A novel role for histone chaperones in the dynamics of non-conventional substrates, the CENP-T/-W complex at the centromere.

The centromere directs chromosome segregation during cell division by coordinating the assembly of the kinetochore, the principle microtubule-binding interface of mitotic chromosomes. Two essential phases in centromere assembly and maturation have been distinguished during the cell cycle; a G1 phase, in which CENP-A (also called CenH3) a centromere specific histone H3 variant, is replenished to establish replication-competent centromeres, and a late-S/G2 phase, in which additional events establish a centromeric chromatin substrate competent for kinetochore assembly. This includes assembly of the CCAN complex, which contains the histone-like CENP-T–CENP-W heterodimers and CENP-S–CENP-X heterotetramer (*1*). The correct and timely deposition of the CENP-T–CENP-W protein complex to centromeres is critical for kinetochore formation, spindle assembly and correct chromosome segregation (*2-5*). However, little is known how the histone-fold CCAN complex is established at the centromere and whether accessory factors such as chaperone proteins help this vital assembly process.

The overall aim of this project was to characterize the assembly of the CENP-T-W complex to the centromere during S-phase with respect to the replicative state of the centromeric chromatin and identify the mechanisms, which are involved in targeting the complex to centromeric chromatin prior to mitosis. Our project has been successfully completed and will be submitted for peer review in a high impact scientific journal in the near future.

In order to identify proteins, which were potentially involved in the deposition of the CENP-T-CENP-W complex to centromeres, we employed a proteomic screening approach. We used a cell line that was expressing a green fluorescent protein (GFP) tagged version of CENP-W. This allowed us to specifically purify CENP-W-GFP and associated interacting proteins. The proteins, which co-purified with CENP-W, were identified by Mass spectrometry. We identified the SSRP1 subunit of the FACT complex as a CENP-W interactor. FACT (facilitates chromatin transcription) is a heterodimeric complex comprising the evolutionarily highly conserved SSRP1 and Spt16 proteins that is required for the chaperoning of histones with DNA (*6*). Co-immunoprecipitation experiments with SSRP1 and anti-GFP antibodies confirm the interaction of both CENP-W and CENP-T with both SSRP1 and Spt16.

We next asked whether the interaction between the FACT complex and the CENP-T-CENP-W complex was direct. An *in vitro* assay using recombinant SSRP1, Spt16 and CENP-T-CENP-W was designed performed. We found that either SSRP1 or Spt16 could bind directly to the histone fold region of the CENP-T-CENP-W. We then further refined the *in vitro* assay to determine specifically which regions of FACT bound to CENP-T-CENP-W. Taken together our data show that the CENP-T-CENP-W complex interacts directly with SSRP1 and Spt16 *in vitro* and *in vivo*.

We next asked whether the interaction between CENP-T-CENP-W and the FACT complex had a functional role. To determine this we depleted HeLa cells of FACT using siRNA. We then looked at the levels of CENP-W and CENP-A, which remained at centromeres. We found that the levels of

CENP-W associated with centromeres were significantly reduced following depletion of FACT. The levels of CENP-A were not significantly reduced. This indicates that the FACT complex may play a role in the deposition of the CENP-T-CENP-W complex to centromeres.

The FACT complex has roles in replication and transcription. Specifically, it travels with the RNA Polymerase II complex during transcription and facilitates the passage of the polymerase through nucleosomes (*7*). WE asked whether inhibiting transcription or replication would also inhibit CENP-W assembly at centromeres. To answer this question, we exploited the CLIP protein *in vivo* labeling system. This system has previously been used to assay the timing of assembly of CENP-A and the CENP-T/-W complex to the centromere(*3*, *7*). Briefly, a quench-chase-pulse experiment was used, whereby the 'old' population of CENP-W-CLIP present in CLIP tag cell lines was quenched using a BC-block, rendering this protein population immune to future labeling by a fluorescent probe. A chase time was used to allow synthesis of 'new' CLIP tagged protein, which was labeled with a fluorescent substrate. Cells, which had assembled CENP-W to the centromere, were assessed using immunofluorescence microscopy. We observed that cells in which transcription had been inhibited using a specific inhibitor of RNA Polymerase II, failed to show assembly of CENP-W at centromeres. This indicates that transcription may play a role in CENP-T-CENP-W assembly at centromeres.

Taken together, we our data lead us to propose a model whereby the FACT complex can bind to the CENP-T-CENP-W complex in vivo and acts in a transcription dependent manner to deposit the CENP-T-CENP-W complex to centromeres. Our findings identify a novel cell cycle-specific chromatin assembly pathway dependent on transcription for assembly of the CENP-T-CENP-W complex to chromatin

Impact and Outlook

Our project has a far-reaching potential impact, as the vital mechanisms involved in the targeting of kinetochore components onto post-replicative CENP-A centromeric chromatin prior to mitosis have not been established. The observation that the essential histone chaperone FACT deposits a non-histone protein complex (CCAN) into chromatin is novel and promises to shed important insights into how the kinetochore is assembled. Investigating the mechanisms underlying this chaperone function at centromeres enhances our understanding of how epigenetic components adapt the cellular machinery to perform the critical task of directing the accurate segregation of chromosomes in cell division.

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