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RNA as a Structural and Regulatory Component of the Centromere

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Abstract

Despite many challenges, great progress has been made in identifying kinetochore proteins and understanding their overall functions relative to spindles and centromeric DNA. In contrast, less is known about the specialized centromeric chromatin environment and how it may be involved in regulating the assembly of kinetochore proteins. Multiple independent lines of evidence have implicated transcription and the resulting RNA as an important part of this process. Here, we summarize recent literature demonstrating the roles of centromeric RNA in regulating kinetochore assembly and maintenance. We also review literature suggesting that the process of centromeric transcription may be as important as the resulting RNA and that such transcription may be involved in recruiting the centromeric histone variant CENH3.

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Centromeric histone H3 (CENH3/ CENP-A): an H3 histone variant found in the chromatin underlying the kinetochore, a defining feature of active centromeres

Histone: a subunit of nucleosomes, which are the primary determinants of chromatin structure

Chromatin: DNA and the associated factors that make up the three-dimensional, orderly structure of chromosomes

Satellite DNA: long arrays of tandem repeats

Chromatin immunoprecipitation (ChIP): a technique to define the DNA binding sites of specific proteins

INTRODUCTION AND A BRIEF HISTORY

In eukaryotes, the transmission of chromosomes from mother cell to daughter cell depends on an intricate interaction between a microtubule-based tethering system and DNA. The DNA at the attachment point is the centromere, and the structure that mediates the tethering of the centromere to the microtubules is the centromere/kinetochore complex, or simply the kinetochore. The kinetochore is composed of as many as 100 different proteins that mediate connections to DNA on one side and tension-sensitive connections to microtubules on the other (reviewed in 52). In addition to physically controlling chromosome movement, the kinetochore also serves as a major signaling center and regulates multiple aspects of cell division (reviewed in 38). Understanding how the centromere-kinetochore complex itself is regulated, i.e., what controls its dynamic structure and interaction with cell cycle regulators, is the subject of much investigation because of its central importance in genetics and the severe consequences of its failure.

Many factors controlling centromere behavior have been identified in recent years, but one has demanded special attention: the highly conserved centromeric histone H3 (CENH3, also commonly known as CENP-A), which is viewed as the primary distinguishing feature of centromeric chromatin (reviewed in 11, 52). Understanding how centromeric chromatin–containing CENH3 is established, maintained, or lost is essential to understanding kinetochores. It is also an avenue into broader questions of chromatin regulation independent of kinetochores, as similar chromatin-based processes can be important in other contexts, such as gene expression and nuclear architecture. In this review, we summarize a variety of evidence showing that RNA or RNA production is an integral part of the centromeric chromatin environment and that centromeric RNA or its downstream effects can have a major impact on the accuracy of chromosome segregation.

The DNA composition of centromeres can vary wildly between species and even between the chromosomes of an individual; however, centromeres are generally depleted of protein-coding sequences other than transposons. Centromeres typically contain long arrays of simple tandem repeats that do not contain recognizable promoters or encode proteins. These tandem repeats have historically been called satellite DNA because their densities often differ from bulk chromatin during centrifugation. As early as the 1960s and 1970s, evidence for transcription of mouse satellites was reported (18, 32), as was the presence of RNA in kinetochores of both plants and animals (7, 10, 56). Detailed characterization of the RNA was not possible in these early studies because of the limitations of the experimental methods available. Part of the difficulty in interpreting these studies came from the typical organization of chromosomes, wherein both the centromere and flanking pericentromere are highly repetitive and difficult to discern from each other (see sidebar, Usage of the Terms Centromere and Pericentromere). Hence, detection of RNA homologous to centromeric DNA does not clearly demonstrate transcriptional activity of centromeres. Nevertheless, at least a fraction of centromere-homologous RNA appears to be associated with centromeres. The first report of centromere-associated RNA involved chromatin immunoprecipitation (ChIP) of maize CENH3 (66) but was supported and advanced

USAGE OF THE TERMS CENTROMERE AND PERICENTROMERE

The term centromere is often used to refer to both centromere and pericentromere; for the sake of clarity, we will use the term centromere to refer specifically to the portion of the chromosome underlying the kinetochore. The term pericentromere is used to refer to the regions on either side where sister chromatids remain attached until anaphase. This region is also known as the inner centromere because as the kinetochores are pulled outward, the attached pericentromeres occupy the inner space between them. It is important to note, however, that these domains may be somewhat fluid and are not strictly defined by DNA sequence.

by similar ChIP studies of mammalian tissue culture cells (16, 25, 71).

Other support for direct interaction between RNA and centromeric chromatin came from studies of CENP-C (centromere protein C), another defining mark of centromeric chromatin. Experiments in both human and maize tissues have shown that CENP-C binds RNA as well as DNA (20, 71). Surprisingly, the domains responsible for RNA binding are not conserved between human and maize; in fact, the protein as a whole is barely similar: The 701-amino-acid maize homolog of CENP-C, CENPCA, has only a short (23-amino-acid) segment of limited homology (63% identical) with the 943-amino-acid human homolog, CENPC1 (19). The conservation of RNA binding despite such difference in amino acid composition, along with detection of RNA physically associated with the centromere, hints at an important, conserved requirement for RNA in centromeric chromatin.

SATELLITE RNA AS A COMPONENT OF THE CHROMOSOMAL PASSENGER COMPLEX

RNA seems to be an integral component of centromeres/kinetochores, but what function, if any, does it have? In vitro experiments with maize CENP-C have indicated that RNA binding facilitates its DNA binding capacity (although a removal of the RNA binding domain had only a mild effect on CENP-C localization) (20). Other evidence from human cell lines suggests that RNA may have a general structural role, particularly in the context of the chromosomal passenger complex (CPC). The key enzyme of the CPC, Aurora B protein kinase, regulates multiple aspects of chromosome dynamics, including kinetochore-microtubule attachment, pericentromeric cohesion, chromosome alignment, and the spindle checkpoint (reviewed in 38, 58). During metaphase, the CPC members Aurora B, Survivin, Borealin, and INCENP localize to the inner centromere, the space between centromeres of sister chromatids that are held close together by pericentromeric cohesion. CENP-C also plays a role in this process because the integrity and proper localization of the CPC depends on an interaction between CENP-C and INCENP (24).

Chromosomal passenger complex (CPC): includes Aurora B kinase, which is an important regulator of sister chromatid separation

RNase treatment of human cultured cells results in both loss of CENP-C and CPC components from centromeres (71). After native CPCs were displaced by RNase, exogenously supplied CENP-C and INCENP could be reassembled if satellite RNA was also cointroduced. Overexpression of mouse centromeric RNA was also shown to compromise CPC integrity, as Aurora B was mislocalized in cells expressing high levels of transgene-encoded α-satellite RNA (9). Cells with overexpressed centromeric RNA also showed abnormalities in the localization of pericentromeric chromatin factors and chromosome segregation. The presumed RNA-associated kinetochore complexes probably include multiple other proteins, including CENP-A (mammalian CENH3) and CPC components, as judged by ChIP, RNA pull-down, and RNase assays of mouse cell extracts (25). In particular, Aurora B, Survivin, and CENP-A could be detected in RNA pull-down experiments with a one-monomer-length (120 nt) satellite RNA bait; and conversely, satellite RNA could be detected in ChIP experiments with antibodies for each of the three proteins. Also in this study, in vitro experiments with immunoprecipitated Aurora B suggested that centromeric RNA promotes its kinase activity. Although the precise interactions between RNA, CENH3, CENP-C, Aurora B, and other CPC members remain to be established, these reports from both plants and animals provide strong evidence that RNA is required for proper function of the centromere.

ASSOCIATION BETWEEN THE CENTROMERE, RNA, AND THE NUCLEOLUS

The nucleolus is a domain of the nucleus best known for being the site of ribosomal RNA synthesis, but it is also a major

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Small RNA: includes various classes of RNAs approximately 20–30 nt in length that function in RNAi-like processes

RNA interference (RNAi): involves siRNAs (short interfering RNAs) guiding repressive proteins via base pairing with RNA targets

contributor to chromatin organization and nuclear architecture (reviewed in 47). Surprisingly, centromeres have been discovered to colocalize with nucleoli (49). Furthermore, several centromeric proteins interact with centromeric RNA in the nucleolus. Human CENP-C physically associates with nucleolar transcription factors, contains a nucleolar targeting domain, and requires satellite RNA to localize to the nucleolus (53, 71). The CPC component INCENP also has a nucleolar targeting domain, and it and other CPC components localize to the nucleolus (2, 57, 71). Likewise, a recently identified centromeric protein, CENP-W, localizes to the nucleolus and associates with the nucleolar RNA-interacting protein B23/nucleophosmin. Independent evidence suggests B23 itself physically associates with centromeric chromatin (27), and the interaction between CENP-W and B23 is sensitive to RNase A and can be rescued by addition of mRNA-enriched RNA extracts (17). These observations raise the possibility that the nucleolus plays a role in assembly of the kinetochore and further support the hypothesis that centromeric RNA is a functionally important component of the system.

RNA POLYMERASE II ACTIVITY AT CENTROMERES

Additional evidence for the importance of centromeric transcription comes from studies of RNA polymerase II (Pol II) and experimental manipulations of its activity. Pol II and several transcription factors localize to mammalian centromeres during mitosis (14). Blocking Pol II activity with α-Amanitin resulted in a loss of satellite RNA, a reduction of CENP-C at centromeres, and an increase in the number of lagging chromosomes. In the budding yeast *Saccharomyces cerevisiae*, a transcription factor called STE12 binds to centromeres and is required for accurate chromosome segregation (50). The Ste12 mutant phenotype can be rescued by ectopically driven (RNA Pol II–based) transcription through the centromere. There is also strong evidence that fission yeast *Schizosaccharomyces pombe* centromeres are transcribed to produce capped 5′ ends and polyadenylated 3′ ends (15). Centromeric RNA was dramatically increased in *S. pombe* mutants with either reduced CENP-A accumulation or compromised RNA turnover, suggesting that centromeric transcripts are quickly processed in normal conditions and that the level of transcript accumulation is connected to CENH3 accumulation. In plants such as maize and rice, not only are low levels of centromeric satellite RNA detectable, but centromeres can also contain genes that look like typical Pol II–transcribed genes based on histone modification profiles and polyadenylation of the transcripts (30, 72). These and other data indicate that plant centromeres are also compatible with Pol II transcription and may be relevant to the interactions between CENP-C and RNA discovered in maize (20).

CENTROMERIC RNA OF UNUSUAL SIZE

Intriguingly, northern analyses in plants and animals have revealed what appears to be centromeric small RNA with sizes larger than any known RNA interference (RNAi)-related small RNA, approximately 40 nt in length. These have been reported to be derived from satellites in maize (20, 66), from satellites in rice (39), and from both satellites and retrotransposons in the tammar wallaby (13). In maize, this species of RNA was found to immunoprecipitate with CENH3, hinting at a role in centromeric chromatin or kinetochore biology (66). However, the functions of these RNA species, as well as their modes of production, are mainly speculative. At this point, we cannot rule out that they are RNA degradation products with no regulatory activity. Furthermore, they have been detected only by northern blots, which do not allow for definitive identification of the signal source. It is important to define these molecules by sequencing, which will identify whether they are really centromeric and whether they show evidence for regulated biogenesis (such as defined endpoints or chemical modifications).

RNA REGULATION OF PERICENTROMERIC CHROMATIN MODIFICATIONS

Molecular Links Between Noncoding RNA and Chromatin Modifications: Lessons From *Schizosaccharomyces pombe* Pericentromeres

Transcription of protein-coding genes occurs in open, accessible chromatin called euchromatin, whereas heterochromatin is highly condensed and characterized by little or no transcription of protein-coding genes. However, in *S. pombe*, screens for mutants that failed to silence a gene embedded in heterochromatin led to the conclusion that the transcription of noncoding RNA can promote heterochromatin formation (22, 67). In brief, *S. pombe* pericentromeric heterochromatin depends on an RNAi-related process in which transcription gives rise to small interfering RNAs (siRNAs) that then guide histone-modifying enzymes and deposition of heterochromatin protein 1 (HP1) (called Swi6 in *S. pombe*), a highly conserved protein associated with loci that are not undergoing mRNA transcription (reviewed in 40). Surprisingly, this heterochromatin pathway is important for the dynamics of cohesion between sister chromatids (6). In addition, the integrity of heterochromatin in *S. pombe* pericentromeres is a major factor in controlling the location of CENH3 (26, 34). Other noncoding RNAs in *S. pombe* help ensure that chromatin modifications stay in their proper domains: Transcription of tRNAs driven by RNA polymerase III at the centromere-pericentromere boundary serves to prevent the spread of pericentromeric heterochromatic modifications into the centromeres (61, 62).

Evidence for RNA-Mediated Pericentromeric Chromatin Regulation in Multicellular Eukaryotes

Work in mammals has also revealed a connection between pericentromeric heterochromatin formation and RNAi. For example, loss of Dicer, a key double-stranded RNase in RNAi, can induce higher levels of long pericentromeric RNA and loss of HP1 from pericentromeres in some but not all contexts (23, 29, 35, 45). Dicer also physically interacts with pericentromeric satellite RNA and with WDHD1, a protein that promotes pericentromeric satellite siRNA accumulation but inhibits longer satellite RNA accumulation (33). Both WDHD1 and the histone lysine demethylase KDM2A, which also inhibits satellite RNA accumulation, are required for HP1 localization to pericentromeres (28, 33). These data suggest a role for satellite transcription and RNAi in the maintenance of pericentromeric heterochromatin, similar to the phenomena described in *S. pombe*. It seems unlikely, however, that the mechanisms are entirely shared because there are major differences in both siRNA distribution and genome architecture between *S. pombe* and other more complex eukaryotes (see sidebar, RNAi-Mediated Heterochromatin as a Determinant of Mitotic Chromosome Behavior: Projecting Too Far from *Schizosaccharomyces Pombe*?) In addition

RNAi-MEDIATED HETEROCHROMATIN AS A DETERMINANT OF MITOTIC CHROMOSOME BEHAVIOR: PROJECTING TOO FAR FROM *SCHIZOSACCHAROMYCES POMBE*?

Although there is ample evidence of small interfering RNA (siRNA)-directed heterochromatin formation in plants, fungi, and animals, the distribution of heterochromatin around centromeres is typically very different from *Schizosaccharomyces pombe*, with siRNA-regulated heterochromatin sometimes absent from the domains defined by cohesion retention and sometimes encompassing much larger regions (see References 30 and 65, and references therein). In addition, RNAi and heterochromatin-defective mutants in animals and plants generally lack overt chromosome segregation defects, and in cases where such defects are observed it is not easy to separate RNAi at pericentromeres from other indirect mutant effects (29). Hence it is unclear to what extent siRNA-regulated pericentromeric chromatin influences chromosome segregation beyond *S. pombe*.

Heterochromatin: condensed chromatin with little mRNA expression

HP1: heterochromatin protein 1

Pericentromeres: regions flanking centromeres, also called inner centromeres because of their location between sister chromatids during metaphase

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to serving as a substrate for RNAi, intact pericentromeric RNA is required for HP1 localization to pericentromeres in mammals (41, 42, 44). Transcription of pericentromeric RNA may also be required to regulate growth and development. For example, mouse pericentromeric satellite RNA is highly expressed at a time during embryogenesis when the paternal pericentromeric chromatin is being established (to remove the spermatogenesis-specific chromatin signature). Strikingly, interference with pericentromeric RNA blocks early development (55). Conversely, another study reported that overexpression of pericentromeric satellite RNA (due to a mutation in the histone H3 variant H3.3) also blocks early development but could be corrected by injecting double-stranded pericentromeric RNA (59). These data suggest that transcription of noncoding repetitive elements giving rise to both siRNAs and longer RNAs can play an important role in determining chromatin structure in a wide range of organisms.

RNA REGULATION OF CENTROMERIC CHROMATIN MODIFICATIONS

Interactions Between RNA Effects on Centromeric and Pericentromeric Chromatin

Centromeres and pericentromeres differ considerably in chromatin structure, and at least in human cells they do not show the same patterns of transcription (23). However, given their close proximity, one might expect that RNA expression/chromatin modifications in pericentromeres would impact centromeres and vice versa. In some species, such as mice and humans, different satellites are associated with each domain (reviewed in 68), whereas in other species, such as maize, there is one dominant satellite and it is preferentially associated with centromeres rather than pericentromeres (70). The pericentromeres would be difficult to identify without the adjacent centromeres in many species, and features such as pericentromeric cohesion may be controlled by centromeric signals (discussed in 30). Hence, it is not surprising that depletion of CENP-C in human cells perturbs both centromeric chromatin modifications and pericentromere chromatin modifications (31). Likewise, overexpression of centromeric satellite RNA induces pericentromeric chromatin aberrations in mammalian cells (9, 28), and HP1 has dual roles in suppressing pericentromeric satellite transcription and promoting the localization of centromeric proteins (36, 48).

Centromeric Transcription and Chromatin Modifications

Although centromeric chromatin is distinguished by the presence of CENH3, it also includes interspersed canonical H3 and its associated N-terminal modifications (8, 64, 74). The relations between H3 modifications and centromeric transcription seem to follow the general patterns discovered in noncentromeric chromatin, but the precise rules have evaded discovery (reviewed in 68). As with pericentromeric satellites, many chromatin-modifying factors associated with transcriptional repression have been reported to localize to centromeres and inhibit the accumulation of centromeric satellite RNA. These include the ubiquitin E3 ligase BRCA1 (75), the histone demethylase KDM2A (28), Dicer (29), and the DNA methyltransferase DNMT3B (31), among others. Although in some cases defects of the centromere or chromosome missegregation have been observed, the specific effects of centromeric transcriptional derepression on the centromere/kinetochore are difficult to separate from nonspecific effects such as genomic instability (75). It is tempting to speculate that directing the activity of specific chromatin-modifying factors to the centromere is a functionally important feature of centromeric transcription.

The repetitive nature of centromeres appears to be particularly conducive to the formation of double-stranded RNA and associated small RNAs with the potential to regulate

chromatin modifications (29, 43). Studies in maize and rice have taken advantage of good centromere assemblies and maps of CENH3 occupancy to confirm that siRNAs are indeed produced in centromeres but at very low levels compared with other parts of the genome (30, 73). It is unclear what function, if any, siRNAs have in regulating chromatin structure at centromeres. One possibility is that they fine-tune levels of longer RNA transcripts available to other RNA-based chromatin regulation mechanisms. Another is that siRNAs are produced in rare or transitive situations in which other mechanisms for regulating RNA levels are impeded.

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Centromeric Transcription and CENH3 Recruitment and Maintenance

Human artificial chromosomes have provided a valuable experimental approach for demonstrating links between chromatin modifications, centromere function, and transcription of centromeric satellites because they can be experimentally manipulated with minimal effects on chromatin elsewhere. Changes in centromeric transcription due to tethering of either transcriptional activators or silencers are associated with chromosome missegregation (12, 46). Furthermore, depletion of an H3 modification associated with RNA Pol II activity (dimethylation of H3 lysine 4) by tethering of the lysine demethylase LSD1 induced loss of centromeric transcription as well as reduced incorporation of newly synthesized CENP-A and mislocalization of the CENP-A chaperone HJURP (5). Similarly, tethering of a transcriptional activator that induced acetylation of H3 lysine 9 and drastic overexpression of satellite RNA resulted in centromere inactivation associated with loss of assembled CENP-A and failure to recruit newly synthesized CENP-A (4). Yet another example of transcription dependence of CENP-A came from the study of a human neocentromere that formed ectopically on a chromosome arm (16). RNAi knockdown of a single retrotransposon within the neocentromere resulted in a decrease of CENP-A coupled with an increased rate of chromosome missegregation.

Neocentromere: a site lacking native centromeric DNA but supporting a kinetochore, often associated with tumors or other genetically unstable contexts

Transcription plays an important role in replication-independent incorporation of the H3 variant H3.3 into chromatin (60). Because CENH3 incorporation is also replication independent, it has been proposed that CENH3 deposition may involve factors that are shared with the H3.3 deposition pathway. H3.3 incorporation has also been proposed to facilitate CENP-A incorporation directly (21). In support of this hypothesis, SSRP1, a factor associated with transcriptional elongation, localizes to centromeres and is required for efficient incorporation of CENH3 in vertebrates (14, 51). SSRP1 is also required for centromeric localization of a chromatin remodeler, CHD1 (51), which has been implicated in the context of transcriptional elongation and in H3.3 incorporation (37, 63). CHD1 is also required for CENH3 incorporation in vertebrates and in *S. pombe* (51, 69). However, these connections are indirect, and perhaps not conserved, because CHD1 in *Drosophila melanogaster* has no obvious connection to CENH3 (54). Nonetheless, it is clear that there is a link between centromeric transcription and CENH3 incorporation, and the idea that centromeric transcription may promote the incorporation of CENH3 via an intermediate step involving H3.3 or its deposition factors is indeed a compelling hypothesis.

CONCLUSIONS AND FUTURE PROSPECTS

As summarized in **Figure 1**, centromeric RNA or its production potentially functions in many capacities related to chromosome segregation, from the structural foundations provided by CENP-C and CENP-A to the signaling roles regulated by Aurora B kinase. Although many details remain to be filled in, basic frameworks are starting to become clear, including the requirement for Pol II activity at centromeres (e.g., see References 14, 50), the involvement of centromeric RNA in both CENP-C and

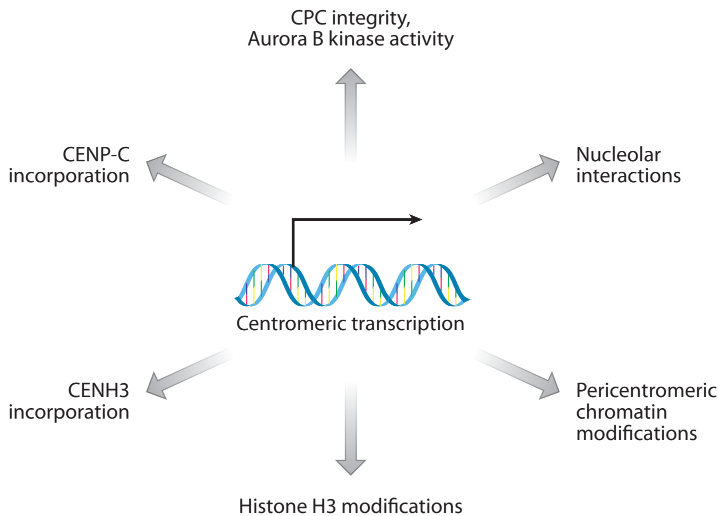

Figure 1

Summary of centromeric phenomena that are affected by either centromeric RNA itself or its transcription. Gray arrows indicate an effect stemming from centromeric transcription. Although the diagram shows all arrows coming directly from transcription, it is also likely that indirect effects explain some of the observations, in that none of these phenomena are entirely independent of each other. Abbreviation: CENH3, centromeric histone H3; CPC, chromosomal passenger complex.

CENH3 incorporation (e.g., see References 5, 16, 20, 71), and the necessity of centromeric RNA for CPC integrity and Aurora B activity (e.g., see References 25, 71).

One area of particular interest is the question of RNA sequence specificity. Multiple studies have concluded that transgene-driven expression of centromeric satellite RNA is sufficient to either recruit centromeric factors or to affect the centromere itself (9, 25, 28, 33, 71). However, kinetochores can also form over neocentromeres, which lack canonical centromere sequences. Consequently, such neocentromeres may either function with *trans*-acting satellite RNA or be mildly defective because of the absence of *cis*-acting RNA (1, 3). Determining whether centromeric RNA acts in *cis* or *trans* will help us understand how the RNA is deposited and retained at centromeres. High-throughput sequencing is another area where much progress can be made, particularly if RNA-seq libraries can be made from coimmunoprecipitates of centromere and kinetochore factors. Characterizing the repertoire of RNA species and their molecular features could answer multiple questions about their biogenesis, likely function, and the degree of sequence specificity required for RNA to fulfill its functions in centromere assembly and kinetochore biology. The large collection of literature already available shows that centromeric RNA is a major contributor to the structure and function of the centromere/kinetochore complex, but mechanisms remain unknown.

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