcription and translation. Naked DNA added to these extracts is rapidly chromatinized, and coordinates the formation of complex structures, such as mitotic spindles capable of segregating chromosomes, and functional interphase nuclei, which carry out nuclear import, DNA repair and DNA replication⁶⁻⁸. Importantly, DNA sequence is of no importance, and transcription is not required for any of these events, resembling the situation in the embryo where transcription is suppressed until the maternal-to-zygotic transition⁹.

Here we establish these extracts as a model system for the analysis of direct nucleosome functions, without complications arising from gene expression changes upon histone manipulation. We developed a method to remove histones H3 and H4 from egg extracts (H3-H4 extracts). H3-H4 extracts are incapable of forming nucleosomes but chromatin functions can be reconstituted by adding back nucleosome arrays generated with recombinant histones. Using this strategy, we were able to systematically profile the roles of nucleosomes and histone modifications in a physiological context. We report the first description of how the composition of chromatin is affected by the absence of nucleosomes, uncover a dependency of spindle assembly on nucleosomes and establish a requirement for nucleosomes in nuclear pore complex (NPC) formation, which we explain by a direct recruitment of ELYS and RCC1 to nucleosomes.

RESULTS

A system for analyzing nucleosome functions in egg extract

The cytoplasm of *Xenopus* eggs contains a large stockpile of core histones in complex with specific chaperone proteins. Histones H3 and H4 are stored as soluble heterodimers, at a concentration that we estimated to be ~6 μ M (Supplementary Fig. 1a). To immunodeplete this large quantity of histones, we screened a panel of monoclonal antibodies that recognize unmodified or modified forms of histone H3 or H4 (Supplementary Fig. 1b). We found that monoclonal antibodies against histone H4 acetylated at Lys5 (H4K5ac) or at Lys12 (H4K12ac) reproducibly depleted 90% of H3 and H4 from egg extracts (Fig. 1a and Supplementary Fig. 1b), consistent with the notion that the majority of H4 in *Xenopus* eggs is diacetylated at these residues¹⁰. Anti-H4K12ac antibodies were exclusively used for the rest of this study. As expected, H3-H4-depleted (H3-H4) extracts were defective for nucleosome formation on naked plasmid DNA as determined by an assay that monitors

plasmid supercoiling as a metric of nucleosome formation (Fig. 1b). The add-back of recombinant histones H3 and H4 rescued supercoiling (Supplementary Fig. 1c), but micrococcal nuclease (MNase) digests indicated that recombinant H3–H4 fail to support proper nucleosome formation in H3–H4 extracts, presumably due to the reduced amounts of histone chaperones (Supplementary Fig. 1b,d).

To bypass the nucleosome assembly process in H3–H4 extracts, we pre-assembled nucleosomes from recombinant histones by salt dialysis on a 19-mer of the 601 nucleosome-positioning sequence¹¹. To determine whether these nucleosome arrays were capable of inducing physiological reactions in H3–H4 extracts, we coupled them, or their naked DNA counterpart, to beads and incubated them with H3–H4 extracts (Fig. 1c). Nucleosome beads, but not the equivalent naked DNA beads, induced spindle assembly in H3–H4 extracts (Fig. 1d,e), demonstrating that H3–H4 extracts support complex chromatin-dependent processes as long as nucleosomes are preloaded onto DNA. Moreover, we conclude that spindle formation requires the presence of nucleosomes.

Nucleosomal regulation of spindle assembly factors

The requirement for nucleosomes in spindle formation could be explained by the nucleosome-dependent binding of two major chromatin-associated activators of spindle assembly, the Ran guanidine exchange factor RCC1 (ref. 12) and the chromosomal passenger complex¹³ (CPC, composed of INCENP, Dasra A, Survivin and the kinase Aurora B; Fig. 2a). Although RCC1 can bind both DNA and histones H2A and H2B^{14–17}, it was completely absent from naked DNA beads recovered from H3–H4 extracts, suggesting that nucleosome interactions are critical for its recruitment (Fig. 2b, each pull down was normalized to DNA amounts, see Supplementary Fig. 2a). Similarly, the CPC was only detected on nucleosome beads, consistent with an interaction between Survivin and histone H3 phosphorylated at Thr3 (H3T3ph)^{18–20} (Fig. 2b). In contrast, the chromokinesin Xkid, which contains helix-hairpin-helix DNA binding domains²¹, bound naked DNA, illustrating the power of our system to distinguish nucleosome-dependent and –independent interactions.

When added to untreated metaphase egg extracts, naked DNA stimulates the kinase activity Aurora B^{18,22}. However, only nucleosome beads, but not DNA beads, were able to induce Aurora B activation in H3–H4 extract, as determined by antibodies against the Aurora B autophosphorylation site at Thr248 (Fig. 2c), suggesting that the interaction of Survivin with H3T3ph is critical for Aurora B activation. We have previously demonstrated that the H3T3 kinase Haspin is necessary for chromatin-induced Aurora B activation¹⁸, but so far it has been technically impossible to demonstrate if H3T3ph is responsible for this process. Thus, we generated nucleosomes with a point mutation at H3T3 and compared Aurora B activity in response to these or wild type nucleosomes in H3–H4 extracts. We found that Aurora B activation was observed in response to wild type nucleosomes but not in response to nucleosomes carrying an H3_T3A mutation (Fig. 2c). In contrast, nucleosomes with a phosphomimetic H3_T3E mutation, which supported interaction with the CPC (Supplementary Fig. 2b), activated Aurora B even in extracts depleted of both Haspin and H3–H4, in which wild type nucleosomes failed to induce Aurora B activation (Fig. 2d),

demonstrating that H3T3 is the sole essential target of Haspin for Aurora B activation. Taken together, our results establish how nucleosomes activate spindle formation and also validate our system to directly test the functional importance of specific histone residues and modifications.

The effect of nucleosomes on chromatin composition

Upon entry into mitosis, chromosomes condense and individualize by the actions of condensins and DNA topoisomerase II²³, but little is known about the impact of nucleosomes on the regulation of mitotic chromatin components. To understand how the landscape of mitotic chromatin composition is affected by the presence or absence of nucleosomes, we carried out a mass spectrometry (MS)-based analysis of the proteins that associate with naked DNA beads or nucleosome beads in metaphase H3–H4 extracts. As before, the amount of DNA beads and nucleosome beads used for each experiment was calibrated so that equal amounts of DNA were recovered (Supplementary Fig. 2a). Proteins identified in two independent replicates were manually grouped into functional categories (Fig. 3a; Supplementary Data Set 1). When relative abundance of proteins copurified with nucleosome beads and DNA beads was displayed in a scatter plot, known stoichiometric components of protein complexes clustered together (Fig. 3b), confirming the validity of our quantification. For most identified proteins, the relative fold enrichment (ratio of nucleosome beads/DNA beads) and relative amount were reproducible between two experiments (Supplementary Fig. 3a-c). To visualize the effect of nucleosomes on selected proteins important for mitotic chromosomes function, these proteins were ranked according to whether they are enriched on DNA or on nucleosomes (Fig. 3c,d). Consistent with our western blot analysis (Fig. 2b, Supplementary Fig. 4a), RCC1 and the CPC were only detected on nucleosomes (Fig. 3b,d).

The quantitative MS analysis of chromatin proteins revealed that while a subset of these proteins exhibit exclusive dependence on the presence or absence of nucleosomes, the effect of nucleosomes for the majority (~ 60%) of proteins is limited to no more than 4-fold (Fig. 3b). For example, both MS and western blot analyses (Fig. 3b,c, Supplementary Fig. 4b-d) showed that condensins effectively bind nucleosome-free DNA beads, and nucleosomes only exhibited a mild, but reproducible, negative impact on condensin binding. This negative effect is somewhat unexpected, since it was recently suggested that Aurora B-mediated condensin phosphorylation, allowing an interaction with H2A, is important for condensin association with chromosomes in fission yeast and human cells²⁴, but is consistent with Aurora B-independent condensin recruitment in *Xenopus* egg extracts²⁵. Both types of beads copurified a substantial amount of signal for both the Ku complex, involved in double stranded break (DSB) repair, and the single-stranded DNA binding complex RPA, which may reflect the existence of DNA ends that were not captured by streptavidin and thus were processed as DSBs²⁶ (Supplementary Fig. 2a). Alternatively, these proteins may display damage-independent association with chromatin. Together, these results suggest that mitotic chromatin is mostly composed of proteins that have capacity to bind histone-free DNA (directly or indirectly), and that nucleosomes have a limited impact on their DNA-binding capacity. Indeed, total amounts of non-histone proteins on DNA

beads did not drastically change by the presence or absence of nucleosomes (Fig. 3a). Thus, DNA itself appears to be a major determinant for the general composition of chromatin.

Nucleosomes showed a strong (more than one order of magnitude) occlusive effect on a small fraction (~10 %) of proteins that bound nucleosome-free DNA beads. These include HMG-box containing proteins, transcription factors with RNA or DNA binding capacity (e.g., PurA, PurB, UBF-1), tRNA synthetases, and the mismatch recognition complex MutSα (MSH2 and MSH6; Fig. 3b, Supplementary Table 1). While it is widely appreciated that nucleosomes have a negative impact on transcription, MSH6 is known to bind histone H3K36me3, a mark enriched during G1 and early S phase²⁷. Therefore, some of these DNA-binding proteins may have evolved to access nucleosomal DNA only within a specific functional context, enriched for specific histone modifications or DNA modifications. We conclude that nucleosomes can inhibit nonspecific interaction of various DNA- or RNA-binding proteins, but these proteins never become major chromatin constituents even in the absence of nucleosomes (Fig. 3a).

Only a few known chromosome structural proteins showed pronounced preference for nucleosomes (Fig. 3c,e, Supplementary Fig. 4a-d). The most abundant proteins in this category were histones H2A–H2B and the embryonic linker histone, H1M (also known as B4). While these histones can bind naked DNA *in vitro*^{28,29}, the strong dependency on nucleosomes that we observed in extracts is likely to reflect the presence of their chaperone NAP1, which prevents nonspecific interactions with naked DNA³⁰⁻³². Topoisomerase IIα (TopoII) also preferred nucleosomes (Fig. 3c,e; Supplementary Fig. 4d). Recently, it was suggested, but not yet proven, that interactions with histone H3 help recruit TopoII to chromatin³³. However, purified TopoII preferred naked DNA in a reconstituted system in the absence of egg extract (Supplementary Fig. 4e), suggesting that post-translational modifications or other factors are important for TopoII's preference for mitotic nucleosomes. Interestingly, TopoII was able to interact with nucleosome-free DNA in interphase egg extracts (Supplementary Fig. 4d), indicating that such modifications or regulating factors act in a cell cycle stage dependently. Altogether, we conclude that proteins that show exclusive binding dependence on nucleosomes are minor constituents of mitotic chromatin assembled in H3–H4 extracts.

Formation of NPCs and of the lamina depends on nucleosomes

Upon exit from mitosis, chromatin-associated signals switch from spindle assembly to nuclear envelope formation. Components of the individual NE units, membranes, the lamina and NPCs, have to be recruited to chromatin, but the critical chromatin modules have not been identified in this process. Our system enables us to determine the importance of nucleosomes for NE formation, since nucleosome beads can assemble functional NEs carrying out nuclear localization signal (NLS)-dependent import in H3–H4 extracts (Supplementary Fig. 5a). Consistent with reports that purified ER vesicles can associate with DNA in the absence of nucleosomes^{34,35}, membranes were recruited to DNA beads independently of nucleosomes (Fig. 4a,b). BAF, an important factor for recruitment of LEM-domain NE proteins and for the formation of closed NEs^{36,37} was recruited preferentially to DNA beads in H3–H4 extracts (Fig. 4c,d). It has been reported that BAF

can interact with DNA³⁸ and histones³⁹, and this result indicates that DNA interactions are predominant in its recruitment to chromosomes. In contrast, recruitment of NPC components, such as FG-NUP nucleoporins that form the NPC diffusion barrier, and Nup96 (a member of the Nup107 complex) that comprises the core scaffold of the NPC⁴⁰, was dependent on nucleosomes (Fig. 4e,f; Supplementary Fig. 5b,c). Consistent with the reported importance of the NLS of lamins for nuclear targeting^{41,42}, lamina assembly, as determined by association of Lamin B3, also depended on nucleosomes (Fig. 4g,h). Together, these results demonstrate that functional NE formation depends on nucleosomes.

Nucleosomes, but not naked DNA, directly recruit ELYS

The earliest known event in NPC formation is chromosomal association of the nucleoporin ELYS (also known as MEL-28), which brings in the Nup107 complex through interactions with its N-terminal domain⁴³⁻⁴⁸. The RCC1-RanGTP pathway is also an important player⁴⁹⁻⁵¹, and its absence from nucleosome free DNA also in interphase (Supplementary Fig. 4d) may explain the observed dependency on nucleosomes in NPC formation. However, it has been shown that RanGTP is dispensable for chromatin recruitment of ELYS, although it does have a stimulatory effect^{45-48,52}. Therefore, we next examined the impact of nucleosomes on ELYS recruitment. ELYS was thought to associate with chromosomes via an AT-hook type DNA binding domain in its C terminus⁴³ but even though the 601 sequence contains an AATT-motif preferred by AT-hooks, we found a strict nucleosome dependence of the interaction of ELYS with chromatin, both by immunofluorescence (Fig. 5a,b), and by pull down from extract (Supplementary Fig. 5d). ELYS was only recruited to nucleosome beads in interphase extracts, but not in M-phase extracts, validating the cell cycle-dependent regulation of NPC formation in our system (Supplementary Fig. 5e).

Full-length ³⁵S-labelled ELYS generated in a reticulocyte lysate recapitulated the interaction with nucleosomes in the absence of egg extract, implying a direct association of ELYS with nucleosomes (Fig. 5c), and this interaction depended on the C terminal 128 residues of ELYS (ELYSC, Fig. 5d-f). This is the portion of ELYS that contains the AT-hook, and also another distinct region, both of which can support chromosome binding in the absence of membranes^{43,44}. Strikingly, ELYSC, purified from bacteria (Supplementary Fig. 6a), exhibited a stoichiometric interaction with purified nucleosomes, but did not noticeably interact with naked DNA (Fig. 5g). We therefore conclude that the C terminus of ELYS mediates interaction with nucleosomes. Although the AT-hook was important for the interaction with nucleosomes (ELYSC^{2A}, Fig. 5d; Supplementary Fig. 6a,b), it was not sufficient (ELYSC³, Supplementary Fig. 6a,c). The very C terminus (ELYSC²) was also unable to bind to nucleosomes (Supplementary Fig. 6a,d), suggesting that the AT-hook like motif and an additional module in the C-terminus act synergistically to support ELYS interaction with nucleosomes.

To investigate which nucleosome component ELYS interacts with, we first removed histone tails by partial proteolysis. Tail-less nucleosomes were still able to interact with ELYSC, suggesting that histone tails are not important for ELYS recruitment to chromatin (Fig. 6a). Next, we determined whether ELYSC could interact directly with core histones in the absence of DNA. Because H3-H4 tetramers require high salt for solubility, we used H3-H4

dimers bound to the histone chaperone ASF1 (fused to human IgG Fc fragment for affinity purification; ASF1-Fc)¹⁸ instead. While ELYS_C failed to interact with H3–H4 (Fig. 6b), it readily interacted with H2A–H2B (Fig. 6c). These findings indicate the importance of nucleosomes in NPC formation by directly binding to ELYS.

Nucleosome-free NPC assembly with DNA-tethered RCC1 and ELYS

It has been shown that high concentration of RanGTP can promote NPC assembly on membrane structures in the absence of chromatin⁴⁹. Therefore, we wondered if local generation of RanGTP by tethering RCC1 to DNA were sufficient to bypass the requirement for nucleosomes in NPC formation. Taking advantage of the fact that the chromokinesin Xkid efficiently bound to nucleosome-free DNA (Fig. 2b), a chimeric RCC1 fused to the DNA-binding domain of Xkid (RCC1-DBD) was translated in metaphase H3–H4 extract containing DNA beads (Fig. 7a,b; Supplementary Fig. 7a,b). When the extract was released into interphase, substantial amounts of FG-NUPs and ELYS were recruited to DNA beads in a manner dependent on RCC1-DBD, but the levels were significantly lower than those normally achieved by nucleosome beads.

Similarly, we attempted to examine the impact of tethering ELYS to DNA by translating a chimeric ELYS whose C-terminal nucleosome-binding domain was replaced with the Xkid DNA binding domain (ELYS C2-DBD; Fig. 7a,b; Supplementary Fig. 7a,b). Likely due to its large size, translation of ELYS C2-DBD was inefficient in egg extracts, and thus exhibited only modest, if any, recruitment of ELYS and FG-NUPs to DNA beads. However, when ELYS C2-DBD and RCC1-DBD were co-translated in H3–H4 extracts, the recruitment of FG-NUPs and ELYS to DNA beads was significantly enhanced when compared to the effect of each individual chimeric protein. Thus, these data suggest that the minimum direct role of nucleosomes in NPC formation is to recruit RCC1 and ELYS.

DISCUSSION

Here we have established a cell-free system that reconstitutes physiological chromatin composition and functions with nucleosomes generated from synthetic DNA and recombinant histones. For the first time, we were thus able to analyze the consequences of the absence of nucleosomes without influencing transcription profiles, and have established that formation of both spindles and functional NEs depend on nucleosomes.

At mitotic exit, it is essential that NEs are formed at, but only at, chromosomes. Our findings demonstrate that functional NE formation is initiated by and restricted to chromosomes by specific interactions with both DNA and nucleosomes (Fig. 8). Previously, it was suggested that chromatin-associated RCC1 locally promotes NPC formation^{49,50}. However, tethering RCC1 to DNA (RCC1-DBD) was able to only partially rescue NPC formation (Fig. 7). This inefficiency may in part reflect the nucleosomes' capacity to stimulate RCC1 (ref. 16), or may indicate the presence of Ran-independent mechanism by which nucleosomes promote NPC formation. Consistent with the latter hypothesis, we demonstrate that the C terminal domain of ELYS directly binds to H2A–H2B and nucleosomes, and that expression of ELYS C2-DBD significantly improved the efficiency

of NPC formation on naked DNA together with RCC1-DBD. Thus, nucleosomes play a direct structural role in NPC recruitment through binding ELYS as well as RCC1.

This requirement of two histone-binding processes for NPC recruitment on chromatin mimics the situation in M phase, where two histone-binding factors, RCC1 and the CPC, collaborate to promote spindle assembly on chromatin (Fig. 8a). Similar to NPC formation, local enrichment of RCC1 is sufficient to promote spindle assembly⁵³, yet dual recognition of chromatin and microtubules by the CPC also independently promotes this process^{13,18,22,54}. This CPC pathway plays a major role in generating a local signal to restrict spindle assembly around chromatin even in the absence of a RanGTP gradient^{22,55}. While the RCC1-RanGTP pathway persists throughout the cell cycle, ELYS and the CPC are specifically enriched on chromatin during interphase and M phase, respectively. Furthermore, persistent CPC on chromatin at the exit from M phase inhibits functional nuclear formation^{56,57}. Thus, in addition to RCC1 that has a capacity to drive completely different chromatin-associated processes depending on the cell cycle stage even in the absence of chromatin^{49,53}, nucleosomes must acquire cell cycle specific navigators that direct the construction of the right architecture at the right cell cycle stage.

It was demonstrated that ELYS is important for post-mitotic NPC formation but is dispensable for insertion of NPCs into membranes once NE formation is complete⁵⁸. Furthermore, on demembrated sperm nuclei exposed to egg cytoplasm, ELYS initially coats bulk chromatin, but eventually accumulates at the nuclear rim⁴⁷. Therefore, ELYS appears to be required for targeting NPCs to chromatin, but nucleosome association of NPCs may not be required for their maintenance at the NE.

If membranes can be recruited to DNA independently of nucleosomes and NPCs can be inserted into preformed membranes via the action of RCC1, why must ELYS interact with nucleosomes? We speculate that nucleosome-dependent NPC formation ensures rapid formation of functional nuclei on chromosomes at the end of mitosis and also prevents NE formation on nucleosome-free DNA fragments derived from missegregated chromosomes or exogenous DNA such as viral DNA. Histones can be stripped from chromosomes in anaphase bridges⁵⁹, and micronuclei generated from chromosome missegregation show defective nuclear organization, including reduced NPC formation, and genome instability. Therefore, the absence of nucleosomes may act as a marker for foreign DNAs that should not promote functional nuclear formation. Furthermore, during fertilization, this mechanism may ensure proper timing of NE formation on the male pronucleus, which derives from sperm chromatin that is void of histones in most vertebrates.

The cell-free nature of our system is compatible with expressed protein ligation⁶⁰ and other similar techniques to generate specific modifications biochemically. Using our system, the proteomic profiling and functional analysis of chromatin proteins whose binding is affected by nucleosomes, histone residues and modifications will be possible. We expect the combination of these approaches to provide an unprecedented understanding of the function and structural organization of chromatin.

METHODS

***Xenopus* egg extracts and protein depletions**

Cytostatic factor (CSF) M phase (cytostatic factor) arrested *X. laevis* egg extracts were prepared as previously described⁶¹. For each μl of extract to be depleted of H3–H4, 2.6 μg of anti-H4K12ac antibody were incubated with 0.25 μl of rProtein A sepharose beads (GE Healthcare) for one hour. Beads were washed and extract was incubated with beads for 38 min under rotation at 4°C. Extract was recovered and incubated for a second time with uncoupled rProtein A sepharose beads (0.17 μl beads/ μl extract) to soak up antibody that had leaked off the original beads. Haspin was depleted as previously described¹⁸. For M phase experiments, beads were incubated in CSF extracts for 80 min at 20°C. For interphase experiments, beads were first preincubated in CSF extracts for 80 min at 20°C, and subsequently released into interphase by the addition of calcium chloride to 0.3 mM at 20°C. Each tube was gently flicked every 20 min. Samples for immunofluorescence and membrane analysis were taken after 90 min. Animal husbandry and protocol, approved by Rockefeller University's Institutional Animal Care and Use Committee, were followed.

Antibodies and western blotting

Monoclonal antibodies against H3N (unmodified K4) and H3C have been described previously^{62,63}. Generation and characterization of mouse monoclonal antibodies against H4K5ac, H4K12ac, and H4N will be described elsewhere (H. K. and N. Nozaki, data not shown). Rabbit Aurora B (5 µg/ml in western blotting), Dasra A (1 µg/ml in western blotting), INCENP (7.4 µg/ml in western blotting) antibodies were used as described^{13,54,64}. Rabbit Aurora B T248ph was detected with antibody #2914 from Cell Signaling Technology (1:200 for western blotting). Rabbit ELYS antibodies⁴⁷ are gifts of Iain Mattaj (1 µg/ml for western blotting; 3.1 µg/ml for immunofluorescence). Rabbit Nup96 antibodies are gifts of Martin Hetzer (1:500 for immunofluorescence). Rabbit H1M⁶⁵ (1 µg/ml for western blotting) and RCC1⁶⁶ (1.8 µg/ml for western blotting) antibodies are gifts of Rebecca Heald. Rabbit HIRA antibodies⁶⁷ are gifts of Gnevire Almouzni (1:10,000 for western blotting). Rabbit N1 antibodies are gifts of David Shechter (1:50,000 for western blotting). Rabbit Orc2⁶⁸ (1:5,000 for western blotting) and Mcm7⁶⁹ (1:9,000 for western blotting) antibodies are gifts of Johannes Walter. Topoisomerase II (1:400 for western blotting) antibodies⁷⁰ are gift of Tatsuya Hirano. Mouse anti α -tubulin antibody was obtained from Sigma (clone DM1A; 1:1,000 for western blotting). Anti SPT16 antibodies were from Cell Signaling (mAB 12191, 1:1,000 for western blotting). FG-NUPs were detected with mouse mAb414 (Covance; 2 µg/ml for immunofluorescence) directly labeled with Alexa Fluor 568 (Invitrogen). H3 was detected with abcam ab1791 (1 µg/ml for western blotting). Haspin was depleted as described^{18,57}. Dppa2 antibodies (5 µg/ml for western blotting) have been described previously⁶⁴. Anti XCAP-G antibodies (1 µg/ml for western blotting) were raised against the last 15 amino acids of XCAP-G as described⁷¹. HA tag was detected with monoclonal antibody 16B12 (1 µg/ml for western blotting). GFP was detected using #A11122 (Life Technologies, 1:1,000).

For western blot detection of proteins, IRDye 680LT goat anti-rabbit IgG (Li-Cor, #926-68021; 1:15,000), IR Dye 800CW goat anti-rabbit IgG (Li-Cor; #926-32211;

1:15,000), IRDye 680LT goat anti-mouse IgG (Li-Cor, #926-68020; 1:15,000) and IR Dye 800CW goat anti-mouse IgG (Li-Cor; #926-32210; 1:15,000) were used as secondary antibodies. The Odyssey Infrared Imaging System (Li-Cor) was used for detection.

Secondary antibodies for immunofluorescence were Alexa Fluor 555 goat anti-rabbit IgG (Life Technologies; # A-21428; 1:1,000) and Alexa Fluor 488 F(ab')₂ fragment of goat anti-rabbit IgG (Life Technologies; #A-11070; 1:1,000).

Plasmids and cDNA clones

His-tagged *Xenopus laevis* H2A, H3.2, H3.3 and H4 expression plasmids are gifts of C. David Allis⁷². Because H3.2 contained a mutation (G102A), this was reverted by quikchange mutagenesis. An untagged *X. laevis* H2B expression plasmid is a gift of Tom Muir. Human ASF1a cDNA was obtained from Thermo Scientific (accession no. BC010878), and was expressed from pET52b as a fusion protein with human IgG1 Fc (cDNA a gift of Jeffrey Ravetch). *X. laevis* ELYS cDNA was obtained from Thermo Scientific (accession no. BC086281). ELYS fragments were expressed as MBP-His-HA fusions. *Xenopus laevis* RCC1 cDNA was obtained from a previously described cDNA library¹³. Detailed cloning procedures and sequences and vector sequences or maps are available upon request.

Protein purification and histone refolding

ASF1-Fc-His was purified on Ni-NTA agarose as follows. ASF1-Fc-His was expressed for 20 h at 18 °C. Cells were spun down and resuspended in lysis buffer (20 mM Tris-Cl [pH 7.5 at 22°C]; 500 mM KCl; 10% glycerol; 30 mM imidazole; 0.1 % Triton X-100; 10 mM 2-mercaptoethanol; 1 mM PMSF; 0.25 mg/ml lysozyme; 10 µg/ml leupeptin; 10µg/ml pepstatin; 10µg/ml chymostatin). Cells were lysed by sonication and the lysate was centrifuged at 42000 rpm in a Ti45 rotor. For each litre of cell culture, 1 ml of Ni-NTA beads (Qiagen) was used. Beads were equilibrated in Wash Buffer 1 (20 mM Tris-Cl [pH 7.5 at 22°C]; 50 mM NaCl; 10% glycerol; 40 mM imidazole; 10 mM 2-mercaptoethanol) and incubated with the clarified lysate at 4°C for 1 h under rotation. Beads were washed extensively with Wash Buffer 1 and 2 (as Wash Buffer 1 but only 100 mM NaCl) and then eluted with Elution Buffer (as Wash Buffer 2 but with 250 mM imidazole). If necessary, the protein was further purified by ion exchange chromatography using a HiTrap Q FF column (GE Healthcare) on an ÄKTA FPLC system (GE Healthcare).

MBP-His-HA-ELYS fragments were expressed in *E. coli* for 20 h at 18°C. Cells were pelleted and resuspended in lysis buffer (1x PBS supplemented with NaCl to 500 mM; 10 mM 2-mercaptoethanol; 10 mM imidazole; 0.05% Triton X-100; 1 mM PMSF; 0.25 mg/ml lysozyme; 10 µg/ml leupeptin; 10 µg/ml pepstatin; 10 µg/ml chymostatin), lysed by sonication, and the lysate was centrifuged at 42000 rpm in a Ti45 rotor. For each litre of cell culture, 1 ml of Ni-NTA beads (Qiagen) was used. Beads were equilibrated in Wash Buffer (1x PBS supplemented with NaCl to 500 mM; 10 mM 2-mercaptoethanol; 10 mM imidazole) and incubated with the clarified lysate at 4°C for 1 h under rotation. Beads were washed extensively with Wash Buffer, subsequently incubated with Prescission protease (GE Healthcare, over night at 4°C) in 1xPBS 10 mM 2-mercaptoethanol. The eluate was

recovered, and beads were washed three times in 1xPBS 10 mM 2-mercaptoethanol. All fractions were combined and dialyzed against 10 mM Na-PO₄ [pH 7]; 150 mM NaCl; 1 mM EDTA; 1 mM DTT. HA-ELYS fragments were separated from Prescission and contaminants by ion exchange chromatography on a HiTrap SP FF column (GE Healthcare) on an ÄKTA FPLC system (GE Healthcare). Fractions were dialyzed against 1x PBS 10 mM 2-mercaptoethanol.

All histones were purified from inclusion bodies. Inclusion bodies were washed once in wash/lysis buffer (50 mM Tris-Cl [pH 8 at 22°C] 100 mM NaCl 10 mM imidazole 10mM 2-mercaptoethanol), once in wash/lysis buffer plus 0.05 % Triton X-100, and once in wash/lysis buffer. Protein was resolubilized from the inclusion bodies by incubation over night in D500 (6 M Guanidine HCL; 500 mM NaCl; 50 mM Tris-Cl [pH 8 at 22°C]; 5 mM 2-mercaptoethanol; 7.5 mM imidazole). For His-tagged histones (H2A, H3.2, H3.3, H4), these were bound to Ni-NTA beads (Qiagen) equilibrated in the same buffer. Beads were washed extensively in D500 and D1000 (as D500 but containing 1 M NaCl), and eluted with Elution Buffer (as D1000 but containing 300mM imidazole). For untagged H2B (this was a necessity because the tags cannot be cleaved off the native H2B N terminus) no further purification beyond preparation from inclusion bodies was carried out.

H3–H4 tetramers and H2A–H2B dimers were purified as follows. Denatured histones were mixed at concentration of ~45 µM in D500 and dialyzed over night in Dialysis Buffer 1 (20 mM MOPS; 500 mM NaCl; 10% glycerol; 1 mM EDTA; 5 mM 2-mercaptoethanol; pH 7 at 22°C). Precipitate was spun down and the mixture was dialyzed twice more against Dialysis Buffer 2 (as Dialysis Buffer 1 but only 5% glycerol) and Dialysis Buffer 3 (as Dialysis Buffer 1 but 2.5% glycerol). Tags were removed by digestion with TEV protease over night at 16°C, resulting in native N termini for all histones. TEV and other contaminants were removed from the tetramers or dimers by gel filtration chromatography on a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) on an ÄKTA FPLC system (GE Healthcare). Peak fractions were recovered, concentrated and stored in the presence of 1 mM TCEP and 50% glycerol at –20°C.

Preparation of 19x601 DNA substrates and nucleosome assembly

Arrays were prepared as described⁷³. pAS696 containing the 19-mer of the 601 positioning sequence separated by 53bp of linker DNA (a gift of Aaron Straight) was digested with HaeII, DraI, EcoRI and XbaI, and the array was separated from the rest of the plasmid by PEG precipitation. Ends were filled in with Klenow fragment (NEB) in the presence of biotin-14-dATP and thio-dTTP and thio-dGTP, resulting in incorporation of biotin label at both ends. The array was separated from unincorporated nucleotides using illustra NICK columns (GE Healthcare).

Nucleosomes were assembled as described previously⁷³. 50 µl mixtures were prepared containing 10 µg of DNA, H2A–H2B dimers and H3.2–H4 tetramers at slight excess over DNA, 2 M NaCl and 1xTE. The exact amounts and ratios of histones were determined empirically for each fresh preparation. The mixtures were transferred into dialysis buttons (Hampton Research) and submerged in 450 ml of High Salt Buffer (10 mM Tris-Cl [pH 7]; 0.2 mM EDTA; 2 M NaCl; 10 mM 2-mercaptoethanol). In an exponential gradient over 72 h

at 4°C, this buffer was exchanged with Low Salt Buffer 1 (as High Salt Buffer but containing only 2.5 mM NaCl). Following this, the reactions were dialyzed against Low Salt Buffer 2 (10 mM Tris-Cl [pH 7]; 0.25 mM EDTA; 150 mM NaCl; 1 mM TCEP). Nucleosome formation efficiency was determined by native gel electrophoresis as described⁷³. Only reactions yielding >90 % nucleosomes were used for experiments.

Coupling of nucleosome arrays and naked DNA to beads

Nucleosomes and naked DNA were coupled to Dynabeads M-280 Streptavidin (Life Technologies). 0.15 µl of beads suspension and nucleosomes totaling 62.5 ng worth of DNA were used for every µl of extract that they would be added to later. Naked DNA was used at conditions giving equal coupling for each type of experiment (determined by microscopy for microscopy experiments, and by phenol extraction/agarose gel electrophoresis for pull down experiments). These were optimized for each experiment and batch of DNA or nucleosomes. For experiments without extract, 270 ng of nucleosome array (and naked DNA giving equal coupling) were coupled to 1.5 µl of beads suspension. In all cases, beads and DNA or nucleosomes were incubated under agitation in 2.5 % polyvinylalcohol 150 mM NaCl (1.5 M NaCl for naked DNA) 50 mM Tris-Cl (pH8 at 22°C) 0.25 mM EDTA 0.05 % Triton X-100. Following coupling, nucleosome arrays were washed extensively.

Plasmid supercoiling assay

pBlueScript was relaxed with *E. coli* Topoisomerase I (NEB), purified by phenol/chloroform extraction and added to extract at a final concentration of 20 ng/µl. Extracts were incubated at 20°C for 2.5 h following which they were diluted 10-fold in Stop buffer I (20 mM Tris pH 8.0 at 22°C; 20 mM EDTA; 0.5% SDS; 50 µg/ml RNase A [Qiagen]) and incubated at 37°C for 25 min. Following this, the samples were diluted 2-fold in Stop buffer II (20 mM Tris pH 8.0 at 22°C; 20 mM EDTA; 0.5% SDS; 1 mg/ml Proteinase K [Roche]), and incubated at 37°C for another 25 min. Samples were then extracted twice with phenol/chloroform/isoamylalcohol and once with chloroform. Samples were ethanol-precipitated, resuspended in 1xTE containing 50 µg/ml RNase A, and incubated at 37°C for 20 min. Products were resolved on a 1% TBE agarose gel in the absence of intercalators at 1V/cm. The gel was stained with 1x SYBR-Safe prior to visualization.

Micrococcal nuclease (MNase) digests

H3.3–H4 tetramers were added to H3–H4 extracts at a final concentration of 3 µM (higher concentrations than that had a strong dominant negative effect on the extracts). As control, mock-depleted extracts and H3–H4 extracts without added H3.3–H4 were treated likewise. Extracts were pre-incubated at 20°C for 1h. Subsequently, relaxed pBlueScript was added at a final concentration of 20 ng/µl, and extracts were incubated for another 2.5 h at 22°C. Extracts were diluted 10-fold in Stop buffer I (20 mM Tris pH 8.0 at 22°C; 20 mM EDTA; 0.5% SDS; 50 µg/ml RNase A [Qiagen]) and incubated at 37°C for 25min. Following this, the samples were diluted 2-fold in Stop buffer II (20 mM Tris pH 8.0 at 22°C; 20 mM EDTA; 0.5% SDS; 1 mg/ml Proteinase K [Roche]), and incubated at 37°C for another 25 min. Samples were then extracted twice with phenol/chloroform/isoamylalcohol and once with chloroform. Samples were ethanol-precipitated, resuspended in 1xTE containing

50 µg/ml RNase A, and incubated at 37°C for 20 min. Samples were diluted 3-fold in MNase buffer (10 mM Hepes pH 8; 50 mM KCl; 1.5 mM MgCl₂; 0.5 mM EDTA; 10% glycerol; 1 mM DTT; 10 mM beta-glycerophosphate; 6.6 mM CaCl₂) and incubated at 20°C for 5 min in the presence of MNase (Worthington) at concentrations of either 0; 0.3 U/µl; 1 U/µl or 3.3 U/µl. Samples were diluted 10-fold in Stop buffer III (20 mM Tris pH 8.0 at 22°C; 20 mM EDTA; 20 mM EGTA; 0.4% SDS; 50 µg/ml RNase A [Qiagen]) and incubated at 37°C for 20 min. Following this, the samples were diluted 2-fold in Stop buffer IV (20 mM Tris pH 8.0 at 22°C; 15 mM EDTA; 15 mM EGTA; 0.25 % SDS; 1 mg/ml Proteinase K [Roche]), and incubated at 37°C for another 25 min. Samples were then extracted twice with phenol/chloroform/isoamylalcohol and once with chloroform. Samples were ethanol-precipitated, resuspended in 1xTE containing 50 µg/ml RNase A, and incubated at 37°C for 20 min. Products were resolved on a 2% agarose TBE gel in the presence of 1x SYBR-Safe (Invitrogen).

Fluorescence microscopy

Spindles were visualized by supplementing extracts with 0.2 µM bovine tubulin labeled with rhodamine succinimidyl ester (Life Technologies; C-1309) and processed for fluorescence microscopy by squashing 1 µl extract with 3 µl fixative (5 mM HEPES, 100 mM NaCl, 50% glycerol, 10% formaldehyde, 1 µg/ml Hoechst 33342 [pH 7.7]) under an 18 × 18 mm square coverslip.

To visualize membranes, 1 µM Vybrant CM-DiI (Life Technologies) was added to extracts and samples were fixed as above. For the analysis in Fig. 4b, 30 individual beads were counted for each sample.

For immunofluorescence of GFP-NLS, beads were incubated in extract containing 5 µM GST-GFP-NLS, and were processed as described previously¹⁸. Briefly, samples were fixed in buffered formaldehyde and spun onto coverslips. Antibodies were diluted in AbDil (10 mM Tris, 150 mM NaCl, 2 % BSA, 0.1 % Triton X-100 [pH 7.4]).

For all other immunofluorescence analyses, samples were processed as follows. 4 µl of extract containing nucleosome beads or DNA beads were removed and added to 90 µl of sperm dilution buffer (SDB, 5 mM HEPES, 100 mM KCl, 1 mM MgCl₂, 150 mM sucrose, pH 8). Beads were recovered on a magnet and washed two more times in SDB. Beads were resuspended in 50 µl SDB containing 2 % formaldehyde and incubated at room temperature for 5 min. Beads were then washed twice in AbDil (10 mM Tris, 150 mM NaCl, 2 % BSA, 0.1% Triton X-100 [pH 7.4]), resuspended in AbDil and transferred to coverslips coated with poly-L-lysine. Antibodies were diluted in AbDil.

Spindles, membranes, and ELYS in Supplementary Fig. 5e were imaged with a Carl Zeiss Axioplan 2 microscope equipped with a Photometrics CoolSnap HQ cooled CCD camera, and controlled by MetaMorph software (Universal Imaging). Images were processed with MetaMorph and Adobe Photoshop.

All other microscopy was imaged using a Delta Vision Spectris (Applied Precision) setup comprised of an Olympus IX71 wide-field inverted fluorescence microscope, and a

Photometrics CoolSnap HQ camera (Roper Scientific). Images were captured at 0.2 μm steps, processed by iterative constrained deconvolution using SoftWoRx (Applied Precision), and analyzed in ImageJ or with MATLAB. Statistical significance was determined by Mann-Whitney U test (two-tailed). When experimental samples were compared to buffer samples that report background signals (Fig. 7b,c), one-tailed tests were applied. Where indicated, individual sections or sum projections are shown (see figure legends).

Binding assays

Peptide binding assays (Supplementary Fig. 2b) were carried out as follows. For each peptide, 100 μl of Dynabeads streptavidin (Life Technologies) were washed twice in wash buffer (20 mM Hepes pH 8; 200 mM NaCl; 1 mM DTT; 0.05% Triton X-100) and resuspended in 100 μl of wash buffer containing the indicated peptides at 20 μM . Beads were incubated at room temperature under rotation for 20 min, washed three times in wash buffer, washed two times in sperm dilution buffer (SDB, 5 mM HEPES, 100 mM KCl, 1 mM MgCl_2 , 150 mM sucrose, pH 8), and resuspended in 15 μl egg extract containing 32 μM nocodazole (Sigma). Samples were incubated with rotation at 4°C for 45 min. Beads were recovered and washed five times in wash buffer, resuspended in 15 μl SDS sample buffer boiled, and the supernatant was loaded on 4-12% Bis-Tris gels (Invitrogen) and analyzed by western blotting or coomassie staining. To prevent the peptides from eluting from the gel during coomassie staining, the gel was fixed with 10% glutaraldehyde for 30 min and washed 3 $\times$ 15 min in water. The gel was quenched with 200 mM M Tris, pH 8 for 30 min, washed in water and stained with GelCode Blue (Pierce). Peptides were synthesized by the Rockefeller University Proteomics Resource Center.

For nucleosome bead and DNA bead pull downs from extract (Supplementary Fig. 5d), beads were incubated with H3–H4 extract (see above), following which the extract was diluted 10 fold in CSF-XB (see above) and beads were recovered. Beads were washed 3 times in CSF-XB containing 0.05% Triton X-100. Bound proteins were eluted with SDS sample buffer and analyzed by gel electrophoresis and western blotting.

For investigating binding to ELYS fragments in the absence of egg extract, nucleosome or DNA beads were incubated in binding buffer (20 mM Hepes pH 7.7; 200 mM NaCl; 0.05 % Triton X-100; 0.5 mM TCEP; pH 7.7) containing 1 μM ELYS fragments and 0.25 ng/ μl BSA for 45 min at 20°C with vigorous agitation. Beads were recovered on a magnet and washed four times in binding buffer. Bound proteins were eluted from the beads by incubation with SDS sample buffer. For the experiments in Fig. 5c and e, ^{35}S -labeled ELYS and ELYS fragments were generated with the TnT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. TnT reactions were diluted 1:10 in binding buffer and beads were incubated in this mixture.

ELYS binding to H3–H4 was performed as follows. ASF1 and H3–H4 were incubated in buffer (20 mM MOPS; 500 mM NaCl; 2 % glycerol; 1 mM EDTA; 1 mM TCEP) at room temperature for 30 min, added to 25 μl of pre-washed suspension of protein A Dynabeads (Life Technologies), and incubated for another 45 min under rotation. Beads were washed and incubated in binding buffer (20 mM Hepes pH 7.7; 200mM NaCl; 0.05 % Triton X-100;

0.5 mM TCEP; pH 7.7) containing 1 μ M ELYS_C and 0.25 ng/ μ l BSA for 45 min at 20 °C with vigorous agitation. Beads were washed in binding buffer, bound proteins were eluted with SDS sample buffer, and analyzed by gel electrophoresis.

To investigate the interaction between ELYS_C and H2A–H2B, 34 μ g of H2A–H2B dimers or BSA (NEB) were incubated with 5 μ l of Affi-Gel 10 beads (BIO-RAD) in 20 mM Hepes pH 7.7 200 mM NaCl for 1h at 4°C under rotation. Beads were washed and quenched by incubation with 100 mM Tris-Cl pH 8 for another hour. Beads were then incubated in binding buffer (20 mM Hepes pH 7.7; 200 mM NaCl; 0.05 % Triton X-100; 0.5 mM TCEP; pH 7.7) containing 1 μ M ELYS_C for 45 min at room temperature under rotation. Beads were washed in binding buffer, bound proteins were eluted with SDS sample buffer, and analyzed by gel electrophoresis.

For investigating binding to recombinant Topoisomerase II α , nucleosome beads, DNA beads or uncoupled beads were incubated in binding buffer (10 mM Tris pH 7.5; 150 mM NaCl; 0.05 % Triton X-100) containing 1.6 μ M recombinant *Xenopus* Topoisomerase II (a gift of Yoshiaki Azuma) and 1 ng/ μ l BSA for 60 min at 20°C with vigorous agitation. Beads were recovered on a magnet and washed three times in binding buffer. Bound proteins were eluted from the beads by incubation with SDS sample buffer.

Expression of RCC1-DBD and ELYS C2-DBD in extract

The DNA binding domain of Xkid (amino acids 544–651)²¹, preceded by a short linker (GGSGGGSG) and followed by a single HA tag was added to the C terminus of RCC1 in pCS2-RCC1 using Gibson assembly. The same sequence was also used to replace amino acids 2281–2408 in pCMV-ELYS (Thermo Scientific, accession no. BC086281). Plasmid were cut with NotI (NEB), purified, and mRNAs were generated by using the mMESSAGE mMACHINE SP6 kit (Life Technologies) containing an extra 1 mM GTP to facilitate long transcript generation, according to the manufacturer's instructions. Purified ELYS cDNA was further treated with *E. coli* poly(A) polymerase (NEB), and purified a second time.

ELYS C2-DBD and RCC1-DBD mRNAs were added to M phase H3–H4 extracts at final concentrations of 300 ng/ μ l and 75 ng/ μ l, respectively, and extracts were preincubated for 80 min at 20°C to allow translation. Then, these extracts were released into interphase by adding 0.3 mM calcium. Ninety minutes after incubation at 20°C, NPC assembly was monitored as before (see above).

Mass spectrometry

Nucleosome beads and DNA beads were incubated in M phase H3–H4 extract for 80 min at 20°C. Tubes were gently flicked every 20 min. The extract was then diluted 1:10 in CSF-XB (100 mM KCl; 1 mM MgCl₂; 50 mM sucrose; 5 mM EGTA; 10 mM Hepes pH 8), and recovered on a magnet for 5 min at 4°C. Supernatant was removed and beads were washed three times in CSF-XB containing 0.05 % Triton X-100. Beads were resuspended in SDS-PAGE buffer and separated by gel electrophoresis.

Standard in-gel digestion was performed without reduction/alkylation prior to trypsinization. Generated peptides were analyzed by LC-MS/MS using a Finnigan Orbitrap XL (Thermo

Scientific, San Jose, CA) mass spectrometer. MS/MS data were extracted using Proteome Discoverer (Thermo, Bremen, Germany) and queried against the NCBI *Xenopus laevis* database using Mascot (Matrixscience, London, UK). Mass tolerance of 20ppm and 0.5 Da were used for peptide precursor and peptide fragments, respectively. Oxidized methionine and N-terminal acetylation were allowed as dynamic modifications, and up to three missed cleavage sites were used. Proteins with 3 peptide spectral matches identified in two biological replicates were included in subsequent analyses. A large number of identified peptides map to two highly similar proteins, most likely due to the structure of the *X. laevis* genome. In these cases, the most abundant protein was retained for subsequent analyses.

Protein quantitation was performed using Proteome Discoverer. Peptide area was calculated by integrating LC-MS peaks corresponding to each identified peptide. Isotope peaks for each peptide were summed to give the total peptide area. Protein area was calculated by averaging the three greatest peptide areas for each protein. To compare protein abundance between nucleosome beads and DNA beads, enrichment values were calculated as the ratio of protein areas between samples. When calculating enrichment values for proteins identified exclusively on nucleosomes or DNA, the detection limit of the LC-MS/MS was used for the unidentified protein. To compare protein abundance between proteins, protein area values for each protein were directly compared. To verify the reproducibility of our abundance determination, we determined whether the patterns were similar between the two replicates for each type of beads (nucleosome vs nucleosome and DNA vs DNA). For both nucleosome beads and DNA beads, reproducibility was very high (Supplementary Fig. 3b,c).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Histone depletion and analysis of mitotic nucleosome functions. **(a)** Western blot analysis of extracts. H3-H4 extracts and mock-depleted extracts. Uncropped images are shown in Supplementary Fig. 8. **(b)** Agarose gel electrophoresis analysis of plasmids purified from *E. coli* or from the indicated extracts. The gel was run in the absence of intercalators. An uncropped image is shown in Supplementary Fig. 8. **(c)** Schematic of the experimental strategy for reconstituting nucleosome functions in H3-H4 extracts. **(d)** Immunofluorescence analysis and quantification **(e)** of mitotic spindle formation in H3-H4 extracts incubated with nucleosome beads or nucleosome beads. Scale bar, 10 μ m. The quantification was done on clusters of 45-75 beads ($n = 14$ for nucleosome beads, $n = 19$ for DNA beads. Experiment repeated twice, counted in detail once). MTs, microtubules.

Figure 2.

The RCC1 and Aurora B dependent spindle assembly pathways both require nucleosomes.

(a) Two chromosome-induced pathways are required for spindle assembly. RCC1 is recruited by interactions with either DNA or H2A-H2B and generates RanGTP.

Chromosome enrichment allows Aurora B activation, and is thought to be mediated by interaction between Survivin and H3T3ph. (b) Western blot analyses of proteins co-purified with nucleosome beads or DNA beads recovered from M phase H3-H4 extracts.

Uncropped images are shown in Supplementary Fig. 8. (c) Western blot analyses of H3-H4 extracts incubated with DNA beads, wild type (wt) nucleosome beads, H3T3A nucleosome beads or with uncoupled beads. AurB, Aurora B. Uncropped images are shown in Supplementary Fig. 8. (d) Western blot analyses of H3-H4 extract or Haspin H3-H4 extracts incubated with DNA beads, wild type (wt) nucleosome beads, H3T3E nucleosome beads or with uncoupled beads. AurB, Aurora B. Uncropped images are shown in Supplementary Fig. 8.

Figure 3. Proteomic profiling of nucleosome beads and DNA beads. **(a)** The abundance of all reproducibly identified proteins on nucleosome or DNA beads from a single LCMS/MS experiment within each functional class were summed and charted. The y-axis represents the integrated LC-MS/MS signal (area). A similar result was obtained from a biologically independent replicate. **(b)** Abundance (LC-MS/MS integration) of each identified protein from a single LC-MS/MS experiment was calculated on DNA and nucleosomes. Proteins belonging to known complexes are colored accordingly. Proteins that were only identified on DNA or nucleosomes are plotted along the axes. Solid lines and dashed lines indicate a 4-fold and 10-fold enrichment, respectively, on DNA or nucleosomes. **(c,d)** For each chromosome structure **(c)** and signaling **(d)** protein, the ratio of protein quantity copurified with nucleosome beads over DNA beads was expressed as a heat map with average values from two biological replicates reported. Proteins enriched on nucleosome beads and DNA beads are marked as yellow and blue, respectively. Proteins showing neutral to mild preference are marked as black. **(e)** The 15 most abundant proteins identified by LC-MS/MS that show at least an order of magnitude enrichment on nucleosome beads over naked DNA beads.

Figure 4.

NPC assembly, but not membrane recruitment requires nucleosomes. **(a)** Fluorescence microscopy analysis and quantification **(b)** of nucleosome beads or DNA beads recovered from interphase H3–H4 extracts containing membrane dye (green). DNA was stained with DAPI (magenta). The quantification was done on isolated single beads ($n = 30$ each). Experiment was repeated twice, counted in detail once. **(c)** Fluorescence microscopy analysis and quantification **(d)** of nucleosome beads or DNA beads recovered from interphase H3–H4 extracts and stained with anti-BAF antibody (green) and DAPI (magenta). Single deconvolved z sections are shown. Each data point is the average intensity per bead of an individual cluster of beads ($n = 12$ for nucleosome beads; $n = 14$ for DNA beads). Median values and interquartile ranges are indicated. $P < 0.02$ (two-tailed Mann-Whitney U test). **(e)** and **(f)** as **c** and **d** but beads were stained with anti FG-NUP antibody (green) and DAPI (magenta). Median values and interquartile ranges are indicated ($n = 20$ for nucleosome beads; $n = 15$ for DNA beads) $P < 0.0001$ (two-tailed Mann-Whitney U test). **(g)** and **(h)** as **c** and **d** but beads were stained with anti Lamin B3 antibody (green) and DAPI (magenta). Sum projections of deconvolved images are shown. Median values and interquartile ranges are indicated ($n = 10$ for nucleosome beads; $n = 12$ for DNA beads) $P < 0.002$ (two-tailed Mann-Whitney U test). All scale bars represent 3 μm.

Figure 5.

Nucleosomes directly recruit ELYS. **(a)** Fluorescence microscopy analysis and quantification **(b)** of nucleosome beads or DNA beads recovered from interphase H3–H4 extracts and stained with anti-ELYS antibody (green) and DAPI (magenta). Single deconvolved z sections are shown. Each data point is the average intensity per bead of an individual cluster of beads ($n = 20$ for nucleosome beads; $n = 15$ for DNA beads). Median values and interquartile ranges are indicated. $P < 0.0001$ (two-tailed Mann-Whitney U test). Scale bar, 3 μm . **(c)** Autoradiography analysis of pull downs of nucleosome beads or DNA beads incubated with buffer containing ³⁵S-labelled ELYS. The uncropped image is shown in Supplementary Fig. 8. **(d)** Description of the ELYS fragments that were generated for this study. ELYS_C^{2A} contains two alanine mutations at R2332 and R2334 within the AT-hook⁴³. Interaction with nucleosome beads *in vitro* is indicated as “+” or “–”. **(e)** Autoradiography analysis and quantification **(f)** of pull downs of nucleosome beads or uncoupled beads incubated with buffer containing the indicated ³⁵S-labelled ELYS constructs. The quantification was normalized to the amount of full length ELYS that was pulled down with nucleosome beads. The uncropped image is shown in Supplementary Fig. 8. **(g)** Coomassie stained gel of a pull down of nucleosome beads, DNA beads or uncoupled beads incubated with recombinant ELYS_C. The uncropped image is shown in Supplementary Fig. 8.

Figure 6.

The C terminal domain of ELYS interacts directly with histones H2A–H2B. **(a)** Coomassie stained gel of nucleosomes incubated without or with trypsin to remove histone tails. The uncropped image is shown in Supplementary Fig. 8. **(b)** Western blot analysis of pull downs of beads containing nucleosomes with or without tails or uncoupled beads incubated with or without ELYS_C. Uncropped images are shown in Supplementary Fig. 8. **(c)** Coomassie stained gel of pull downs of beads containing ASF1-Fc–H3–H4 or ASF1-Fc incubated with or without recombinant ELYS_C. The uncropped image is shown in Supplementary Fig. 8. **(d)** Coomassie stained gel of pull downs of affi-gel resin coupled to recombinant H2A–H2B or BSA incubated with recombinant ELYS_C. FT, flow through. The uncropped image is shown in Supplementary Fig. 8.

Figure 7.

Targeting RCC1 and ELYS to DNA is sufficient for NPC assembly in the absence of nucleosomes. **(a)** Immunofluorescence analysis of nucleosome beads or DNA beads recovered from interphase H3–H4 extracts containing the mRNAs indicated on the top and stained with anti-ELYS antibody (green), anti FG-NUP antibody (magenta) and DAPI (blue). Single deconvolved z sections are shown. Scale bar, 3 μ m. **(b)** and **(c)** Quantification of **a**. Each data point is the average intensity per bead of an individual cluster of beads ($n = 20$ for each set). Median values and interquartile ranges are indicated. Numbers directly on top of each data set indicate P values for comparisons with buffer treated extracts containing DNA beads (one-tailed Mann-Whitney U test). Numbers on top of lines indicate P values for comparisons of the indicated data sets (two-tailed Mann-Whitney U test). Representative data from one of two biological replicates are shown.

Figure 8.

Roles of nucleosomes in spindle assembly and nuclear envelope assembly. **(a)** During M phase, RanGTP generated by nucleosome-associated RCC1, liberates microtubule assembly proteins from importin β . The chromosomal passenger complex (CPC) interacts with H3T3ph and microtubules to inhibit microtubule destabilizing proteins. **(b)** Upon transition from M phase to interphase, membranes are recruited by direct interactions between membrane proteins and DNA or DNA-bound proteins, such as BAF. High concentration of RanGTP, generated by RCC1, promotes fusion of nuclear membranes and insertion of nuclear pore complexes (NPCs). This process is synergized by direct recognition of H2A–H2B by ELYS, which promotes NPC formation by recruiting the Nup107 complex.