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Non-coding murine centromeric transcripts associate with and potentiate Aurora B kinase

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ABSTRACT

Non-coding RNAs are emerging as key players in many fundamental biological processes, including specification of higher-order chromatin structure. We examined the implication of RNA transcribed from mouse centromeric minor satellite repeats in the formation and function of centromere-associated complexes. Here we show that the levels of minor satellite RNA vary during cell-cycle progression, peaking in G2/M phase, concomitant with accumulation of proteins of the chromosomal passenger complex near the centromere. Consistent with this, we describe that murine minor satellite RNA are components of CENP-A-associated centromeric fractions and associate with proteins of the chromosomal passenger complex Aurora B and Survivin at the onset of mitosis. Interactions of endogenous Aurora B with CENP-A and Survivin are sensitive to RNaseA. Likewise, the kinase activity of Aurora B requires an RNA component. More importantly, Aurora B kinase activity can be potentiated by minor satellite RNA. In addition, decreased Aurora B activity after RNA depletion can be specifically rescued by restitution of these transcripts. Together, our data provide new functional evidence for minor satellite transcripts as key partners and regulators of the mitotic kinase Aurora B.

INTRODUCTION

The centromeres of eukaryotic chromosomes are genomic regions featuring a unique and specific chromatin architecture, necessary for proper chromosome segregation during mitosis. The common trait of centromeres in all species is the presence of nucleosomes containing a specific variant of histone H3, the centromere protein A (CENP-A) (1). Flanking pericentromeric regions are devoid of CENP-A, but exhibit a high density of histone H3 tri-methylated on its lysine 9 (H3K9Me3), consistent with the heterochromatin nature of these domains (2). In addition to CENP-A, numerous proteins identified as essential for centromere assembly and function occupy centromeric regions in a constitutive manner (3). In contrast, the Chromosomal Passenger Complex (CPC), composed of Aurora B kinase and its regulatory subunits inner centromere protein (INCENP), Survivin and Borealin, shows dynamic changes in its subcellular localization in a cell-cycle-dependent manner (4).

Sequences of centromeric DNA repeats are not conserved among species, but transcripts originating from them have been described in a broad range of organisms (5–12). It remains unclear how these RNA participate in the formation and/or stabilization of large-scale centromeric chromatin structures. However, in many chromatin complexes, including pericentromeric heterochromatin, RNA is an integral component (13,14).

The repeats of minor satellite DNA on mouse chromosomes are found at the primary constriction, adjacent to repeats of major satellite that define pericentromeric regions. We previously described new RNA transcribed from minor satellite repeats and their accumulation on chromocenters, which are clusters of several centromeres, and proposed that they might participate in the formation of specific centromeric complexes (15). We now report that minor satellite transcripts are integral components of the CENP-A chromatin fraction and associate with endogenous CPC proteins Aurora B, Survivin and INCENP, at the onset of mitosis. Moreover, these transcripts potentiate Aurora B kinase activity on its mitotic substrate, the histone H3. Together with the cell-cycle regulated accumulation of minor satellite RNA during

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G2/M transition, our data provide new insights into the functional implication of non-coding minor satellite RNA in favouring Aurora B specific interaction with CENP-A-associated chromatin domains and enzymatic function at the onset of mitosis.

MATERIALS AND METHODS

Cell culture and synchronization

Murine Erythroleukemic (MEL) cells were grown in RPMI-1640 medium (Gibco) supplemented with 10% Fetal Calf Serum (FCS, Invitrogen). Exponentially growing MEL cells were stained with 5 μg/ml of Hoechst 33342 (Sigma) and sorted in different phases of the cell cycle by Fluorescence Activated Cell Sorting (FACS) (Epics ALTRA, Beckman Coulter) on the basis of their DNA content. Exponentially growing MEL cells were arrested in mitosis by 12 h treatment with 100 ng/ml of nocozadole (Sigma).

Murine NIH/3T3 fibroblasts were grown in DMEM (Gibco) supplemented with 10% FCS and were synchronized in mitosis by mechanical shake off.

Cell-cycle stage was determined by flow cytometry following cell fixation with 70% cold ethanol and propidium iodide-staining PI (Sigma) 20 μg/ml, 0.1% Triton X-100, 0.2 mg/ml RNaseA (Sigma).

MEL cells have a near-diploid karyotype whereas NIH/3T3 cells contain hyper-tetraploid cells. Since transcription of satellite sequences can be induced in stress conditions (15–17), culture conditions were controlled to ensure these cells have not undergone any stresses in culture (lack of nutriments, heat shock, etc.).

RNA extraction and semi-quantitative RT-PCR

For cell-cycle analysis, total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA was removed by digestion with 2 U of DNaseI (Ambion). cDNA were synthesized from an equivalent of 500 000 cells (Figure 1A) or 100 000 cells (Figure 1B and C), using random hexamers and Superscript II reverse transcriptase (Invitrogen), and amplified by PCR. The amount of input cDNA was normalized to the signal obtained using primers that amplify the mouse β-actin transcripts. All PCR assays were calibrated to 23 cycles to stay in a range of linear amplification for minor satellite RNA. When using lower number of cells (100 000 cells) an additional hybridization step was performed rather than increasing the number of cycles.

The primers used in PCR or RT-PCR analysis were:
Mouse minor satellite:
forward 5′-GAACATATTAGATGAGTGAGTTAC-3′
and
reverse 5′-GTTCTACAAATCCCGTTTCCAAC-3′
Mouse β-actin:
forward 5′-AAGAGCTATGAGCTGCC-3′ and
reverse 5′-ACTCCTGCTTGCTGATCC-3′

Primers used to amplify mouse major satellite and ribosomal DNA are described in ref. (18).

For Southern blot hybridization, the minor satellite fragment used as a probe corresponds to region 20–100 of the consensus sequence (19) shown in Supplementary Figure S4.

Antibodies

The primary antibodies used in the present study were directed against CENP-A (ab33565, Abcam), H3K9Me3 (Upstate), H3Ac (Upstate), HP1γ (Euromedex), HDAC1 (2E10; Upstate), Aurora B (ab2254, Abcam), Survivin (ab469, Abcam), INCENP (ab36453, Abcam), H3K9Me3-S10ph (ab5819, Abcam) and Sir2 (Upstate).

Nuclear extracts and immunoprecipitation

Cells were resuspended in hypotonic buffer [10 mM Tris pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, complete protease inhibitors (Roche) in presence of 2 mM Ribonucleoside Vanadyl Complex-RNase Inhibitor (VRC, New England BioLabs]. After disruption of cytoplasmic membranes with a dounce, nuclei were extracted in ice-cold lysis buffer (10 mM Tris–HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM $NaVO_4$, 10% glycerol, 1% NP40, 1 mM DTT and complete protease inhibitors) in presence of 1 U/μl RNaseOut (Invitrogen) and sonicated (three pulses of 20 s, 5 W, Ultrasonic processor, Bioblock Scientific). Magnetic protein-G beads (Dynabeads) were pre-incubated with lysis buffer containing 200 μg/ml bovine serum albumin (Pierce) and 0.2 mg/ml yeast tRNA (Ambion). Nuclear extracts were incubated for 2 h at 4°C with antibody bound to the beads, in lysis buffer containing 15 μg/ml yeast tRNA, 1 U/μl RNaseOut. Beads were washed twice with lysis buffer adjusted to 300 mM NaCl and once with lysis buffer in presence of VRC.

For RNA NChIP, precipitated RNA was extracted with Trizol reagent, digested with DNaseI, and the totality of precipitated RNA was reverse transcribed. To assess presence of minor satellite RNA in the precipitate, 1 μl of the resulting cDNA was used for PCR analysis and 30 cycles of PCR were performed.

For co-IP analysis, recovered proteins were analysed by western blot using the indicated antibodies. RNaseA treatment was performed with 100 μg/ml RNaseA (Ambion) on nuclear extracts or after IP.

RNA pull-down

A scheme of the procedure is shown in Supplementary Figure S4. One hundred and twenty base pair repeat unit of minor satellite cDNA was generated by PCR amplification and cloned into EcoRI-BamHI sites of pcDNA3 vector (Invitrogen). Minor satellite RNA was *in vitro* transcribed in both orientations using T7 or SP6 RiboMax large-scale production system (Promega). Four hundred picomoles of RNA probe was annealed with 500 pmol of biotinylated oligonucleotide in 50 mM KCl, 1 U/μl RNaseOUT (Invitrogen), for 1 h at RT. Different types of biotinylated oligonucleotides were tested for their ability to precipitate minor satellite RNA (Supplementary Figure S4): a 2′-*O*-methyl RNA oligonucleotide

complementary to regions 1–27 of minor satellite consensus sequence and biotinylated in 5′ (lane 1), a DNA oligonucleotide complementary to regions 1–27 of consensus sequence and biotinylated in 5′ (lane 2), a DNA/locked nucleic acids (LNA) mixmer oligonucleotide complementary to regions 4–27 of consensus sequence and biotinylated in 3′ (lane 3), a DNA/LNA mixmer oligonucleotide complementary to regions 89–115 of consensus sequence and biotinylated in 3′ (lane 4) and a DNA/LNA mixmer oligonucleotide complementary to regions 4–27 of consensus sequence and biotinylated in 5′ (lane 5). As shown in Supplementary Figure S4, the DNA/LNA mixmer oligonucleotide complementary to regions 4–27 of consensus sequence and biotinylated in 3′ (lane 3) retained more efficiently minor satellite RNA and was further used in all experiments. Streptavidin magnetic beads (Dynabeads) were washed according to the manufacturer's instructions twice in 0.1 M NaOH, 0.05 M NaCl and once in 0.1 M NaCl and then incubated with the RNA/oligonucleotide complex in binding/washing buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 M NaCl) for 30 min at RT. Pre-incubated beads were washed twice in binding/washing buffer and added to 4 mg of nuclear extract in RNA binding buffer (10% glycerol, 10 mM HEPES pH 7, 150 mM KCl, 1 mM EDTA, 0.5% TritonX-100) containing 15 μg/ml yeast tRNA, 1 mM DTT, 240 U RNaseOut for 2 h at 4°C. RNA-bound proteins were washed twice with RNA binding buffer adjusted to 300 mM KCl and once with RNA binding buffer before protein denaturation. Precipitated proteins were then resolved on a gradient-like SDS–PAGE (Prosieve, Cambrex), transferred on a Hybond C-extra membrane (Amersham) and blotted with the indicated antibodies.

Kinase assay

Immunoprecipitated Aurora B kinase was included into a 20 μl reaction containing 1 μg of canonical core histones (Upstate), 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM $NaVO_4$, 1 mM NaF, 0.5 μl of [γ-^{32}P]ATP (PerkinElmer Life Sciences) and increasing amounts of *in vitro* transcribed minor satellite RNA or β-tubulin RNA. Protein samples were separated by 12% SDS–PAGE and phosphate incorporation determined by phosphorimager (Typhoon 8600, Amersham Pharmacia Biotech). For rescue experiment, RNaseA treatment was performed after IP and kinase reactions were carried out in presence of 1 U/μl RNaseOut and *in vitro* transcribed minor satellite or β-tubulin RNA.

RESULTS

Levels of minor satellite transcripts vary with cell cycle

We previously reported that minor satellite transcripts accumulate on chromocenters in a subset of cycling cells, suggesting that levels of these RNA may vary during cell cycle (15). We therefore analysed the levels of these RNA in MEL cells sorted in G0/G1, S or G2/M phase (Figure 1A). Semi-quantitative RT–PCR using primers amplifying minor satellite transcripts was normalized to the signal of a RT–PCR reaction amplifying mouse β-actin transcripts, whose levels do not change during the cell cycle. RT–PCR analysis of minor satellite RNA showed a long ladder of discrete bands differing by about 120 bp consistent with transcription through multiple tandem repeats of the 120-nt minor satellite unit, and revealed that the levels of minor satellite transcripts were barely detectable in G0/G1 phase, increased in S phase and peaked in G2/M phase (Figure 1A).

MEL cells were then synchronized in G0/G1 phase using FACS sorting and released in culture for 9 h. Flow cytometry analysis confirmed the synchronous progression of the sorted population through early and late S phase, then G2 and mitosis, at 3, 6 and 9 h, respectively after release in culture (Figure 1B). In agreement with the results described above, RT–PCR analysis on total RNA isolated at various time points after release in culture showed that the levels of minor satellite RNA were below detection levels in G0/G1 sorted cells, started to increase 3 h after release in culture, concomitantly with entry of MEL cells into S phase, accumulated gradually during the course of S phase and reached a maximum in G2/M phase (Figure 1B). We then performed similar experiments in a different murine cell line. NIH/3T3 cells were synchronized in mitosis by mechanical shake-off and cultured over a 2 h time course that was sufficient for the majority of the cells to complete mitosis and enter the subsequent G1 phase of the cell cycle (Figure 1C). Minor satellite transcripts strongly accumulated in mitotic NIH/3T3 cells and became undetectable as early as 2 h after mitosis, when the cells re-entered the next G1 phase (Figure 1C).

Taken together, these results indicated that the pool of minor satellite RNA varies significantly during cell cycle, from barely detectable in G1 phase to a distinct peak in G2/M.

CENP-A chromatin fraction contains minor satellite RNA

Our initial characterization of minor satellite transcripts subcellular location by RNA-FISH revealed that these RNA localize to chromocenters, suggesting that they belong to specific ribonucleoprotein complexes located at centromeric or pericentromeric regions (15). To further characterize centromeric RNA-containing complexes we isolated subnuclear fractions enriched in chromocenters by discontinuous sucrose gradient (Supplementary Figure S1), as described in the Supplementary Data. Chromocenters were separated from dispersed fragments of low density chromatin in a fraction characterized by enrichment in DNA sequences and proteins specifically found at centromeric regions, i.e. CENP-A (Supplementary Figure S1B) and centromeric minor satellite DNA sequences (Supplementary Figure S1C and D). In addition, the CPC proteins Aurora B and Survivin were predominantly recovered in the chromocenter fraction. In contrast, histone deacetylase HDAC1 was recovered in both low density and chromocenter fractions, consistent with its subnuclear localization in MEL cells (20), whereas the repressor Sir2 was recovered only in low density chromatin fractions, also consistent with its previously reported subnuclear location (21) (Supplementary

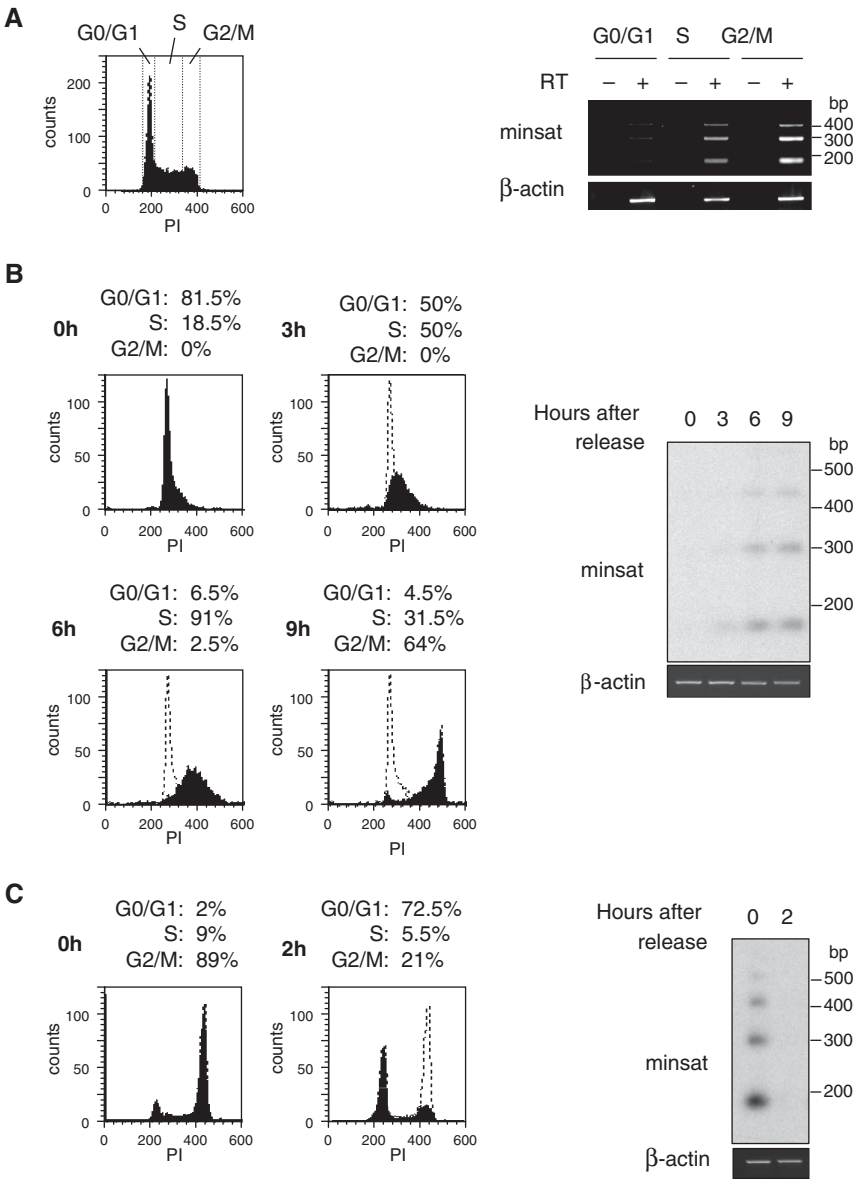

Figure 1. Minor satellite transcripts accumulate in G2/M phase of the cell cycle. (**A**) FACS plot showing the gates used to sort MEL cells at different stages (G0/G1, S, G2/M) of the cell cycle (left panel). Levels of minor satellite RNA (minsat) from each fraction were evaluated by RT–PCR, in reactions containing (+) or not (−) RT (right panel), and normalized to the signal of a RT–PCR reaction amplifying β-actin transcripts, which levels do not change during cell cycle. (**B**) Cell-cycle distribution of PI-labelled MEL cells after FACS-sorting in G0/G1 phase and release in culture for 9 h (left panel). Total RNA at time 0, 3, 6 and 9 h was analysed by RT–PCR followed by hybridization with a minor satellite probe (right panel). Control RT-PCR reaction amplifying β-actin transcripts are shown below. (**C**) FACS analysis of PI-labelled NIH/3T3 cells after mitotic shake off and release into culture (left panel). Total RNA at time 0 and 2 h was analysed by RT–PCR followed by hybridization with a minor satellite probe (right panel). Control RT–PCR reactions amplifying β-actin transcripts are shown below.

Figure S1B). In those conditions, RT-PCR analysis on total RNA isolated after nuclear fractionation showed that minor satellite RNA were specifically enriched in the chromocenter fraction (Supplementary Figure S1E).

To further characterize minor satellite RNA-containing ribonucleo-complexes located at centromeric regions, we performed Native Chromatin Immunoprecipitation (NChIP) using antibodies directed against CENP-A. As controls, we used antibodies directed against H3K9Me3, a heterochromatin mark found at pericentromeric regions, or against acetylated H3 (H3Ac), an activating mark mainly associated with euchromatic regions. DNA or RNA were then isolated from the precipitated material and analysed by PCR or RT-PCR. The immunoprecipitated material was first tested for the presence of centromeric minor and pericentromeric major satellite DNA sequences. To assess the NChIP background, we used primers specific for ribosomal DNA. Under these conditions, CENP-A indeed exclusively co-precipitated with minor satellite DNA, whereas both murine minor and major satellite DNA sequences associated with H3K9Me3 chromatin fractions (Supplementary

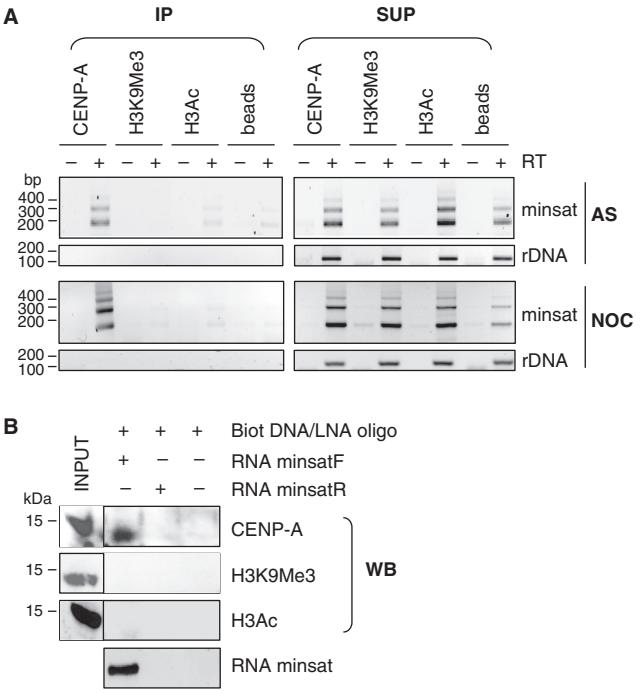

Figure 2. Minor satellite RNA associate with CENP-A-containing chromatin. (**A**) RNA NChIP from nuclear extracts of asynchronously growing (AS) or nocodazole-arrested (NOC) MEL cells using antibodies against CENP-A, H3K9Me3 or H3Ac. Following DNaseI digestion, minor satellite RNA (minsat) and rDNA transcripts (rDNA) were analysed by RT-PCR, from the immunoprecipitated (IP) or supernatant fractions (SUP), in reactions containing (+) or not (−) RT. Control reactions in the absence of immunoprecipitating antibody (beads) were used as a negative control. (**B**) RNA pull-down experiments using *in vitro* transcribed minor satellite RNA (minsatF), followed by western blot (WB) analysis with the indicated antibodies. As a control, a similar experiment was performed with *in vitro* transcribed minor satellite RNA from the reverse strand (minsatR), therefore not complementary to the biotinylated DNA/LNA oligonucleotide used, or in the absence of RNA.

Figure S2), in agreement with previous reports (2,18). In addition, association between these repetitive sequences and H3Ac was significantly lower consistent with the hypoacetylated status of these regions (22). In contrast, control ribosomal DNA (rDNA) was only detected in the H3Ac fraction (Supplementary Figure S2). To further assess the association of minor satellite transcripts with murine centromeric fractions, NChIP was performed from asynchronous and nocodazole-arrested MEL cells, using the same antibodies, in the presence of RNase inhibitor, and followed by a DNaseI treatment of the collected material. Following nocodazole treatment 86% of MEL cells accumulated in G2/M phase (Supplementary Figure S3, left and middle panel), a stage at which minor satellite RNA strongly accumulated in the cells (Supplementary Figure S3, right panel). RT-PCR analysis of precipitated RNA revealed that minor satellite RNA were specifically associated with the CENP-A immune complex in both asynchronous and nocodazole-arrested MEL cells (Figure 2A). In contrast, minor satellite RNA were not associated with H3K9Me3 or H3Ac chromatin fractions (Figure 2A), even in G2/M arrested cells, a stage at which minor satellite RNA strongly accumulated in the cells (Supplementary Figure S3, right panel). As a negative control, abundant control rDNA transcripts were not precipitated with any of the antibodies used, whereas RT-PCR performed on supernatants from NChIP reactions showed correct amplification of minor satellite RNA and rDNA transcripts in all samples, indicating that RNA was not degraded (Figure 2A).

These results provide evidence that minor satellite transcripts preferentially associate with the CENP-A chromatin fraction *ex vivo*.

To confirm association of minor satellite RNA with CENP-A chromatin, RNA pull-down assays were performed, using minor satellite RNA transcribed *in vitro* from a 120-nt repeat unit (minsatF), bound to a complementary biotinylated oligonucleotide, and incubated with MEL cells nuclear extracts. We first tested several types of oligonucleotides for their efficiency to pull down minor satellite transcripts *in vitro*, and chose a 3′ biotinylated compound DNA/LNA oligonucleotide (Supplementary Figure S4). After pull-down using streptavidin-coated magnetic beads, and verification that the oligonucleotide efficiently retained minor satellite RNA (Figure 2B, bottom line), the associated proteins were analysed by western blot. Immunoblot analysis of minor satellite RNA-associated histone H3 species revealed that these RNA preferentially precipitated CENP-A, but not H3K9Me3 nor H3Ac (Figure 2B). CENP-A was not precipitated in various control experiments in which centromeric RNA was omitted (Figure 2B, far right column) or reactions treated with RNaseA prior or after precipitation (not shown). As an additional control for the specificity of the DNA/LNA oligonucleotide, we used *in vitro* transcribed RNA from the reverse strand (minsatR), therefore not complementary to the oligonucleotide used, which did not precipitate CENP-A (Figure 2B). Together, our experiments showed that CENP-A was detected in the precipitated material only when minor satellite RNA were efficiently precipitated.

Minor satellite RNA associate with proteins of the CPC in G2/M phase

The association of minor satellite transcripts with CENP-A-associated chromatin together with their steady-state levels peaking in G2/M phase suggests that they could play a role in the assembly of centromeric complexes during G2/M transition. The CPC exhibits a highly dynamic localization throughout the cell cycle. During G2 phase and metaphase, the complex localizes to centromeres, transfers to the central spindle at the onset of anaphase, and finally flanks the midbody during telophase and cytokinesis (4). To determine whether proteins of the CPC associate with minor satellite transcripts, we performed RNA pull-down assays using *in vitro* transcribed 120-nt minor satellite RNA, and analysed the presence of the kinase Aurora B, its obligate co-activator Survivin and the inner centromere protein INCENP, in the precipitated material. RNA pull-down assays revealed that Aurora B, Survivin and, to a lesser extent, INCENP, were efficiently precipitated when the oligonucleotide was complementary

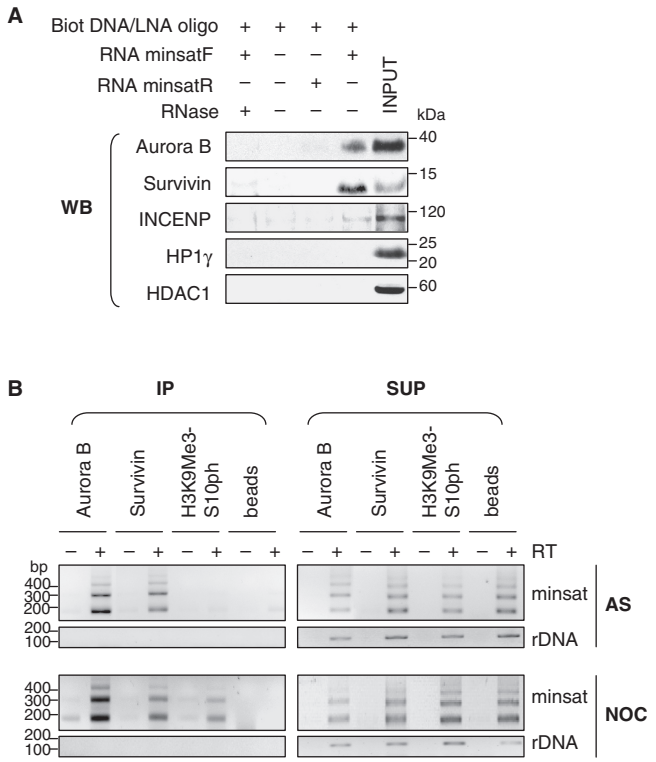

Figure 3. Association of minor satellite RNA with proteins of the chromosomal passenger complex. (**A**) *In vitro* transcribed minor satellite RNA (minsatF) was used in RNA pull-down experiments, followed by western blot (WB) analysis with the indicated antibodies. As controls, similar experiments were performed in presence of RNaseA, in absence of minor satellite RNA, or using an RNA not complementary to the biotinylated oligonucleotide used (minsatR). (**B**) RNA NChIP from nuclear extracts of asynchronously growing (AS) and nocodazole-arrested (NOC) MEL cells using antibodies against Aurora B, Survivin and H3K9Me3-S10ph. As a negative control, a similar experiment was performed in the absence of immunoprecipitating antibody (beads). Following DNaseI treatment, immunoprecipitated (IP) RNA and RNA from supernatant fractions (SUP) were analysed by RT-PCR, in reactions containing (+) or not (−) RT, for the presence of minor satellite RNA (minsat) and rDNA transcripts (rDNA).

to the minor satellite RNA (Figure 3A, minsatF). No signal was observed when the reaction was carried out in presence of RNaseA or when the RNA was omitted from the reaction (Figure 3A, two left panels). As described above, an additional control consisted in using *in vitro* transcribed RNA not complementary to the oligonucleotide used in the pull-down assay (minsatR). Interestingly, although heterochromatin protein HP1γ and the histone deacetylase HDAC1 associated with chromocenters in MEL cells (20), they were not retained on minor satellite transcripts (Figure 3A).

To validate these results *ex vivo*, we immunoprecipitated Aurora B and Survivin from asynchronous MEL cells extracts. RT-PCR analysis of the co-precipitated RNA resulted in specific amplification of minor satellite RNA from both Aurora B and Survivin immunoprecipitated material, whereas they were not detected in control reaction performed in absence of immuno-precipitating antibody (Figure 3B). In addition, no association was observed between these proteins and the abundant rDNA transcripts (Figure 3B). Similar experiments were conducted with nocodazole-arrested MEL cells. NChIP data revealed that minor satellite RNA were significantly associated with Aurora B and Survivin in nocodazole-arrested cells whereas rDNA transcripts were not precipitated (Figure 3B). Phosphorylation of H3 on its serine 10 by Aurora B kinase initiates at centromeric regions in late G2 interphase cells, and further spreads throughout the condensing chromatin. We found that minor satellite RNA co-precipitated with the H3K9Me3-S10ph in nocodazole-arrested cells (Figure 3B). In all experiments, RT-PCR from supernatant fractions showed correct amplification of both minor satellite RNA and rDNA transcripts, indicating that RNA was not degraded.

These data are consistent with a model in which minor satellite RNA could provide a scaffold to recruit and/or stabilize passenger proteins at centromeric regions in G2/M phase.

The assembly of Aurora B/Survivin complex and Aurora B kinase activity require an RNA component

The observation that the CPC and minor satellite RNA have the potential to form a protein–RNA complex, prompted us to test the functional relevance of this association. Assembly of the CPC is a prerequisite for its correct localization, kinase activity of Aurora B and substrate specificity during G2/M transition (23). We first examined the ability of Aurora B to form a complex *in vivo* with Survivin, in presence or absence of RNA. We tested the sensitivity of the interactions to RNaseA treatment, before immunoprecipitation of Aurora B complexes and omission of RNase inhibitors during the process, or after immunoprecipitation of Aurora B complexes. Under these conditions, the amount of recovered Aurora B was not affected by RNaseA treatment (Figure 4A). In contrast, analysis of co-immunoprecipitated material showed that the interaction of Aurora B with Survivin was significantly diminished after RNA depletion compared to control experiments where RNA was protected from degradation (Figure 4A). In addition, we tested whether association of Aurora B with CENP-A-associated chromatin was dependent on an RNA molecule. Interestingly, our results showed that RNaseA treatment completely abolished the interaction between Aurora B and CENP-A (Figure 4A).

We then asked whether the enzymatic activity of Aurora B kinase was also sensitive to RNA depletion. Aurora B was immunoprecipitated from MEL cells extracts and examined for its ability to phosphorylate histone H3 *in vitro*, a physiological substrate of this kinase, in presence or absence of RNaseA treatment. The results showed that endogenous Aurora B complex displayed high specific kinase activity when assayed against purified histones and that its activity significantly decreased after RNaseA treatment (Figure 4B).

Thus, our data revealed that, in the context of the native immunoprecipitated Aurora B complex, RNA/protein interactions are crucial for interactions of Aurora B with its protein partners CENP-A and Survivin. In addition,

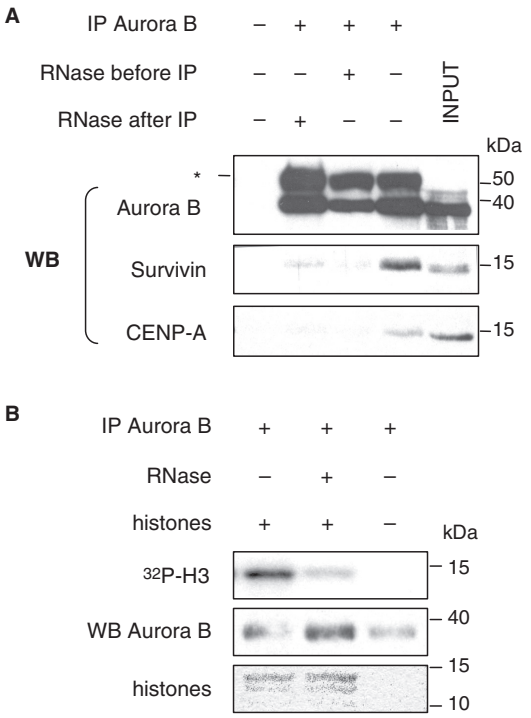

Figure 4. Interactions of Aurora B with Survivin and CENP-A and its kinase activity are sensitive to RNA depletion. (**A**) Co-IP experiment using an anti-Aurora B antibody. RNaseA treatment was performed before or after IP of Aurora B. Input lysates (INPUT) and precipitated proteins in the absence of immunoprecipitating antibody (first line) are shown as positive and negative controls, respectively. Co-precipitated Survivin and CENP-A were visualized by western blotting (WB). Asterisk indicates IgG heavy chain which is detected by the secondary antibody. (**B**) *In vitro* kinase activity of immunoprecipitated Aurora B from MEL nuclear extracts, treated (+) or not (−) with RNaseA, using core histones as exogenous substrate. Specific histone H3 phosphate incorporation (^{32}P-H3) was revealed by autoradiography, Aurora B protein levels were determined by western blotting and histones visualized by Ponceau red staining.

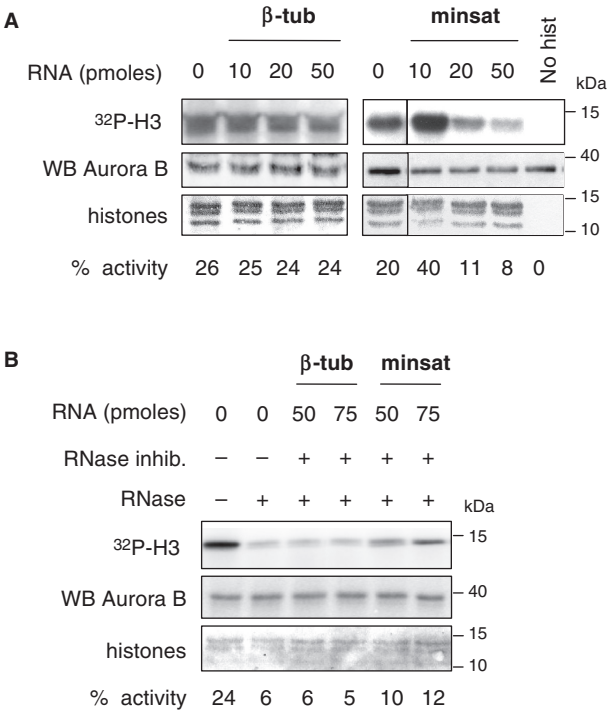

Figure 5. Minor satellite RNA potentiate endogenous Aurora B kinase activity. (**A**) Kinase activity of immunoprecipitated Aurora B in presence of increasing amounts (10, 20, 50 pmol) of *in vitro* transcribed minor satellite RNA (minsat). Control reactions were performed in presence of increasing amounts (10, 20, 50 pmol) of *in vitro* transcribed β-tubulin RNA (β-tub) or in absence of histones (No hist). (**B**) Kinase activity of endogenous Aurora B after RNaseA treatment and rescue assay with addition of *in vitro* transcribed minor satellite RNA (50, 75 pmol), in presence of RNase inhibitors. As a negative control, the same amount of *in vitro* transcribed β-tubulin RNA (50, 75 pmol) was used in the rescue assay. Specific histone H3 phosphate incorporation (^{32}P-H3) was quantified by phosphorimager, Aurora B protein levels were determined by western blotting and histones visualized by Ponceau red staining.

Aurora B kinase activity appeared to be sensitive to RNaseA treatment.

Kinase activity of Aurora B is enhanced in presence of minor satellite RNA

Based on the findings that Aurora B associates with minor satellite transcripts and that its kinase activity is sensitive to RNaseA treatment, we performed experiments in which the kinase assay was realized in the presence of increasing amounts of *in vitro* transcribed minor satellite RNA. Interestingly, Aurora B kinase activity showed a two-fold increase in presence of minor satellite RNA, in conditions where the estimated amounts of RNA and kinase were equivalent (10 pmol), whereas addition of higher amounts (20–50 pmol) of minor satellite RNA impaired the activity of the endogenous kinase (Figure 5A, right panel). These effects were specific to minor satellite RNA since an unrelated control RNA showed no such effects under the same conditions (Figure 5A, left panel). Importantly, the decreased Aurora B kinase activity after RNaseA treatment was substantially rescued by addition of large amounts of RNase inhibitors combined to addition of increasing amounts of *in vitro* transcribed minor satellite RNA (Figure 5B). By contrast, in the same conditions, addition of increasing amount of control RNA did not have any effect on Aurora B kinase activity (Figure 5B).

Together, these data show that decreased Aurora B kinase activity after RNA depletion can be specifically rescued by restitution of minor satellite transcripts in the reaction, and suggest that minor satellite transcripts may regulate Aurora B enzymatic activity.

DISCUSSION

We report a functional role for RNA transcribed from minor satellite repeats as part of ribonucleoprotein complexes of murine centromeric chromatin, key factors in mediating interactions between protein components of the CPC and in potentiating the activity of the mitotic kinase Aurora B.

We have previously characterized RNA transcribed from minor satellite repeats found at murine centromeres,

and showed by RNA-FISH (15) or using biochemically purified chromocenters (this study) that they associate with chromocenters. Native ChIP experiments aimed to selectively precipitate centromeric chromatin fractions, based on their unique content in the histone H3 variant CENP-A, revealed that murine minor satellite RNA specifically precipitated with CENP-A-associated chromatin fractions. Interestingly, another case of centromere-encoded RNA co-precipitated with CENP-A exists in maize (10). Other examples of non-coding RNA specifically associated with defined chromatin domains from which they are transcribed have been reported. For example, Xist RNA is transcribed from and associates with the inactive X-chromosome (24). Worth mentioning, Xist also associates with a histone variant, macroH2A, deposited at facultative heterochromatin formed on the inactive X-chromosome (25). A more recent example described telomeric-repeat-containing RNA enriched at telomeric heterochromatin and involved in the maintenance of telomere integrity (26,27). Thus, transcripts emanating from specific chromosomal domains in conjunction with definite epigenetic marks may contribute to the specification of their higher-order chromatin organization. However, whether transcription across these regions, or transcripts themselves, define distinct chromatin domains with differential histone modifications or variants remains to be clarified.

Although several experimental evidence support that transcription across centromeric repeats, and/or remodelling of nucleosomes, contributes to centromere formation and deposition of the histone variant CENP-A (7,10,28), other data uncovered the implication of non-coding RNA in stabilizing the binding of structural non-histone proteins to chromatin (29). For example, binding of HP1 to heterochromatin is sensitive to digestion with RNaseA (13) and requires the contribution of the hinge domain, known to bind RNA *in vitro* (14). Therefore, minor satellite transcripts themselves, and not transcription *per se*, may be involved in formation of ribonucleoprotein complexes located at centromeres and in centromere specification. CENP-A may serve as a docking platform for centromeric RNA-dependent loading of centromeric complexes. Consistent with this, we found that minor satellite RNA associate with proteins of the CPC and that interactions between endogenous passenger proteins Aurora B and Survivin within CENP-A chromatin require an RNA component.

A particular feature of centromeric chromatin domains is that many of their associated proteins display dynamic changes in distribution patterns during cell cycle (30). In particular, the chromosomal passenger proteins such as Aurora B and Survivin, and to a lesser extent INCENP, accumulate near centromeres in early G2 (31–33). In correlation with this dynamic organization of centromeric domains, our analysis of minor satellite RNA levels revealed that the pool of centromeric RNA greatly varies during cell-cycle progression. Indeed, minor satellite transcripts accumulated gradually during the course of S phase, reached a maximum at G2/M phase and became undetectable early after mitosis, when cells re-enter the next G1 phase. Likewise, a cell-cycle regulation has also been reported for pericentromeric heterochromatin transcription both in mouse (34) and in fission yeast (35), resulting in accumulation of major transcripts in late G1/S phase. In contrast, we detected low levels of minor satellite RNA in G1 and S phase. Together with the observations that they associate with CENP-A-associated chromatin domains, and reach high levels in G2/M phase, this favours the hypothesis that minor satellite transcripts are key players in the assembly of protein complexes at the centromere, before mitosis, rather than in CENP-A deposition that was suggested to occur in G1 phase (36).

Indeed, we found that minor satellite transcripts levels greatly increased during G2/M phase, concomitant with assembly of Aurora B and Survivin to centromeres (32). At this phase, our NChIP experiments demonstrated that centromeric transcripts associate with proteins of the CPC, Aurora B and Survivin, as well as with their mitotic substrate histone H3 phosphorylated on its serine 10 (37). In agreement with the recent observations that human centromeric α-satellite transcripts are components of protein complexes located at centromeres of metaphase chromosomes (38), our data suggest that non-coding minor satellite RNA are implicated in the formation of specific centromeric complexes during G2/M transition. In addition, we provided the first experimental evidence that an RNA component favours interactions between Aurora B and its partner Survivin, as well as with CENP-A, supporting a structural role for centromeric RNA in stabilizing centromeric-associated complexes. We propose that interaction with minor satellite transcripts may represent an additional mechanism for both Aurora B proper association within CENP-A chromatin domains and enzymatic function. Minor satellite RNA could play an indirect role by providing a permissive chromatin environment for the productive interaction of Aurora B and Survivin with centromeric domains. Alternatively, minor satellite RNA could favour the interaction between Aurora B and Survivin required for the function of this complex (39,40).

We previously reported that sustained expression of minor satellite transcripts resulted in dramatic changes in localization of Aurora B, which failed to target centromeric regions of mitotic chromosome (15). In addition, deregulated accumulation of minor satellite transcripts led to impaired centromeric function and abnormal chromosome segregation (15). Consistent with this, our present data suggest a critical role for centromeric transcripts in centromere identity and function. Their implication in formation of centromere-associated complexes at specific phases of the cell cycle together with centromere failure when centromere satellite transcripts accumulate in stressed cells (15–17) argues that levels of these transcripts must be tightly regulated.

More importantly, our data put forward a functional relationship between Aurora B kinase activity and minor satellite RNA. We demonstrated that Aurora B kinase activity is sensitive to RNA depletion, probably due to disruption of the Aurora B/Survivin complex, required for Aurora B activity (39,40). Of importance, Aurora B kinase activity can be potentiated by addition of minor

satellite transcripts, in conditions where the estimated amounts of RNA and kinase are equivalent. In addition, its decreased activity after RNA depletion can be specifically rescued by restitution of minor satellite RNA in the kinase assay. Recently, RNA molecules have emerged as active participants in regulating, catalysing and controlling biological processes, a role first ascribed to proteins. Several examples described RNA molecules bound to proteins and regulating their activity, subcellular location and interactions with protein partners (41). Thus, it is conceivable that specific functions and enzymatic activity of Aurora B are controlled not only by its proteins partners but also by interactions with non-coding minor satellite transcripts.

Until now, posttranslational modifications and protein components have been postulated to be the targeting actors of the CPC (42,43). We now added the evidence that both interaction of Aurora B with CENP-A-associated chromatin and kinase activity might be controlled by non-coding RNA transcribed from the domain where this enzyme is recruited and active at a specific stage of the cell cycle.

Altogether, our data added centromeric transcripts to the ever-growing list of functional non-coding RNA (44) and provide new insights into the implication of minor satellite RNA in the establishment of a functional centromere, by regulating Aurora B association with CENP-A-associated domains and enzymatic function.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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