An X-chromosome RNA-binding motif protein (RBMX) is required for proper kinetochore formation

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Abstract

RBMX is an RNA-binding motif protein identified as a component of purified human metaphase chromosomes. We have analyzed the functional significance of RBMX by electron microscopy (EM) in combination with RNA interference (RNAi). We revealed that depletion of RBMX results in the accumulation of mitotic cells. EM investigation further showed that RBMX depletion leads to an increase in the fuzzy ball structure that is reminiscent of the prekinetochore. It is most probably due to the result of disruption at early stages of kinetochore maturation, so that kinetochore differentiation into the authentic trilaminar morphology is disturbed. The present EM data illuminated the importance of RBMX in kinetochore formation for subsequent stable microtubules interaction.

Keywords:
RBMX (RNA-binding motif protein), hnRNP G (heterogeneous nuclear ribonucleoprotein G), kinetochore, EM, RNA interference

Introduction

During mitosis, two complete sets of chromosomes carrying identical genetic information are apportioned to a pair of daughter cells. Error-free sister chromatid separation is orchestrated by the attachment of the sister kinetochores of each chromosome to microtubules emanating from opposite spindle poles (O’Connell and Khodjakov 2007; Tanaka 2008). The mammalian kinetochore is a small and yet an elaborate structure, providing physical attachment to the microtubules, force generation and spindle assembly checkpoint (SAC) signaling that delays anaphase onset until all chromosomes are attached to the spindle (Cheeseman and Desai 2008). This intricate cellular machinery comprises of more than 120 components (Ohta et al. 2010), and an ever-increasing number of proteins are being implicated in, and changing our understanding of the kinetochore function and structure.

Early electron microscopic (EM) studies with conventional chemical fixative methods established the classical trilaminar model of kinetochore structure (Brinkley and Stubblefield 1966; Comings and Okada 1971). The inner kinetochore forms the interface with chromatin; the middle kinetochore is a less electron-dense interzone; and the outer kinetochore is a flexible network that interacts with multiple kinetochore microtubules (Dong et al. 2007). In the absence of microtubule attachment, a moderately dense fibrillar corona extended from the outer kinetochore is visible (Cheeseman and Desai 2008; McEwen and Dong 2010). Numerous studies have shown that kinetochores are formed transiently on the centromere during mitosis (Roos 1973; Rieder 1982), suggesting that kinetochores underwent an assembly/disassembly cycle (Brenner et al. 1981; He and Brinkley, 1996). Correlative light and electron microscopy (CLEM) reveals that kinetochores are visible on the surface of the primary constrictions as roughly circular patches of finely fibrillar materials as cells enter the prophase and gradually differentiate into the trilaminar morphology that is visible until anaphase (Roos 1973).

hnRNP G (heterogeneous nuclear ribonucleoprotein G) is implicated in the splicing control of several pre-mRNAs (Glisovic et al. 2008), and hnRNP G promotes the expression of tumor-suppressor Tnix and protects the fidelity of DNA end-joining activity (Shin et al. 2008). The relatively low abundance hnRNP G protein is unique among hnRNPs (Kanhoush et al. 2010) for being glycosylated (Soulard et al. 1993). In human cells, the gene coding for hnRNP G is located in the X chromosome, and therefore it is also known as RBMX (RNA-binding motif protein, X chromosome). RBMX is subject to X inactivation (Soulard et al. 1993), and is critical for proper neural development of zebrafish and frog embryos (Tseng-Ayusai et al. 2005; Dichmann et al. 2008). Multiple processed copies of RBMX are present in the human genome,
suggested that RBMX has multiple roles (Lingenfelter et al. 2001). Our previous efforts in elucidating chromosome higher order structure based on their constituent proteins enabled us to identify over 200 proteins by proteome analysis of human metaphase chromosomes (Uchiyama et al. 2005). RBMX was identified as an abundant protein in isolated chromosomes, suggesting its function in mitosis (Fukui and Uchiyama 2007; Takata et al. 2007). However, the function of RBMX in mitotic chromosomes has not been well investigated.

We have now shown that RBMX is essential for mitotic progression in human cells, suggested that RBMX is involved in kinetochore formation. This conclusion is reinforced by ultrastructural analysis of kinetochores in RBMX-depleted cells. RBMX is required for kinetochore plate development and stable microtubule attachment; prerequisites for accurate chromosome congression and segregation.

Materials and Methods

Cell culture

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL) supplemented with 10% fetal-bovine serum (FBS; Equitech-Bio) at 37°C and 5% CO₂.

Antibodies

For immunoblotting, primary antibodies were goat polyclonal anti-RBMX antibody (1:1000; Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin antibody (1:10000; Sigma). Secondary antibodies were alkaline phosphatase anti-mouse IgG (1:2000; Vector Laboratories) and alkaline phosphatase anti-goat IgG (1:2000; Vector Laboratories).

siRNA methods

HeLa cells were transfected at a final concentration of 100 nM with RBMX-siRNA (5’-uca aga gga uau agc gau att-3’) using Lipofectamine 2000 according to the manufacturer’s instructions. Cells transfected with Lipofectamine alone were used as a control. Cells were collected 48h post-transfection for analyses.

Immunoblotting and gel electrophoresis

Cells (siRNA or mock-transfected) grown in 24-well plates were collected and lysed in 2x Laemmli Sample Buffer (Santa Cruz Biotechnology) with equal amounts of PBS buffer. Protein extracts were fractionated on 10% polyacrylamide gels and then transferred onto PVDF membranes. The immunoblots were blocked with 1% BSA-TBST (0.1% Tween 20, 25 mM Tris-HCl pH 7.4, 137 mM NaCl, 25 mM KCl) and labeled with the primary and secondary antibodies. The immunoreactive protein bands were detected by NBT/BCIP solution (Roche) diluted in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 1 mM MgCl₂).

Fluorescence microscopy

HeLa cells grown on coverslips were transfected with the target siRNA as above, fixed with 4% PFA (paraformaldehyde) diluted with PBS (phosphate buffered saline, pH 7.4) for 15 min at 37°C, and stained with Hoechst 33342 (Sigma) for mitotic index calculation.

Electron microscopy

Sample preparation and EM analyses were based on those of Lee et al. (2011). In brief, HeLa cells grown on plastic coverslips (mono-layer) were fixed for 1 h in 3% glutaraldehyde and 0.2% tannic acid in PBS buffer at room temperature. Post-fixation was in 2% OsO₄ for 20 min. The cells were dehydrated through an increasing ethanol series and infiltrated with epoxy resin (Quetol 812). The resins were polymerized at 37 °C for 12 h, 45 °C for 12 h and 60 °C for 48 h. Plastic coverslips were removed, and cells of interest embedded in the resin were chosen under an optical microscope and trimmed to ~ 1.0 mm². Samples were cut into 70-80 nm thick serial sections, parallel with the coverslip direction, with an ultramicrotome equipped with a diamond knife (ULTRACUT E, Reichart-Jung). The sections were stained with uranyl acetate and lead citrate and examined by a transmission electron microscope (JOEL, JEM-1200EX).

For EM analyses, cells that apparently aligned at metaphase plate were chosen for control, and RBMX RNAi cells were chosen based on the phenotypes of poor chromosome alignment. Alternatively, colcemid treated cells were chosen randomly both in the control and RBMX RNAi. All kinetochores observed were included in the analyses regardless of their appearance. Only a few sections, containing chromosome-rich regions, were examined for individual cells. As the boundary between individual chromosomes is not obvious, and sister kinetochore appearances can sometimes

Figure 1. Mitotic defects in RBMX disruption. A Depletion of RBMX by RNAi treatment. β-actin was used as loading control. RBMX has a molecular weight of 43 kDa. B Low magnification electron micrograph of a control cell. C Typical phenotype of RBMX disrupted cells. Scale bar: 10 µm.
be seen differently depending on kinetochore fiber attachment, kinetochores were analyzed individually rather than as a pair of kinetochores of a chromosome. Several adjacent serial sections were analyzed to classify individual kinetochores, because kinetochore morphology varies even between adjacent serial sections. Therefore kinetochore is classified as trilaminar once the canonical layered structure is visible in any of the adjacent serial sections for an individual kinetochore, even if the structure is rather fuzzy in other sections.

Results

Mitotic progression was impaired by RBMX depletion.

To assess the functional roles of RBMX in mitosis, we employed an RNAi-mediated gene-silencing approach to knockdown RBMX from the cells. Immunoblot analysis of HeLa cultures subjected to RBMX siRNA treatment for 48 hours revealed a decline in the expression level of RBMX of less than 20% when compared with mock transfected cells (Figure 1A). Mitotic index was increased to more than 3-fold (13.1±3.3%) of the control (4.4±0.2%). A high degree of alignment defects (74.3±6.8%, compare to 4.7±4.6% in the control) was observed as we assayed for the mitotic profiles in cells lacking RBMX, as shown in the lower magnification electron micrographs (Figure 1B, C).

RBMX depletion compromises kinetochore formation

Chromosome congression defects are a common feature of aberrant microtubule association. Often, irregular kinetochore formation couples with chromosome-to-spindle attachment defects. Thus, we evaluated whether kinetochores assemble properly in RBMX RNAi cells with electron microscopy using conventional chemical fixation. First, we treated the cells with colcemid to examine the kinetochore structure. 34 kinetochores from 4 control cells and 31 kinetochores from 8 RBMX RNAi cells were examined (Fig. 2). Kinetochores were classified into three groups, i.e. well-formed (Class 3), poorly-formed (Class 2), and fuzzy ball (Class 1), based on the classification scheme we have proposed (Lee et al. 2011). Because inner plates are not always visible in colcemid treatment (Roos 1973), kinetochores with an electron-dense outer layer are considered well-formed. In the control, nearly 90% of the kinetochores were well-formed (Fig. 2A). In contrast, nearly 70% of the kinetochores in RBMX RNAi cultures failed to construct the layered structure and appeared as fuzzy balls (Fig. 2B, 2C), although outer plates were constructed to some degree in some kinetochores (Fig. 2B).

Next, we examined whether the kinetochore aberrations affected microtubules interaction. We imaged adjacent serial sections of 5 control cultured cells (Fig. 1B) and 11 RBMX-depleted cultured cells (Fig. 1C). The kinetochores were again classified based on our classification scheme, and each group was further divided into two categories, i.e. with or without microtubule attachment. Inner plates are visible in most of the well-formed kinetochores. Fig. 3A shows the frequency of kinetochore disorganization in RBMX RNAi cultures. 58 and 56 kinetochores were examined in the control and RBMX RNAi cultures, respectively. In the control, robust microtubules attachment (white arrowheads) was observed, regardless of the kinetochore structures (Fig. 3A-F). When RBMX is knockdowned, kinetochore-microtubule interactions were significantly reduced, especially for poorly-formed or fuzzy ball kinetochores (Fig. 3A, 3G-L). These data indicate that RBMX is essential for proper kinetochore formation for subsequent stable microtubule interactions to achieve chromosome congression.

Discussion

RBMX has been identified in screenings of mitotic chromosome components in both human (Uchiyama et al. 2005; Takata et al. 2007) and DT40 cell lines (Ohta et al. 2010), suggesting its involvement in the mitotic events. In this study, we found that cells lacking RBMX show particularly aberrant chromosome congression, suggesting that they are defective with respect to microtubule capture. EM studies support this conclusion. Authentic trilaminar kinetochores are notably rarer in RBMX RNAi cultures. Fuzzy ball structures, resembling the prekinetochores
RBMX is required for kinetochore formation

(Roos 1973; Lee et al. 2011) often observed in early mitotic stages, suggest that kinetochore maturation is highly disrupted. Normally, most of the kinetochores interact with microtubules after nuclear envelope breakdown, even when the trilaminar structure has not been fully constructed (data not shown; Roos 1973). Only a few kinetochores in RBMX RNAi cells associate with microtubules; those with a particularly low population appeared as fuzzy balls (Fig. 3A).

The observation of well-formed kinetochore in RBMX RNAi cells was not surprising, as DeLuca et al. (2005) showed that trilaminar kinetochores were observed even when Hec1-Nuf2, the major components constructing the outer kinetochore plates were depleted. We think that it was because RBMX was knockdown but not knockout from the cells, therefore some kinetochores may develop
even the proteins level is very low in that cell. Fuzzy ball structures observed in the control may be derived from several reasons: differences in kinetochore maturation, microtubule interactions and angle of the kinetochores. Well-formed kinetochores increased in colcemid treatment, suggested that differences in kinetochore maturation and microtubule association contributed to some degree in the fuzzy ball appearance in the control. These may also be the case in RBMX RNAi cells, because fuzzy ball structures were slightly decreased, although well-formed kinetochores remained low, and microtubule attachments were fewer without colcemid treatment. It was shown by Roos (1973) that kinetochores appeared as fuzzy balls in paraequatorial sections. Therefore, fuzzy ball in the control may also caused by kinetochore angle during sectioning. Nevertheless, the fuzzy balls in the control are relatively low, and the percentages of well-formed kinetochores in colcemid treated cells were consistent with the previous report (Deluca et al. 2005).

In conclusion, we have shown that RBMX plays critical role in proper kinetochore formation where subsequent stable microtubules attachment is attained. Failure to do so induced mitotic arrest (data not shown) with high degree of alignment defects, resulted in the increasing of mitotic index. Detailed characterization of the mechanism is under investigation and will be reported elsewhere.

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