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Cell cycle-dependent regulation of Aurora kinase B mRNA by the Microprocessor complex

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ABSTRACT

Aurora kinase B regulates the segregation of chromosomes and the spindle checkpoint during mitosis. In this study, we showed that the Microprocessor complex, which is responsible for the processing of the primary transcripts during the generation of microRNAs, destabilizes the mRNA of *Aurora kinase B* in human cells. The Microprocessor-mediated cleavage kept Aurora kinase B at a low level and prevented premature entrance into mitosis. The cleavage was reduced during mitosis leading to the accumulation of *Aurora kinase B* mRNA and protein. In addition to *Aurora kinase B* mRNA, the processing of other primary transcripts of miRNAs were also decreased during mitosis. We found that the cleavage was dependent on an RNA helicase, DDX5, and the association of DDX5 and DDX17 with the Microprocessor was reduced during mitosis. Thus, we propose a novel mechanism by which the Microprocessor complex regulates stability of *Aurora kinase B* mRNA and cell cycle progression.

1. Introduction

The RNase III enzymes, Drosha and Dicer, play central roles in the generation of microRNAs (miRNAs). In the nucleus, the primary transcript (pri-miRNA) is cleaved at the stem of a hairpin structure by the Microprocessor, a multimeric complex composed of Drosha, and an RNA-binding protein, Dgcr8 [1–5]. In addition to the generation of miRNAs, a number of reports suggest these enzymes process other classes of RNAs. Phenotypic differences between Drosha- or Dicer-deficient cells have indicated that some miRNA-independent functions are regulated by these RNaseIII enzymes, such as Microprocessor-mediated destabilization of mRNAs [6–8].

The first mRNA that was identified as a target of the Microprocessor is one of its components, *Dgcr8* [9]. The Microprocessor recognizes and cleaves hairpin structures at the 5′ UTR and the coding region of *Dgcr8* mRNA. This self-regulation by the negative feedback loop contributes to the homeostatic control of miRNA generation. More recently, analyses of transcriptional or proteomic changes caused by ablation of Drosha or Dicer expression indicate that a number of mRNAs are posttranscriptionally regulated by the Microprocessor [6–8]. Other than the self-regulation of *Dgcr8*, the process by which Microprocessor-mediated cleavage is regulated in different cell types, or at different stages has not been determined.

Aurora kinase B (AURKB), a member of Aurora family of protein kinases, regulates chromosome–microtubule interactions, cohesion, spindle stability and cytokinesis during mitosis [10]. Consistent with its roles in the regulation of mitosis, overexpression or stabilization of the kinase in rodent and human cells have been reported to induce aneuploidy [11–14]. As aneuploidy is one of the common feature of human cancers, the overexpression of the AURKB observed in various types of cancers indicates that dysregulation of AURKB may contribute to tumorigenesis [11,12,14–16].

In this study, we have demonstrated that *AURKB* mRNA is destabilized by Microprocessor-mediated cleavage at the coding region. Constitutive cleavage maintained the expression of AURKB at a low level while knockdown of DGCR8 reversed it. The cleavage was reduced during mitosis with a concomitant increase of *AURKB* mRNA and protein. We found that association of DGCR8 with RNA helicases, DDX5 and DDX17, was reduced during mitotic arrest. Thus, we propose a novel mechanism for the Microprocessor, regulation of cell cycle progression by targeting an mRNA.

Abbreviations: TDF, teratoma-derived fibroblast; UTR, untranslated region; miRNA, microRNA; siRNA, small interfering RNA; CLIP-seq, CrossLinking-ImmunoPrecipitation and sequencing; RT-PCR, reverse transcription-polymerase chain reaction; hESC, human embryonic stem cell.

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2. Materials and methods

2.1. Cell culture and transfection

The generation and maintenance of TDFs were described before [17]. The TDFs at passage 10–15 were used for all of the experiments. TDFs were transfected with 100 nM siRNAs by electroporation. TDFs growing exponentially were trypsinized and collected by centrifugation. The cell pellet was re-suspended with 300 μl of Gene Pulser Electroporation Buffer (Bio-Rad) with 100 nM of siRNA and transferred to a 4-mm electroporation cuvette. Electroporation was carried out using Gene Pulser Xcell with exponential decay protocol (250 V, 500 μF, 1000 ohm). siRNA against AURKB was purchased from Cell Signaling. Other siRNAs were purchased from Thermo Scientific. For transfection of HeLa S3 cells, lipofectamine LTX with Plus reagent (Invitrogen) was used for plasmid, and lipofectamine 2000 was used for siRNA.

2.2. Ligation-mediated RT PCR assay

Total RNA was isolated using Trizol (Invitrogen) and further purified with RNeasy mini kit (Qiagen). 500 ng of total RNA was ligated with 1 pmol of pre-adenylated adaptor (5′-NNN CGTCGTGT AGGGAAAGAGTGTAGATC-3′) using 1 unit of T4 RNA ligase 1 and 2 unit of truncated ligase 2 K227Q (NEB) at 16 °C for 2 h. The ligated RNA was converted to cDNA with RT primer (5′-CCTGTGGTCGTAG-CATCAGCTACTACACTCTTTCCCTACACGA-3′) and SuperScript III reverse transcriptase (Invitrogen). 2 ng of cDNA was used for PCR. Sequences of PCR primers for human AURKB mRNA used were as follows: P1, 5′-GAGAGTGCATCACACAACGAGACCT-3′; P2, 5′-GGG CAGCCGTTCCGAG-3′; P3, 5′-CCTGTGGTCGTAGCATCAGCT-3′. For mouse Aurkb mRNA, the sequences of P1 and P2 primers were 5′-GAGAGCCCTAGCCACAGTGAGACGT-3′ and 5′-ACCTGAAGTTCCCC TCTTCT-3′, respectively.

2.3. Quantitation of mitotic cells

48 h after plating, exponentially growing HeLa S3 cells or TDFs were harvested by trypsinization and fixed in 90% ethanol for 24 h. Mitotic cells were identified by staining with propidium iodide for DNA content and antibody recognizing histone H3 phosphorylated at Ser10. The antibody was purchased from Cell signal and the staining was carried out following the manufacturer's instruction.

2.4. DNA construct

The coding region of AURKB was amplified from a human cDNA library and subcloned into pCDNA3.1 with tandem HA-epitope sequences at the N-terminus. Silent mutations at the hairpin sequence were introduced by PCR.

2.5. Immunoprecipitation and Western blotting

HeLa S3 cells were lysed by freeze/thaw in a solution of 20 mM TrisHCl (pH 7.6), 140 mM NaCl, 1 mM EDTA, 0.1% NP40, 10 mM NaF, 0.2 mM sodium vanadate, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Roche). Then, 500 μg of lysate was incubated with 1 μg of anti-DGCR8 antibody bound to protein G Dynabeads (Invitrogen). The immunoprecipitate was washed 3 times with lysis buffer. Anti-DGCR8 (A302-469A for immunoprecipitation and A302-468A for Western blot), anti-DDX5 and anti-DDX17 antibodies were purchased from Bethyl laboratories. Anti-AURKB antibodies were purchased from Cell Signaling.

2.6. Quantitative reverse transcription PCR assay

The primer sequences of quantitative PCR were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). The sequences of the primer pairs were as follows: GAPDH, 5′-ATGGGGAA GGTGAAGGTCG-3′, 5′-GGGGTCATTGATGGCAACAATA-3′; DGCR8, 5′-GCAGAGGTAATGGACGTTGG-3′, 5′-AGAGAAGCTCCGTAGAAGTT AA-3′; DDX5, 5′-AGAGAGGCGATGGGCCTATTT-3′, 5′-CTTCAAGCGA CATGCTCTACAA-3′; AURKB, 5′-CAGTGGGACACCCGACATC-3′, 5'-GTACACGTTTCCAAACTTGCC-3′; mouse Aurkb, 5′-CAGAAGGAGAAC GCCTACCC-3′, 5′-GAGAGCAAGCGCAGATGTC-3′.

The sequences of the primer pairs for the pri-miRNAs were as follows: Pri-miR-21, 5′-TGGGGTTCGATCTTAACAGG-3′, 5′-GGTGTT GCCATGAGATTCAA; Pri-miR-34a, 5′-CACCATGCCTGGCTAATTTT-3′, 5′-CCCCAGCACCTGTTGATAAG-3′; Pri-miR-143, 5′-AGGCCACAG ACAGGAAACAC-3′, 5′-CCAACTGACCAGAGATGCAG-3′; Pri-miR-195, 5′-AAATCTCCAGGGCAGTTTCA-3′, 5′-GCCTGTTCCCTCTTCTCT CC-3′; Pri-miR-200a, 5′-GCCTGTGTGCAGTCTCAGG-3′, 5′-CCAGCA CTGTCCGGTAAGAT-3′.

For the quantitation of the cleavage products, the first primers of each primer set and the P3 primer were used.

3. Results and discussion

In a separate study, we carried out CrossLinking-ImmunoPrecipitation and sequencing (CLIP-seq) of a human embryonic stem cell (hESC) line, H1, using an antibody against DGCR8 to investigate the process of the miRNA biogenesis *in vivo* (paper in preparation). In addition to the primary transcripts of miRNAs, several mRNAs were identified with high confidence that could be cleaved by the Microprocessor complex. *AURKB* mRNA was one of the putative substrates and no miRNA has been annotated to the region. It was also included in the 2256 putative target mRNAs of the Microprocessor complex [18]. The CLIP-reads indicated that two cleavage sites were located within exon 8 of the mRNA. The sequence between the putative cleavage sites predicted to form a hairpin structure with a 2-nt overhang at the 3′ end, a canonical structure of Microprocessor-mediated processing (Fig. 1A).

To confirm specific cleavage at the predicted sites, we employed a ligation-mediated reverse transcription-PCR (RT-PCR) assay (Fig. 1B). In this assay, an adapter was ligated to the 3′ end of purified RNAs. cDNAs were generated by reverse transcription. Amplification of the cDNA with a primer that binds to the upstream region of the putative cleavage site and a second primer that is complementary to the 5′ end of the reverse transcription primer yielded a specific product close to the expected 177 bp. Sequencing of the amplified product confirmed the specific cleavage at the predicted site (Fig. 1C).

To verify whether microprocessor-mediated cleavage affects the stability of *AURKB* mRNA, hESCs were transfected with control siRNA or siRNA against DGCR8. The transfected cells were harvest after 24 h to minimize any secondary effect caused by perturbation of the Microprocessor activities. Ligation-mediated RT-PCR was employed to assess the amount of cleaved product relative to non-cleaved mRNA. As expected, we observed a decrease in the amount of cleaved product after knockdown of DGCR8 (Fig. 1D). To assess the contribution of the Microprocessor-mediated cleavage to the total amount of *AURKB* mRNA, quantitative RT-PCR assay was performed. Despite the reduced cleavage, knockdown of DGCR8 in hESCs had a modest effect on quantity of *AURKB* mRNA. Thus, the biological implication of the Microprocessor-mediated destabilization in hESC was not clear. We reasoned that the embryonic stem cells may be inadequate to observe the Microprocessor-mediated regulation of the mitotic checkpoint kinase as hESCs show inefficiency in cell cycle checkpoints and tend to gain extra

copies of chromosome 12 and 17, which indicates that mitotic checkpoint is not proficient in the culture condition [19]. We decided to use a differentiated cell line that shows efficient cell cycle checkpoints. Fibroblast cells were derived from teratomas (teratoma-derived fibroblasts, TDFs) that were generated by injecting H1 hESCs into immunocompromised mice. In the TDF cells, we observed that *AURKB* mRNA was increased by ~60% after knockdown of DGCR8 while the cleavage product was decreased (Fig. 1D and E). Consistent with the increased mRNA level, the amount of AURKB protein was also increased after knockdown of DGCR8 (Fig. 1F).

As AURKB is involved in regulation of mitosis, we sought to determine whether cleavage of *AURKB* mRNA is dependent on the cell cycle. To measure the quantity of full-length *AURKB* mRNA, we used oligo-dTs as reverse transcription primers to generate a cDNA library from TDF cells arrested at the mitotic phase by treatment with nocodazole. We observed that the amount of *AURKB* mRNA increased during mitotic arrest (Fig. 2A). Similar results was obtained with HeLa S3 cells arrested by nocodazole. To determine if the increase of *AURKB* mRNA occurs in normal mitotic phases, mitotic cells were collected from untreated HeLa S3 cells by shake-off (mitotic shake-off). The quantity of *AURKB* mRNA was elevated in untreated mitotic cells indicating that the increase of the mRNA is a part of normal cell cycle regulation and not restricted to the arrest induced by activation of the mitotic checkpoint.

To verify if the Microprocessor-mediated cleavage is correlated with the quantity of *AURKB* mRNA, we used the same ligation-mediated RT-PCR described above (Fig 1B). While the relative quantity of the uncleaved mRNAs increased at mitotic phases, this was inversely correlated with the quantity of the cleavage products (Fig. 2B). Consistent with stabilization of *AURKB* mRNA, the protein levels were also increased in mitotic cells collected by shake-off or arrested by nocodazole treatment (Fig. 2C). Thus, the reduced

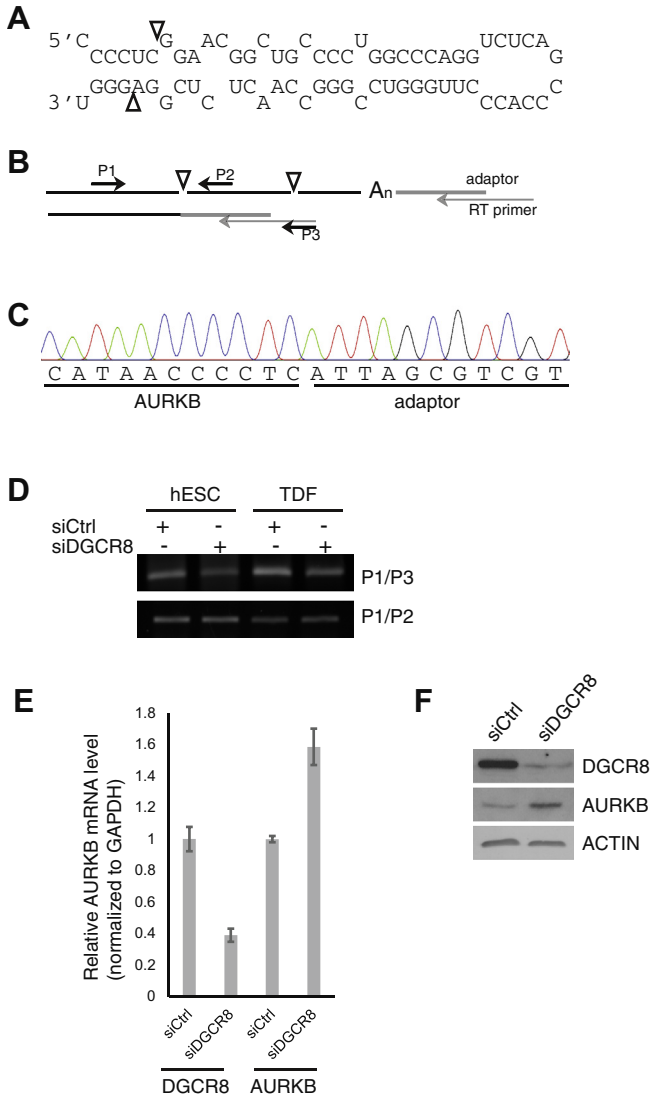

Fig. 1. The Microprocessor complex cleaves *AURKB* mRNA. (A) Predicted secondary structure of a sequence covered by DGCR8-CLIP reads in *AURKB* mRNA. The hairpin sequence is located within the last exon (exon 8). Open triangles indicates cleavage sites predicted by DGCR8-CLIP. (B) Detection of cleavage products by ligation-mediated RT-PCR. Pre-adenylated adaptor (grey line) was ligated to the 3′ end of RNA. Reverse transcription proceeds from an RT primer (grey arrow) hybridized to the adaptor sequence. Primers (P1, P2 and P3) are represented as black arrows. Binding sites of P1 and P2 are located in exon 7 and exon 8, respectively. P3 has the same sequence as the 5′ half of the RT primer. (C) The cleavage site was confirmed by subcloning and sequencing the amplified product. (D) Decrease of cleavage products after knock-down of DGCR8 in hESC and TDF. P1/P3 primer pair amplified the cleavage product and P1/P2 pair amplified intact mRNA. (E and F) Increase of *AURKB* mRNA and protein were also observed after DCGR8 knock-down in TDF.

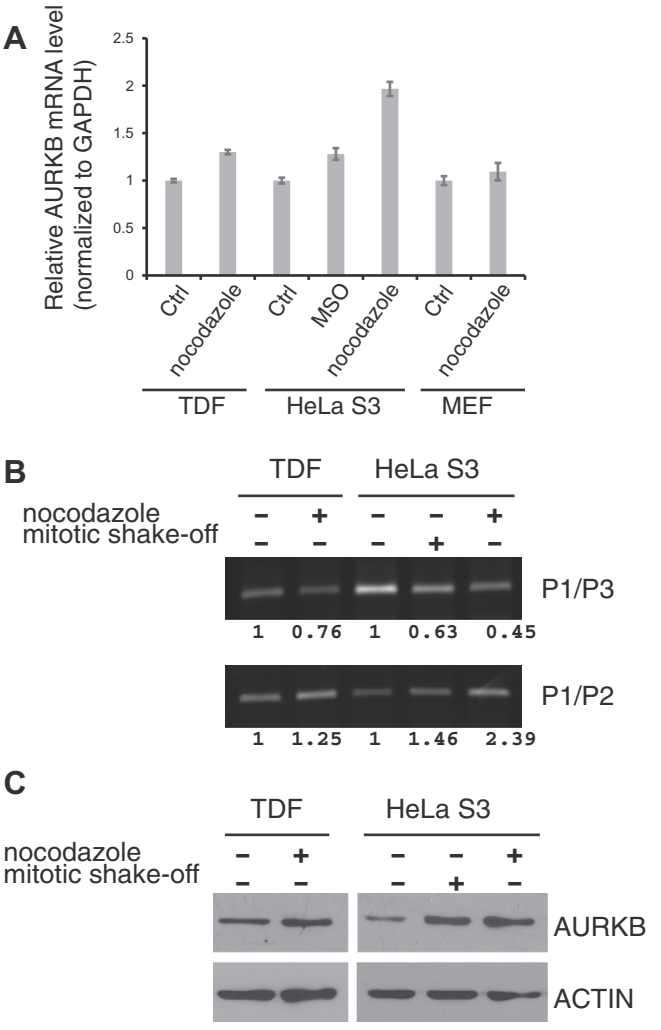

Fig. 2. Cell cycle-dependent cleavage of *AURKB* mRNA by the Microprocessor. (A) Increase of poly-adenylated *AURKB* mRNA at the mitotic phase. TDFs and MEFs were treated with 200 nM nocodazole for 18 h. HeLa S3 cells were treated for 12 h. Mitotic HeLa S3 cells were isolated by mitotic shake-off (MSO) from untreated culture. (B) Decreased cleavage of *AURKB* mRNA during mitosis. Ligation-mediated RT-PCR assay was carried out as described in Fig. 1B. (C) Increase of AURKB protein during mitosis.

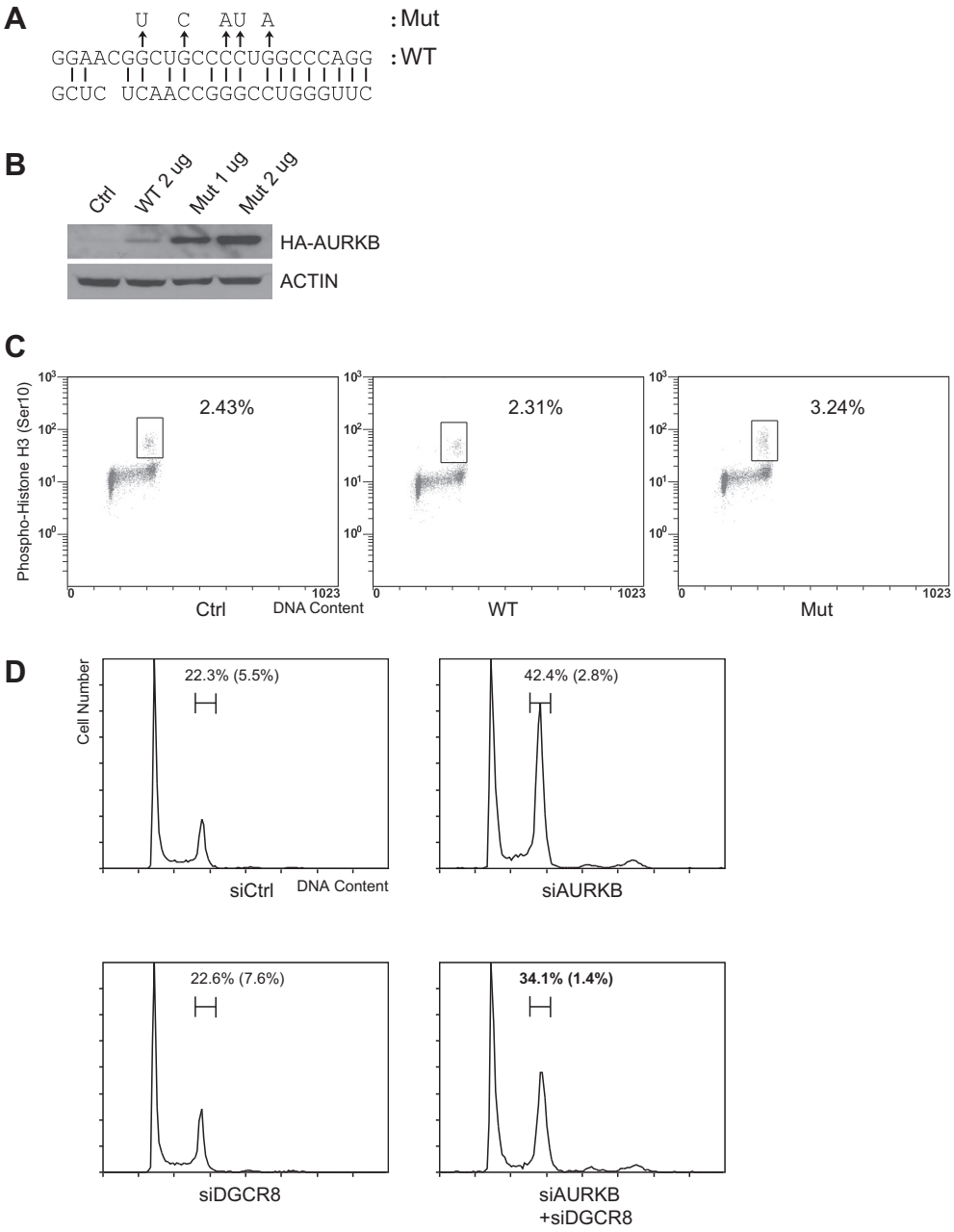

Fig. 3. Physiological role of the Microprocessor-mediated regulation of AURKB mRNA. (A) Sequences of wild type (WT) and the hairpin mutant (Mut). The mutated sequences are indicated by arrows. The stems of the hairpin were shown. (B) Effect of the stability of the hairpin structure on the Microprocessor-mediated cleavage. HA-tagged wild type AURKB or a hairpin mutant were ectopically expressed in HeLa S3. (C) Increased mitotic cell population by the expression of AURKB from the mutant construct. Phosphorylation of histone H3 at Ser10 was used as a mitotic marker. (D) Rescue of mitotic phenotypes by siRNA-mediated knock-down of AURKB. The proportion of G2/M cells are indicated. The numbers in the parenthesis indicate proportion of mitotic cells among the post-replication cell populations.

cleavage of *AURKB* mRNA during mitosis is correlated with increase of AURKB protein level.

In contrast to the human cells, mouse embryonic fibroblasts (MEFs) treated with nocodazole did not show significant increase of *Aurkb* mRNA (Fig. 2A). We carried out the ligation-mediated RT-PCR with mouse specific primers to detect cleavage products from MEFs. Even with nested PCR, we did not observe any specific products. Comparison of the human and the mouse mRNA sequences indicates that the region corresponding to the hairpin was not conserved between the two species and the mouse sequence was not supposed to form a stable hairpin structure. Thus, the Microprocessor-mediated regulation of *AURKB* mRNA seems to be a relatively recent invention during the course of evolution. Compared to mouse cells, human cells have more elaborate mechanisms to prevent the accumulation of genetic alterations, including aneuploidy. For example, disruption of the p53 gene in human cells does not lead to aneuploidy, while p53-null mouse fibroblasts accumulate abnormalities within a few passages [20,21]. The Microprocessor-mediated control of *AURKB* mRNA could be an added safety measure of human cells to prevent the appearance of aneuploidy and cancer.

As we confirmed the regulation of AURKB mRNA by the Microprocessor, we investigated its physiological roles. HeLa S3 cells were transfected with a plasmid construct that expresses HA-tagged AURKB or a mutant construct which have five silent mutations at the stem region of the hairpin (Fig. 3A). The expression

from the mutant construct was noticeably higher even with reduced amount of introduced DNA (Fig. 3B). This result indicates that the hairpin structure is critical for the Microprocessor-mediated cleavage and keeping the expression of AURKB protein at a low level. Next, the transfected cells were cultured with G418 to obtain stably transformed cells. Consistent with higher level of HA-AURKB, HeLa S3 cells with the mutant construct showed higher proportion of mitotic cells indicating accelerated entry into

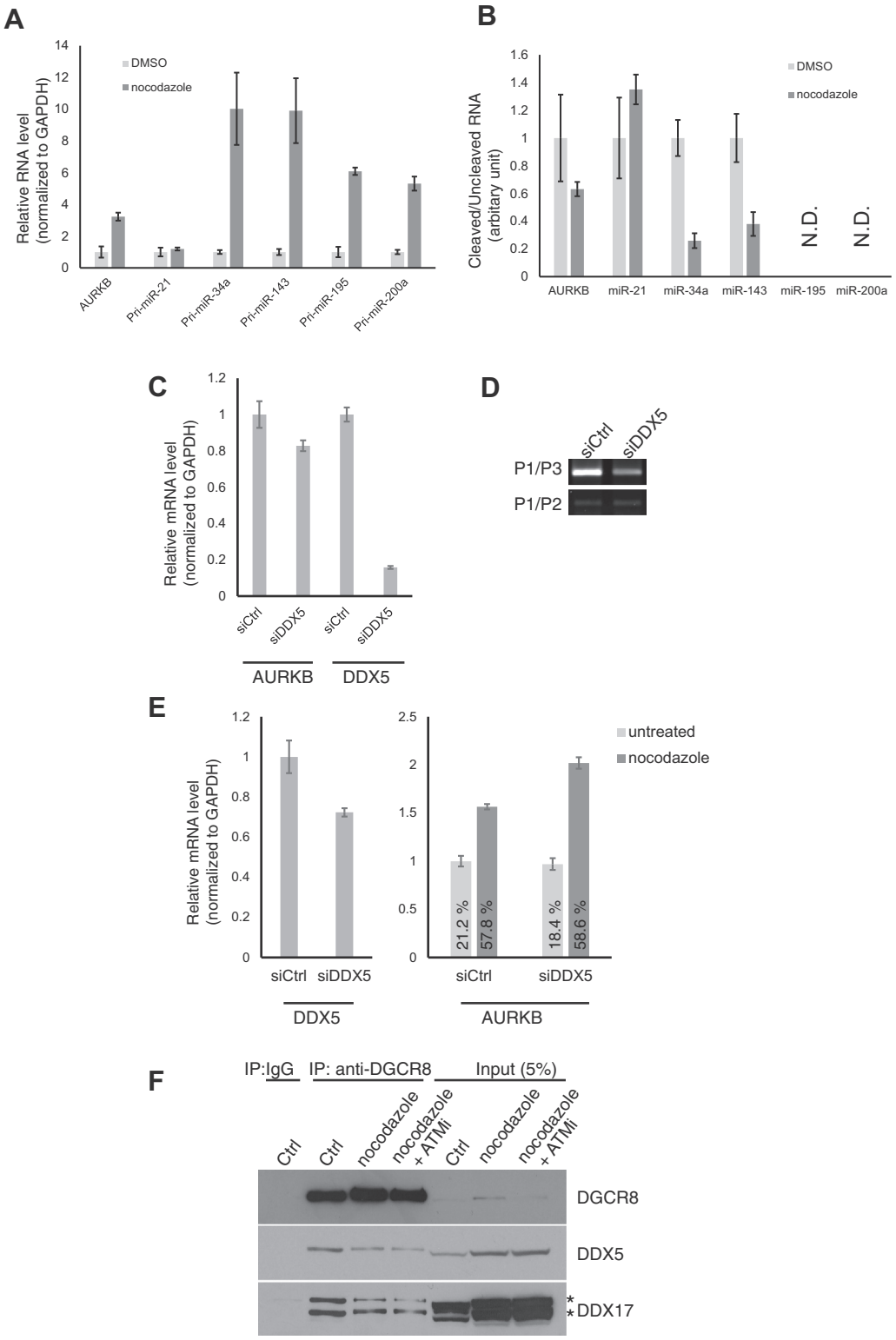

Fig. 4. Regulation of the activity of the Microprocessor complex during mitosis. (A and B) Processing of the pri-miRNAs during mitosis. After the ligation-mediated reverse transcription, the amount of the pri-miRNAs and the cleavage product was measured by quantitative PCR. (C) Role of DDX5 in the regulation of AURK mRNA stability. TDFs were transfected with siRNA mixture against DDX5. mRNA levels were measured by quantitative RT-PCR. (D) The cleavage were measured by the ligation-mediated RT-PCR. (E) HeLa S3 cells transfected with control siRNA or siRNA mixture against DDX5 were treated with nocodazole and the quantity of *AURKB* mRNA were measured by quantitative RT-PCR. The numbers indicate proportion of G2/M cells. (F) Association of DDX5 and DDX17 with DGCR8 during mitosis. The Microprocessor complex was immunoprecipitated with anti-DGCR8 antibody and probed with anti-DGCR8, anti-DDX5 and anti-DDX17 antibodies. The asterisks indicate the two isoforms of DDX17.

mitotic phase (Fig. 3C). In contrast, the wild type construct had minimal effect on proportion of mitotic cells. But it induced appearance of multi-nuclear cells, indicating that the increased level of the AURKB during mitosis inhibited cytokinesis (Supplementary Fig. 1). These results indicate that downregulation of AURKB by the Microprocessor before mitotic entry is important in preventing premature launch of the mitotic program which may lead to the appearance of genetic instability.

To further confirm the physiological roles of the Microprocessor-mediated cleavage of AURKB mRNA, TDFs were transfected with siRNAs against DGCR8, AURKB, or both (Fig. 3D). Knock-down of DGCR8 increased proportion of mitotic cells in post-replication cell populations, which is consistent with increased level of AURKB. Simultaneous knock-down of the AURKB abrogated the mitotic phenotype. Conversely, knock-down of AURKB increased G2/M cells indicating reduced entry into mitotic phase. Simultaneous knock-down of DGCR8 reduced the arrest.

Next, we investigated if the cell cycle-dependent destabilization by the Microprocessor complex is restricted to *AURKB* mRNA. We analyzed the amount of the primary transcript of miR-21 (pri-miR-21) and several pri-miRNAs randomly picked. Similar to *AURKB* mRNA, the primary transcripts, except pri-miR-21, were increased during the mitotic arrest (Fig. 4A). Also, the amount of the cleaved product relative to the uncleaved product was decreased implying that the reduced cleavage by the Microprocessor could have contributed to the increase of the primary transcripts (Fig. 4B). In contrast to other primary transcripts, the amount of pri-miR-21 and its cleavage product were unaffected by the mitotic arrest. It has been reported that without TGF-β or BMB signaling, the processing of pri-miR-21 is inefficient [22]. Thus, the impairment of processing during mitosis could have barely affected the already inefficient processing of pri-miR-21.

Because the Microprocessor-mediated degradation of *AURKB* mRNA is decreased at mitosis, we next investigated how the activity of the Microprocessor complex is regulated at mitosis. Biochemical analysis of Drosha-associated proteins revealed that in addition to smaller Drosha–Dgcr8 complex, there are larger complexes with other protein components in addition to the di-subunit complex [4]. Among the associated proteins of the larger complex, DEAD-box RNA helicase subunits, DDX5 and DDX17, are reportedly responsible for the recognition of a subset of primary miRNAs [23]. Recently, the association of tumor suppressor p53 and SMAD proteins with DDX5/DDX17 has been reported to regulate processing of subsets of primary miRNAs [22,24]. It is not clear how the association of these proteins with DDX5/DDX17 affect the activities of the Microprocessor complex. In addition, DDX5 has been reported to be phosphorylated by cyclin-dependent kinases during mitosis [25]. Thus, we investigated whether DDX5 is involved in the cleavage of *AURKB* mRNA. TDFs were transfected with siRNA against DDX5 and the level of *AURKB* mRNA was examined. In contrast to the knock-down of DGCR8, we did not observed any increase in the mRNA level of *AURKB* (Fig. 4C). Instead, the amount of degradation product relative to the uncleaved mRNA was reduced by knock-down of DDX5 as shown by the ligation-mediated RT-PCR assay (Fig. 4D). DDX5 has been shown to regulate a number of cellular processes including translation, transcription, splicing, cell cycle progression and development [26]. Thus, we reasoned that the pleiotropic effect on cellular physiology by knock-down of DDX5 could have obscured the effect on the level of *AURKB* mRNA. However, the reduction of cleaved product compared to full-length RNA was obvious in this experiment. To further confirm that DDX5 is involved in the regulation of *AURKB* mRNA stability, HeLa S3 cells were transfected with siRNA against DDX5. The knock-down of DDX5 reduced the expression by approximately 30% and barely affected cell-cycle profile. Also, the level of *AURKB* mRNA was unaffected by the knock-down. But the increase of the mRNA by mitotic arrest was more robust in the HeLa S3 cells with reduced DDX5 expression further confirming the involvement of DDX5 in destabilization of *AURKB* mRNA (Fig. 4E).

Because the degradation of *AURKB* mRNA is reduced by knock-down of DDX5, we investigated if the association of DDX5 with the Microprocessor complex is altered during mitosis. The Microprocessor was immunoprecipitated with anti-DGCR8 antibody and probed with anti-DGCR8, anti-DDX5 and anti-DDX17 antibodies. As shown in Fig. 4F, the association of DDX5 and DDX17 with DGCR8 was reduced at mitotic phase. Recently, ATM, a protein kinase that phosphorylates hundreds of substrates upon DNA damage-mediated activation, have been reported to be phosphorylated and activated by AURKB during mitosis [27,28]. But ATM-dependent phosphorylation of DGCR8, DDX5 or DDX17 was not detected and treatment of ATM inhibitor had no effect on the association of DGCR8 with DDX5 and DDX17 (Fig. 4F).

In this study, we showed that regulation of *AURKB* mRNA stability by the Microprocessor complex is cell cycle-dependent and responsible for the accumulation of the AURKB protein at mitosis. During mitotic exit, AURKB proteins are degraded by anaphase promoting complex/cyclosome (APC/C) [13,29]. Thus, the Microprocessor, together with APC/C, contribute to the confinement of the high kinase activity during the mitotic phase. Correlation between the overexpression of AURKB and the appearance of aneuploidy indicate that tight control of the expression of AURKB is important in maintaining genetic stability. In this regard, we proposed a novel role of the Microprocessor in the maintenance of genetic stability and the prevention of cancer.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.104.

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