Functional Interaction between Chfr and Kif22 Controls Genomic Stability

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Proper activation of checkpoint during mitotic stress is an important mechanism to prevent genomic instability. Chfr (Check point protein with FHA (Forkhead-associated domain) and RING domains) is a ubiquitin-protein isopeptide ligase (E3) that is important for the control of an early mitotic checkpoint, which delays entry into metaphase in response to mitotic stress. Because several lines of evidence indicate that Chfr is a potential tumor suppressor, it is critically important for us to identify Chfr substrates and understand how Chfr may regulate these substrates, control mitotic transitions, and thus, act as a tumor suppressor in vivo. Here, we report the discovery of a new Chfr-associated protein Kif22, a chromokinesin that binds to both DNA and microtubules. We demonstrated that Kif22 is a novel substrate of Chfr. We showed that Chfr-mediated Kif22 down-regulation is critical for the maintenance of chromosome stability. Collectively, our results reveal a new substrate of Chfr that plays a role in the maintenance of genome integrity.

Chfr (Check point protein with FHA and RING domains) is an early mitotic checkpoint protein that delays entry into metaphase in response to mitotic stress (1, 2). The checkpoint function of Chfr requires both of its FHA and RING domains. The exact role of FHA domain in Chfr function is largely unknown. Chfr via its RING domain transfers both lysine 48-linked and lysine 63-linked polyubiquitin chains to its target proteins, which either promotes the degradation of target proteins or alters their function (3, 4). Recently, a PAR-binding zinc finger motif, which binds directly to polyADP-ribosylated substrates catalyzed by PARP1, was identified at the C-terminal region of Chfr (5). This PAR-binding zinc finger motif was reported to be required for Chfr function in anaphase checkpoint (2, 5).

Chfr delays the cell cycle progression at mitosis by inactivating cyclin B1-bound Cdc2 and then exporting them from the nucleus (6). Further, mechanistic studies have suggested that the inactivation of Cdc2 may be due to a negative regulation of Plk1 by Chfr (3). Polyubiquitination of Plk1 by Chfr negatively regulates the Plk1 protein levels, which delay the inactivation of Cdc2 inhibitory Wee1 kinase and the activation of Cdc25 phosphatase and thus maintain Cdc2 at its inactive state.

Several lines of evidence indicate that Chfr is a potential tumor suppressor. Loss or down-regulation of Chfr has been reported in several types of cancers including primary breast, lung, esophagus, colon, and gastric carcinomas (1, 7, 8). To investigate directly whether Chfr loss contributes to tumorigenesis, our laboratory has generated Chfr knock-out mice, which were cancer-prone and developed spontaneous tumors (9). The increased tumor incidence in Chfr null mice is likely due to a failure in maintaining chromosomal stability, which occurs at least partially due to the overexpression of a key mitotic kinase Aurora A (9). Chfr physically interacts with Aurora A and promotes its ubiquitination and degradation; thus, higher protein levels of Aurora A in Chfr null mice may contribute to chromosomal instability and eventually tumorigenesis. Therefore, our current hypothesis is that Chfr may regulate the stability of several of its substrates including Aurora A, and thus, control mitotic progression and prevent chromosomal instability. In this study, we reported the identification of another Chfr substrate as chromokinesin protein Kif22 and revealed that Kif22 overexpression also contributes to chromosomal instability observed in Chfr-deficient cells.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length Chfr and Kif22 were cloned into an S-protein/FLAG/STB (streptavidin-binding protein) triple-tagged destination vector using the Gateway cloning system (Invitrogen). Various Kif22 deletions (D1–D8), Chfr deletions (∆FHA, ∆RING, and ∆Cys), and point mutations were generated by PCR-based site-directed mutagenesis and verified by DNA sequencing. Full-length Chfr and Kif22 were also cloned to Myc-tagged mammalian expression destination vector and GST-tagged and MBP-tagged bacterial expression vector. An HA-tagged ubiquitin construct was used in vivo ubiquitination assays.

Cell Culture—HeLa and 293T cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Human mammary epithelial cells (HMEC) were maintained in MEGM complete medium supplemented with bovine pituitary extract. 293T-Chfr, 293T-Kif22, MCF7-control shRNA, MCF7-Chfr shRNA, T47D-control shRNA, and T47D-Chfr shRNA stable cell lines were maintained in complete RPMI medium supplemented with 2 μg/ml puromycin. HeLa-Myc Chfr stable cell lines were maintained in the presence of 2 μg/ml puromycin.
Mouse monoclonal anti-Chfr antibodies were produced by hybridoma fusions. Rabbit anti-Aurora B antibodies were raised previously in the laboratory by immunizing rabbits with GST-Aurora B fusion protein. Antisera were affinity-purified using AminoLink plus immobilization and purification kit (Pierce).

Tandem Affinity Purification—293T cells were transfected with plasmid encoding triple-tagged Chfr, and 3 weeks later, puromycin-resistant colonies were selected and screened for Chfr expression. Chfr stable cells were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 50 mM β-glycerophosphate, 10 mM NaF, 1 μg/ml of pepstatin A and aprotinin (10 μg/ml) on ice for 20 min. Cell lysates were incubated with streptavidin-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. The bound proteins were washed three times with NETN and then eluted twice with 2 mg/ml biotin (Sigma) for 30 min at 4 °C. The eluates were incubated with S-protein-agarose beads (Novagen) for 1 h at 4 °C and then washed three times with NETN. The proteins bound to S-protein-agarose beads were resolved by SDS-PAGE and visualized by Coomassie Blue staining. The identities of eluted proteins were revealed by mass spectrometry analysis performed by the Taplin Biological Mass Spectrometry Facility at Harvard.

Transfection, Immunoprecipitation, and Immunoblotting—293T cells and HeLa cells were transfected with various plasmids using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. For immunoprecipitation assays, whole cell lysates were incubated with 2 μg of specified antibody bound to either protein A-Sepharose beads or protein G-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. The immunocomplexes were then washed with NETN buffer four times and applied to SDS-PAGE. Immunoblotting was performed following standard protocols.

In Vivo Ubiquitination Assay—HeLa Cells were transfected with various combinations of plasmids as shown in Fig. 3, A and B, along with HA-tagged ubiquitin. At 24 h after transfection, cells were treated with MG132 (4 μM) for 6 h, and whole cell
extracts prepared by NETN lysis were used to immunoprecipitate the substrate protein. The analysis of ubiquitination was performed by immunoblotting using anti-HA antibodies.

**GST Pull-down Assays**—GST-tagged Chfr or control GST bound to glutathione-Sepharose beads (Amersham Biosciences) were incubated with 293T cell lysates expressing FLAG-tagged Kif22 or a bacterially expressed recombinant MBP-Kif22 for 1 h at 4 °C, and the washed complexes were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, and the interactions were analyzed by Western blotting.

**RNA Interference**—Control small interfering RNA (siRNA) and the smart pool siRNAs against Kif22 and Chfr were purchased from Dharmacon Inc. Transfection was performed twice 30 h apart with 200 nM siRNA using Oligofectamine reagent according to the manufacturer’s protocol (Invitrogen), and the protein expression was analyzed by immunoblotting 72 h after transfection. Control shRNA and Chfr shRNA target sets were purchased from Open Biosystems, and stable clones of MCF-7 and T47D cells were made by transfecting with Lipofectamine and puromycin selection for 3 weeks.

**Immunofluorescence Staining**—Cells grown on coverslips were fixed with 3% paraformaldehyde solution in phosphate-buffered saline (PBS) containing 50 mM sucrose at room temperature for 15 min. After permeabilization with 0.5% Triton X-100 buffer containing 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM MgCl₂, and 300 mM sucrose at room temperature for 5 min, cells were incubated with a primary antibody (α-tubulin, CENPA, γ-tubulin, Chfr, and Kif22) at 37 °C for 20 min. After washing with PBS, cells were incubated with either fluorescein isothiocyanate-conjugated or rhodamine-conjugated secondary antibody at 37 °C for 20 min. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. After a final wash with PBS, coverslips were mounted with glycerin containing para-phenylenediamine. Images were acquired by using a Nikon ECLIPSE E800 microscope.

**Cell Cycle Analysis**—T24 cells were arrested in G₀ phase by contact inhibition and were released into fresh medium. At the indicated time points, cells were lysed, and the levels of Chfr and Kif22 during different phases of the cell cycle were analyzed by Western blotting. Cell cycle progression was monitored by fluorescence-activated cell sorter analysis.

**Metaphase Spreads**—Metaphase chromosome spreads were performed as described before (9). Briefly, cells treated with colcemid for 8 h were collected, washed with PBS, and treated with 75 mM KCl at room temperature for 15 min. The treated cells were then fixed in fresh methanol-acetic acid (3:1) solution and dropped onto glass slides. Cells were allowed to air dry and were stained with Giemsa (5%) and visualized under the microscope.

**RESULTS**

**Kif22 Is a Chfr-associated Protein**—In an attempt to better understand the molecular mechanisms underlying the tumor suppressor function of Chfr, we established a 293T derivative cell line stably expressing a triple epitope (S-protein, FLAG, and streptavidin-binding peptide)-tagged version of Chfr (SFB-Chfr). A two-step affinity purification using streptavidin-Sepharose beads and S-protein-agarose beads followed by mass spectrometry analysis allowed us to identify several Chfr-interacting proteins (Fig. 1A and supplemental Table 1). Among them, we repeatedly isolated Kif22 as a major associated protein in the purified Chfr complex. Kif22 (also known as hKid) is a mitotic motor protein with both DNA and microtubule binding activities and thus plays a positive role during spindle assembly and proper chromosome segregation in mitosis (10–12). We first confirmed that endogenous Kif22 but not Kif11 specifically associates with Chfr (Fig. 1, B and C). Co-immunoprecipitation (Fig. 1, D and E) and GST pulldown assays (Fig. 1, F and G) further confirmed a direct interaction between these two proteins. Further, immunostaining of Chfr and Kif22 revealed overlapping localization of both proteins during interphase and early mitotic phases, but they appear to localize differently during late mitotic phases (supplemental Fig. S1). To further
Functional Interaction between Chfr and Kif22

understand how these two proteins may function together, we examined whether Chfr and Kif22 expression would be coordinately regulated during cell cycle. T24 cells were arrested in G0 phase by contact inhibition, and the synchronized cells were allowed to reenter the cell cycle by releasing them into fresh medium at the appropriate density. As shown in Fig. 1H, the levels of Kif22 and Chfr were tightly regulated and inversely correlated during different phases of the cell cycle, suggesting a potential functional link between these two proteins.

Chfr contains N-terminal FHA, a middle RING, and C-terminal cysteine-rich domains (1). The FHA domain, known to be a phospho-peptide-binding motif, is important for Chfr the N termini and the C termini because only a deletion mutant (D4) that lacks both termini of Kif22 failed to bind to Chfr (Fig. 2D). The interaction of Chfr with the N terminus of Kif22 appears to be weaker than its binding to the C terminus of Kif22 (Fig. 2D).

Kif22 Is a Novel Substrate of Chfr—At least in the case of Aurora A, we know that Chfr uses its C-terminal cysteine-rich domain as a substrate recognition domain, which recruits substrates for ubiquitination and degradation (9). To map the Kif22-binding region on Chfr, we used Myc epitope-tagged wild-type and a series of Chfr deletion mutants (Fig. 2A). Immunoprecipitation with anti-Myc antibody revealed that endogenous Kif22 interacted with full-length Chfr, ΔFHA, and ΔRING Chfr mutants but not with the Δcysteine-rich domain mutant of Chfr (Fig. 2B), suggesting that the C-terminal cysteine-rich domain of Chfr is essential for mediating its interaction with Kif22.

On the other hand, Kif22 also has several functional domains that are critically important for its mitotic function during spindle assembly and proper chromosome alignment in metaphase (11, 16). We generated expression constructs for FLAG-tagged Kif22 and a series of N-terminal or C-terminal deletion mutants that lack different Kif22 functional domains (Fig. 2C). To map the Chfr-binding region on Kif22, we co-expressed these constructs along with full-length Myc-tagged Chfr. The immunoprecipitation results suggest that Chfr interacts with Kif22 through both checkpoint function in response to mitotic stress (1, 13, 14), but the identities of the proteins interacting with Chfr FHA domain are still unknown. The RING finger domain, a characteristic domain of ubiquitin-protein isopeptide ligase (E3), also plays a crucial role in Chfr function via its ability to ubiquitinate substrates such as Aurora A and Plk1 (3, 9, 15). The C-terminal cysteine-rich domain of Chfr is required for its binding to substrates and therefore targeting its substrates for degradation (9). To map the Kif22-binding region on Chfr, we used Myc epitope-tagged wild-type and a series of Chfr deletion mutants (Fig. 2A). Immunoprecipitation with anti-Myc antibody revealed that endogenous Kif22 interacted with full-length Chfr, ΔFHA, and ΔRING Chfr mutants but not with the Δcysteine-rich domain mutant of Chfr (Fig. 2B), suggesting that the C-terminal cysteine-rich domain of Chfr is essential for mediating its interaction with Kif22.

FIGURE 3. Chfr ubiquitinates Kif22 and promotes its degradation. A, Myc-tagged full-length (FL), ΔRING (ΔR), or ΔCys-rich (ΔC) domain mutants of Chfr were expressed in HeLa cells along with FLAG-Kif22 and HA-ubiquitin (HA-Ub). The levels of Kif22 ubiquitination were evaluated by anti-HA immunoblotting (WB) following immunoprecipitation (IP) of Kif22. FL, ΔFHA, and ΔRING Chfr mutants but not with the Δcysteine-rich domain mutant of Chfr (Fig. 2B), suggesting that the C-terminal cysteine-rich domain of Chfr is essential for mediating its interaction with Kif22.

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Kif22 is essential for Chfr-dependent Kif22 polyubiquitination. This is evident by the efficient ubiquitination of Chfr (Fig. 3C) and the dependency of the proteasome-dependent pathway during Chfr-mediated Kif22 down-regulation and its dependence on the proteasome inhibitor MG132. Co-transfection of Kif22 but not Chfr binding-deficient mutant of Kif22 (D4) along with Chfr reduced the steady-state levels of Kif22 protein (Fig. 3D). Pretreatment with MG132 prevents Chfr-mediated Kif22 down-regulation, suggesting the involvement of the proteasome-dependent pathway during Chfr-mediated Kif22 degradation. Taken together, these data suggest that Kif22 is a bona fide in vivo substrate of Chfr.

To further confirm the Chfr-mediated Kif22 down-regulation and its dependence on the proteasome pathway, we performed a co-transfection experiment with Chfr and Kif22 in the presence or absence of a proteasome inhibitor MG132. Co-transfection of Kif22 but not Chfr binding-deficient mutant of Kif22 (D4) along with Chfr reduced the steady-state levels of Kif22 protein (Fig. 3D). Pretreatment with MG132 prevents Chfr-mediated Kif22 down-regulation, suggesting the involvement of the proteasome-dependent pathway during Chfr-mediated Kif22 degradation. Taken together, these data suggest that Kif22 is a bona fide in vivo substrate of Chfr.

**Aberrant Expression of Kif22**

**Results in Abnormal Mitotic Spindles and Promotes Genomic Instability**—Kif22 is known to be required for proper mitotic spindle organization and normal chromosomal alignment on the metaphase spindle (17). In addition, our laboratory has previously reported that the loss of Chfr leads to multiple mitotic defects (9). Thus, we tested whether the Chfr-mediated Kif22 regulation would play a role in controlling mitotic progressions, especially during mitotic spindle assembly.

HMECs expressing control shRNA displayed normal bipolar mitotic spindles, whereas some of the cells with Chfr knockdown exhibited abnormal mitotic spindles with multiple poles and disorganized array of microtubules that failed to organize a proper metaphase plate (Fig. 4, A and B). Similar to Chfr domain deletion mutants, indicating that Kif22 is a substrate of Chfr, and both the intact Chfr E3 ligase activity and its substrate recognition domain are required for efficient Chfr-mediated Kif22 ubiquitination (Fig. 3A). Conversely, full-length Kif22, a Kif22 mutant (Fig. 2C, D2) with intact N-terminal Chfr interaction region but devoid of the C-terminal Chfr-interacting region, and a Kif22 mutant (Fig. 2C, D5) with intact C-terminal Chfr interaction region but devoid of the N-terminal Chfr-interacting region were efficiently ubiquitinated by Chfr (Fig. 3B). Another Kif22 mutant (Fig. 2C, D4), which lacks both the N-terminal and the C-terminal Chfr-binding regions, could not be ubiquitinated by Chfr (Fig. 3B), again supporting the notion that a specific interaction of Chfr with Kif22 is essential for Chfr-dependent Kif22 polyubiquitination.
Functional Interaction between Chfr and Kif22

DISCUSSION

In this study, we have shown that Kif22 physically interacts with Chfr and is a newly identified Chfr substrate. Kif22 is a plus-end-directed microtubule-based motor protein that plays a role in bipolar organization of spindle microtubules and chromosome movement (11, 18), which are important for chromosome segregation during mitosis. We speculate that the spindle disorganization and abnormal metaphase chromatid alignment observed in Chfr-deficient cells could be at least partially explained by the up-regulation of Kif22 in these cells. Importantly, analogous to Chfr expression, proper control of Kif22 expression is also important for the maintenance of chromosomal stability. Thus, we propose that in addition to previously identified Chfr substrates (Plk1 and Aurora A), Kif22 also plays a role in the maintenance of chromosomal stability. Moreover, chromosomal instability observed in primary cells with Kif22 overexpression may suggest a potential previously unidentified involvement of Kif22 in tumorigenesis that warrants further investigation.

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shRNA, transient overexpression of wild-type Kif22 but not DNA-binding domain-deleted (ΔDBD) Kif22 mutant (Fig. 4E) also resulted in cells with abnormal mitotic spindles (Fig. 4, A and B), suggesting that Chfr loss may lead to Kif22 overexpression and thus cause spindle disorganization. Upon knocking down of Chfr or Kif22 overexpression, we also observed aberrant arrangement of kinetochores (Fig. 4C) and the presence of abnormal number of centrosomes (Fig. 4D). A partial knock down of Kif22 (Fig. 4E) alleviated mitotic spindle defects observed in Chfr knockout cells (Fig. 4, A–D).

Previously, our laboratory has also reported chromosomal instability in cells derived from Chfr knock-out mice (9). Similar to our previous findings using Chfr null mouse embryonic fibroblasts, the analysis of metaphase spreads revealed that Chfr down-regulation in HMECs results in abnormal chromosome numbers when compared with cells transfected with control shRNA (Fig. 4, F and G). Interestingly, Kif22 overexpression also results in chromosomal instability, analogous to cells with Chfr knockdown. These results suggest that at least one mechanism for Chfr functions in the maintenance of chromosomal stability and that tumor suppression could be through its regulation of Kif22 protein levels because both Chfr down-regulation and Kif22 overexpression result in chromosomal instability, which is a hallmark of tumorigenesis.