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Report

MST2- and Furry-Mediated Activation of NDR1 Kinase Is Critical for Precise Alignment of Mitotic Chromosomes

Shuhei Chiba,[1] Masanori Ikeda,[1] Kokichi Katsunuma,[1] Kazumasa Ohashi,[1] and Kensaku Mizuno[1,*]
[1]Department of Biomolecular Sciences
Graduate School of Life Sciences
Tohoku University
Sendai, Miyagi 980-8578
Japan

Summary

The precise alignment of chromosomes on the metaphase plate prior to the onset of anaphase is essential for ensuring equal segregation of sister chromatids into two daughter cells, and defects in this process potentially cause chromosome instability and tumor progression [1–3]. NDR1 is an evolutionarily conserved serine/threonine kinase whose activity is regulated by MST kinases, Furry (Fry), and MOB [4]. Although the NDR1 signaling pathway is implicated in cell division and morphogenesis in yeast and invertebrates [4–16], the mechanisms of NDR1 activation and the functional significance of the NDR1 pathway in mammalian cells are largely unknown. Here, we show that NDR1 is required for accurate chromosome alignment at metaphase in HeLa cells; depletion of NDR1, Fry, or MST2 caused mitotic chromosome misalignment. Chromosome misalignment in MST2-depleted cells was corrected by expression of active NDR1. The kinase activity of NDR1 increased in early mitotic phase and was dependent on Fry and MST2. We also provide evidence that Fry binds to microtubules, localizes on the spindle, acts as a scaffold that binds to both NDR1 and MOB2, and synergistically activates NDR1 with MOB2. Our findings suggest that MST2-, Fry-, and MOB2-mediated activation of NDR1 is crucial for the fidelity of mitotic chromosome alignment in mammalian cells.

Results and Discussion

Knockdown of NDR1 or Fry Causes Chromosome Misalignment

NDR1 is a member of the highly conserved NDR and LATS subfamily of serine/threonine kinases [4]. Orthologs of mammalian NDR1, including *Drosophila* Trc, *C. elegans* Sax-1, *S. pombe* Orb6p, and *S. cerevisiae* Cbk1p, are implicated in the control of cell division and morphogenesis and are genetically linked to MST kinases, Fry, and MOB [4–16]. NDR1 is activated by MST-mediated phosphorylation of Thr-444 and by autophosphorylation of Ser-281 [17, 18]. MOB proteins bind to and activate NDR1 [19, 20]. Fry is a large protein that contains multiple HEAT or Armadillo-like repeats [5, 9, 10, 12]. In yeast and *Drosophila*, Fry associated with NDR1 [5, 14]; however, it is unknown how Fry activates NDR1. Although it was reported that NDR1 plays a role in centrosome duplication [21], the functional significance of NDR1 and its regulators in mammalian cells is still only poorly understood.

To explore the roles of NDR1 and Fry in mammalian cells, we examined the effects of their knockdown on mitotic spindle organization in HeLa cells. Short-hairpin RNAs (shRNAs) targeting NDR1 and Fry suppressed the expression of each protein (Figure S1, available online). HeLa cells cotransfected with CFP-histone H2B (CFP-H2B) and shRNAs were synchronized and stained for CFP-H2B and α-tubulin. In control shRNA-treated cells, all chromosomes were aligned on the metaphase plate of the bipolar spindle in metaphase or segregated concurrently toward opposite spindle poles in anaphase (Figure 1A, Movie S1). In contrast, in NDR1 or Fry shRNA-treated mitotic cells, chromosomes were broadly scattered in the spindle, with several chromosomes positioned close to the spindle poles (Figure 1A, Movies S2 and S3). Whereas most NDR1 or Fry shRNA-treated cells retained a bipolar spindle structure (Figure 1A), a minority of cells displayed a deformed spindle with fragmented spindle poles (Figure S2), suggesting that NDR1 and Fry play some roles in maintaining spindle pole integrity. Although it was reported that NDR1 is required for centrosome duplication [21], NDR1 knockdown neither enhanced monopolar spindle formation nor caused a failure of centriole duplication (Figure S3), indicating that NDR1 is not significantly involved in normal centriole or centrosome duplication, at least in HeLa cells. We focused on the roles of NDR1 and Fry in chromosome positioning. Chromosome mispositioning was observed in 67% and 88% of NDR1- and Fry-depleted cells, respectively (Figure 1B). Cotransfection of NDR1 shRNA with shRNA-resistant wild-type (WT) NDR1, but not with kinase-dead NDR1(D212A), decreased the number of cells with chromosome mispositioning (Figure 1C), indicating that the kinase activity of NDR1 is required for proper chromosome positioning.

To distinguish whether the aberrant chromosome positioning in NDR1- or Fry-depleted cells is a consequence of chromosome alignment errors or segregation errors, we analyzed chromosome movements by time-lapse microscopy after treating cells with the proteasome inhibitor MG132, which causes cells to arrest at the metaphase-anaphase transition (Figures 1D and 1E). Whereas more than 90% of control cells exhibited complete chromosome alignment on the metaphase plate until 30 min after nuclear envelope breakdown, more than 60% of NDR1- or Fry-depleted cells displayed misaligned chromosomes even 60 min after this event. These results indicate that NDR1 and Fry are required for precise chromosome alignment prior to anaphase onset.

For examination of kinetochore localization, HeLa cells synchronized in metaphase with MG132 were stained with anti-CENP-A (Figure 1F). In comparison to control cells, in which all kinetochores aligned on the equator of the bipolar spindle, NDR1- or Fry-depleted cells exhibited a broad distribution of kinetochore signals in the spindle. Of these NDR1- or Fry-depleted cells, 75%–80% had misaligned kinetochores, compared to 12% of control cells (Figure 1G). Whereas the kinetochores aligned on the metaphase plate in either control cells or NDR1- or Fry-depleted cells were attached to spindle microtubules (MTs) from opposite poles

*Correspondence: kmizuno@biology.tohoku.ac.jp

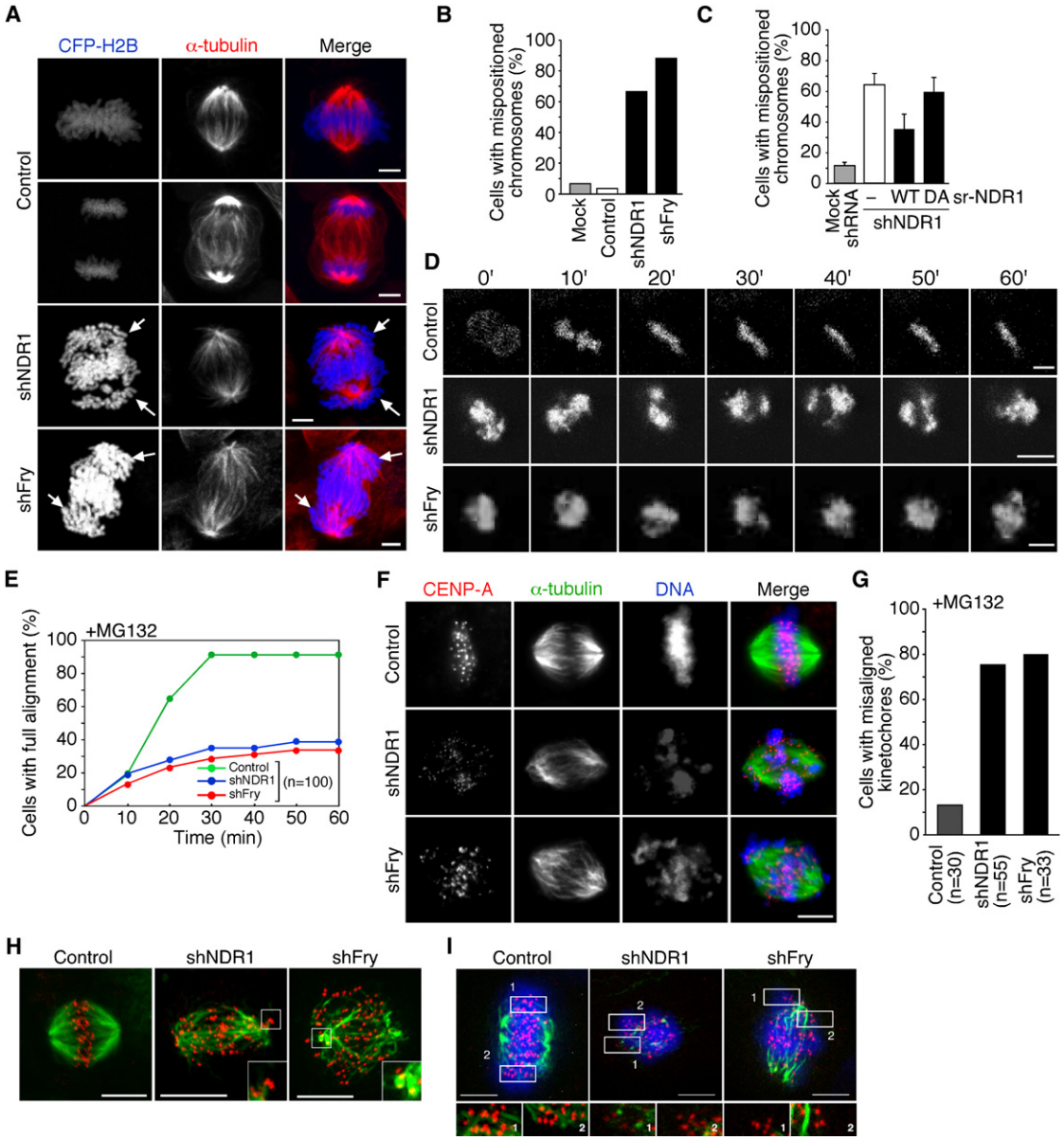

Figure 1. NDR1 and Fry Are Critical for Chromosome Alignment

(A) NDR1 or Fry knockdown causes chromosome mispositioning. HeLa cells were cotransfected with CFP-H2B and shRNA plasmids at the ratio of 1:19. Cells were cultured in thymidine-containing medium for 24 hr, released from thymidine for 12 hr, then fixed and stained for CFP-H2B (blue) and α-tubulin (red). Scale bar represents 5 μm. Arrows indicate mispositioned chromosomes. See also Movies S1–S3.

(B) Percentages of cells with mispositioned chromosomes. Data were obtained from at least 60 cells.

(C) The kinase activity of NDR1 is required for rescue of the effect of NDR1 shRNA. HeLa cells were cotransfected with CFP-H2B, shRNA-resistant (sr-) NDR1 (WT or D212A), and NDR1 shRNA plasmids at the ratio of 1:5:14 and analyzed as in (A). Data are means ± SD of triplicate experiments (more than 100 cells for each experiment).

(D) Time-lapse analyses of chromosome movements. HeLa cells cotransfected with CFP-H2B and shRNAs were treated with MG132 3–6 min before the beginning of image acquisition. The time after nuclear-envelop breakdown is indicated. Scale bar represents 10 μm.

(E) Effects of shRNAs on chromosome alignment. Chromosome movements were analyzed by time-lapse microscopy, as in (D). Each point represents the cumulative percentage of cells with full alignment of chromosomes on the metaphase plate at the time indicated on the abscissa.

(F) NDR1 or Fry knockdown causes kinetochore misalignment. HeLa cells transfected with shRNAs were treated with MG132 for 1.5 hr, permeabilized, fixed, and stained for α-tubulin, CENP-A, and DNA. Scale bar represents 10 μm.

(G) Percentages of cells with misaligned kinetochores. Cells having kinetochore(s) located outside the central 35% of the spindle were counted.

(H) Misaligned kinetochores in NDR1 or Fry shRNA cells are mono-oriented. HeLa cells treated as in (F) were stained for α-tubulin (green) and CENP-A (red). Insets show magnified images of white boxes. Scale bar represents 10 μm.

(I) Instability of kinetochore MTs in NDR1 or Fry shRNA cells. HeLa cells transfected with shRNA plasmids were pretreated on ice for 10 min before fixation. Cells were stained for α-tubulin (green), CENP-A (red), and DAPI (blue). Insets show magnified images of white boxes. Scale bar represents 5 μm.

(bi-orientation), the misaligned kinetochores near the poles in NDR1- or Fry-depleted cells were mono-oriented (Figure 1H). When HeLa cells treated with MG132 were briefly exposed to low temperature, which induces disassembly of unstable MTs [22], almost all kinetochores in control cells were still attached to spindle MTs, but the unaligned kinetochores in NDR1- or Fry-depleted cells were not (Figure 1I). This suggests that either kinetochore-attached MTs or kinetochore-MT interactions are less stable in NDR1- or Fry-depleted cells as compared to control cells. Previous studies indicated that depletion of kinetochore components essential for kinetochore-MT attachments, such as Nuf2 and Hec1, caused severe defects in chromosome alignment, whereas depletion of regulators of MT dynamics, such as ch-Tog (also named XMAP215) and HURP, often caused partial defects in chromosome alignment [23–25]. Thus, NDR1 and Fry might be involved in chromosome alignment through regulation of MT dynamics and reorganization.

Fry Localizes on Spindle MTs

Next, we analyzed the localization of Fry during the cell cycle with a Fry antibody that specifically recognizes endogenous Fry (Figure S4). Immunostaining revealed that Fry was diffusely distributed in the cytoplasm in interphase but accumulated on separating centrosomes in prophase, on spindle poles and spindle MTs during prometaphase to anaphase, and on the distal sections of the midbody during telophase to cytokinesis (Figure 2A). After treatment with nocodazole, Fry was diffusely distributed in both interphase and mitosis (Figure S5). In contrast, after treatment with taxol, Fry localized on MTs in interphase and near the taxol-induced MT asters in mitosis (Figure S5). These results indicate that Fry localization depends on MT assembly. Additionally, Fry colocalized with acetylated MTs in mitotic HeLa cells (Figure S6). In vitro MT cosedimentation assays revealed that all three Fry fragments coprecipitated with MTs (Figure 2B), indicating that Fry has the potential to directly bind to MTs via its multiple MT-binding sites.

Fry Binds To and Activates NDR1

We then analyzed whether Fry binds to NDR1. YFP-Fry expressed in COS-7 cells was pulled down with GST-NDR1, but not with control GST (Figure 2C). Both WT and kinase-dead Myc-NDR1 were coprecipitated with endogenous Fry (Figure S7), indicating that NDR1 interacts with Fry independently of its kinase activity. A coprecipitation assay using HA-NDR1 and a set of deletion mutants of Myc-Fry revealed that Fry binds to NDR1 via its N-terminal region (amino acids 1–730) (Figure S8). We also examined the effect of Fry overexpression or knockdown on the kinase activity of endogenous NDR1. The kinase activity of NDR1 increased about 1.8-fold in Fry-expressing cells and significantly decreased in Fry-depleted cells, compared to that in control cells (Figures 2D and 2E). Accordingly, Fry plays a critical role in activating NDR1 in cultured cells.

Cell-Cycle-Dependent Activation of NDR1

The important role of NDR1 for chromosome alignment in mitosis prompted us to investigate whether NDR1 activity changes during the cell cycle. HeLa cells were synchronized at early S or early mitotic phase, and the kinase activity of NDR1 was measured. The relative kinase activity of NDR1 in mitotic cells increased about 2.2-fold and 4.4-fold, compared with that in asynchronous and S phase cells, respectively (Figure 3A). We further analyzed changes in the NDR1 activity during mitosis. HeLa cells were synchronized at early mitotic phase with nocodazole and released for 0–240 min. Analysis of cell morphology and staining for tubulin and DNA indicated the major cellular stage at each time after release: 0 min (prometaphase), 45 min (metaphase), 90 min (telophase), 180 and 240 min (G1 phase) (data not shown). The kinase activity of NDR1 at 0 and 45 min increased to 2.3- to 2.5-fold, compared with that in interphase cells, and then gradually decreased as cells progressed to later stages of mitosis (Figure 3B), indicating that NDR1 is activated in early stages of mitosis (prometaphase and metaphase) and then is gradually inactivated to the basal level in later stages.

For examination of the role of Fry in the mitotic activation of NDR1, the kinase activity of NDR1 was analyzed in Fry-depleted cells. Fry knockdown considerably decreased the kinase activity of NDR1 in interphase and mitosis (Figure 3C), indicating that Fry is required for NDR1 activity in both phases. We also analyzed the binding of NDR1 to Fry in interphase and mitotic HeLa cells. The amount of NDR1 coprecipitated with Fry increased in mitosis, compared with that in interphase (Figure 3D), suggesting that Fry binding is involved in the mitotic activation of NDR1.

MST2 Is Required for Mitotic NDR1 Activation and Chromosome Alignment

Given that MST2 is activated in mitosis [26], we hypothesized that MST2 is involved in the mitotic activation of NDR1. When MST2 was depleted by shRNA, mitosis-specific activation of NDR1 was abrogated (Figure 3E). Thus, MST2 plays a crucial role in the mitotic activation of NDR1. We then analyzed whether MST2 depletion affects chromosome alignment in metaphase. When HeLa cells cotransfected with MST2 shRNA and CFP-H2B were treated with MG132, 52% of MST2 shRNA cells displayed chromosome misalignment, a phenotype similar to that of NDR1- or Fry-depleted cells (Figures 3F and 3G). Cotransfection of shRNA-resistant WT MST2, but not of kinase-dead MST2(K56R), corrected chromosome misalignment induced by MST2 shRNA (Figure 3G), indicating that the kinase activity of MST2 is required for chromosome alignment. Given that MST kinases activate NDR1 by Thr-444 phosphorylation [17], we examined the effects of expression of phosphorylation-mimic (T444E) and nonphosphorylatable (T444A) mutants of NDR1 on MST2 shRNA-induced chromosome misalignment. Intriguingly, expression of WT NDR1 or NDR1(T444E), but not of NDR1(T444A), markedly suppressed MST2 shRNA-induced chromosome misalignment (Figure 3H). Taken together, these results suggest that MST2 plays a crucial role in precisely aligning chromosomes by activating NDR1 via Thr-444 phosphorylation during mitosis.

Fry and MOB2 Synergistically Activate NDR1

MOB proteins bind to and activate NDR1 [19, 20]. To examine how Fry and MOB activate NDR1, we analyzed the effects of single or combined expression of Fry and MOB proteins on the kinase activity of endogenous NDR1 (Figure 4A). Treatment of cells with okadaic acid (OA), which activates MST kinase, increased the kinase activity of NDR1, as reported previously [27]. In OA-treated cells, the kinase activity of NDR1 was augmented 2.7-fold and 5.4-fold by individual expression of Fry and MOB2, respectively, compared to that of control cells. Intriguingly, coexpression of MOB2 and Fry further increased NDR1 activity (9.5-fold), indicating that MOB2 and Fry synergistically activate NDR1 and that both proteins are required

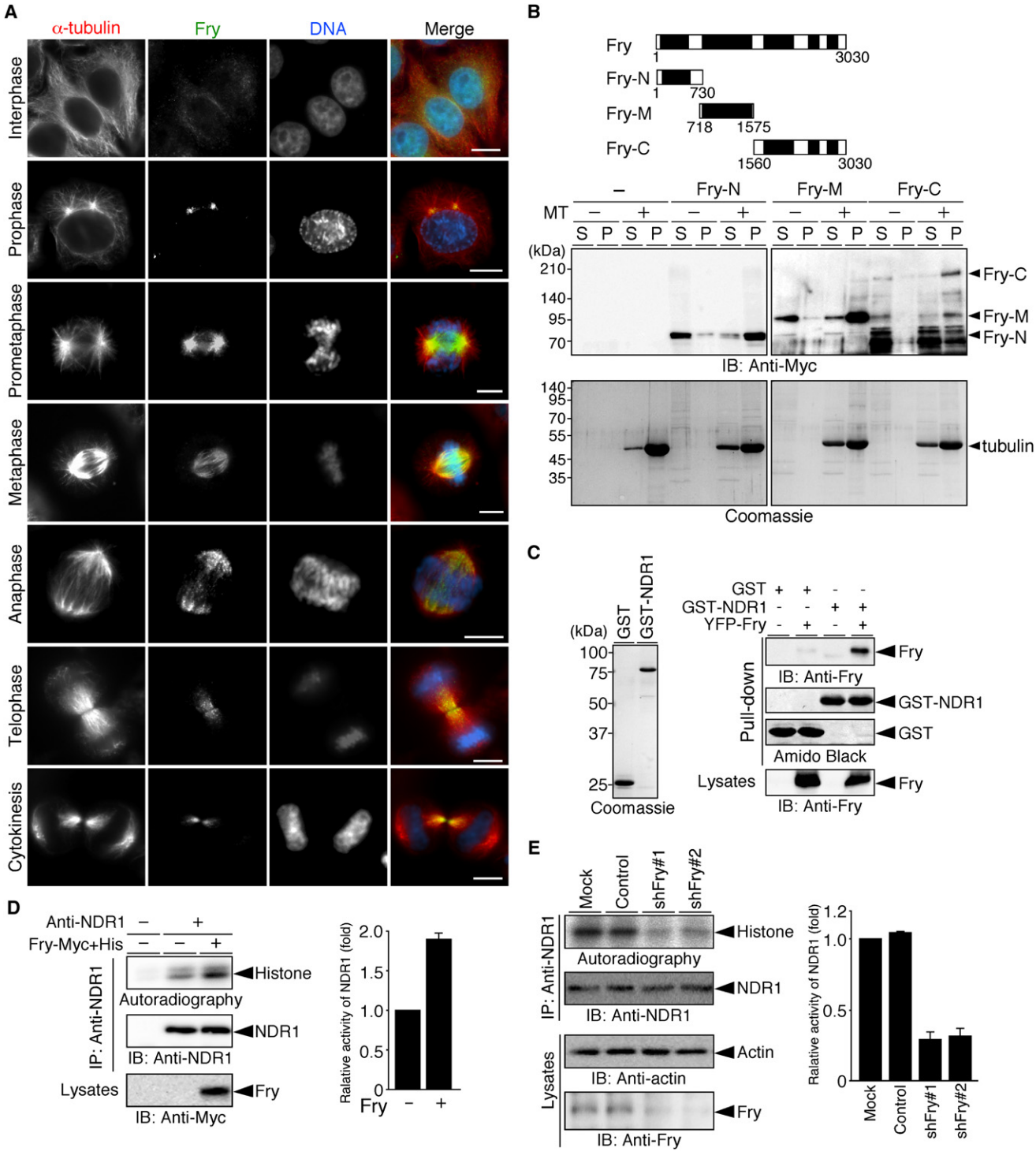

Figure 2. Fry Localizes on Spindle MTs and Activates NDR1

(A) Cell-cycle-dependent localization of Fry. HeLa cells were fixed and stained with anti-Fry (green) and anti-α-tubulin (red) and with DAPI for DNA (blue). Scale bar represents 10 μm.

(B) MT cosedimentation assays. Structures of mouse Fry and its deletion mutants are schematically shown. The regions conserved among species are denoted by black bars. Fry-(Myc+His) mutants expressed in Sf21 insect cells were purified and incubated with preassembled MTs. After centrifugation, equal amounts of supernatant (S) and pellet (P) fractions were analyzed by immunoblotting with a Myc antibody. Tubulins were analyzed by Coomassie staining.

(C) Fry binds to NDR1. (Left) Coomassie staining of purified GST and GST-NDR1. (Right) Pull-down assay of YFP-Fry using GST- or GST-NDR1-immobilized beads.

(D) Expression of Fry increases the kinase activity of NDR1. HeLa cells were transfected with Fry-(Myc+His). Endogenous NDR1 was immunoprecipitated and subjected to kinase assay, with histone H1 used as a substrate. The relative kinase activity of NDR1 is shown as the means ± SE of triplicate experiments.

(E) Knockdown of Fry decreases the kinase activity of NDR1. U2OS cells were infected with retrovirus coding for control or Fry shRNAs. The relative kinase activity of NDR1 was measured as in (D).

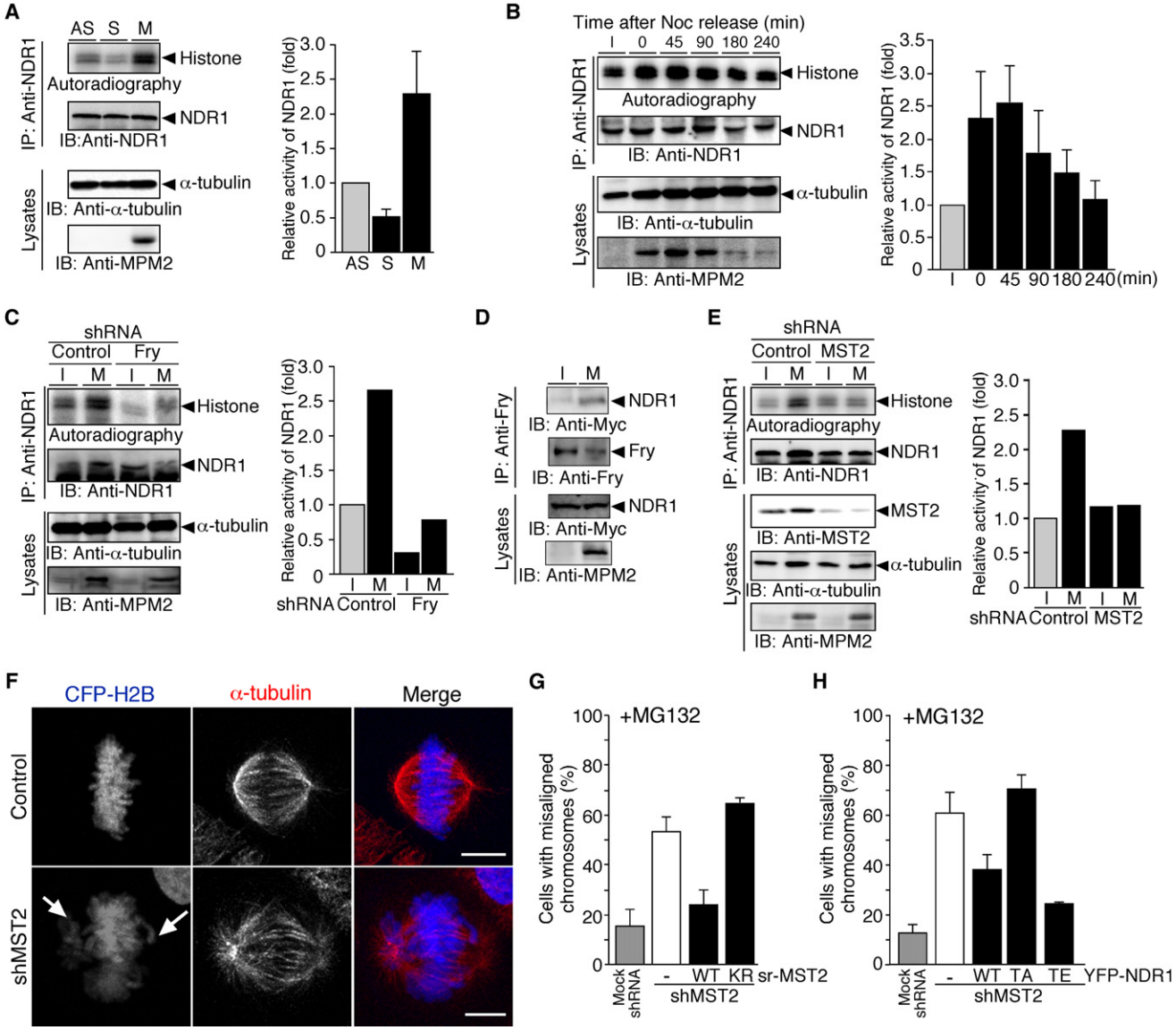

Figure 3. Mitotic Activation of NDR1 and the Role of MST2 in Chromosome Alignment

(A) Mitotic activation of NDR1. HeLa cells were asynchronous (AS) or synchronized at early S phase by a double thymidine block and at early mitotic phase with nocodazole, as described [30]. NDR1 was immunoprecipitated and subjected to an in vitro kinase assay. Mitosis was confirmed by immunoblot with a MPM2 antibody. The relative kinase activity of NDR1, normalized to the band intensity on the NDR1 immunoblot, is shown as the means ± SD of triplicate experiments.

(B) Changes in NDR1 activity during mitosis. HeLa cells were arrested with nocodazole and released for the indicated times, as described previously [30]. NDR1 was immunoprecipitated from lysates from interphase cells (I) and cells released for the indicated times after the removal of nocodazole, and the relative kinase activity of NDR1 was analyzed as in (A).

(C) Fry is required for NDR1 activity. HeLa cells were transfected with control or Fry shRNA. NDR1 activity was measured as in (A).

(D) NDR1 binding to Fry increases in mitosis. HeLa cells were transfected with Myc-NDR1. Fry was immunoprecipitated from lysates of interphase (I) and mitotic (M) cells, and Myc-NDR1 was analyzed by immunoblotting with a Myc antibody.

(E) MST2 is required for mitotic activation of NDR1. HeLa cells were treated with control or MST2 shRNA, and the kinase activity of NDR1 was measured as in (A).

(F) Knockdown of MST2 causes chromosome misalignment. HeLa cells transfected with CFP-H2B and control or MST2 shRNA were treated with MG132 for 1 hr. Cells were stained for CFP-H2B (blue) and α-tubulin (red). Arrows indicate the misaligned chromosomes. Scale bar represents 10 μm.

(G and H) Quantitative analyses of cells with misaligned chromosomes. HeLa cells were cotransfected with CFP-H2B and the indicated plasmids and treated as in (F). The percentages of cells with misaligned chromosomes among total CFP- and YFP-positive cells are shown as means ± SD of triplicate experiments.

for full activation of NDR1. Similar amounts of MOB2 were co-precipitated with NDR1, irrespective of OA treatment (Figure 4A), indicating that the MOB2-NDR1 interaction is not related to phosphorylation. In contrast to MOB2, MOB1 (MOBKL1B) only weakly bound to NDR1, and expression of MOB1 did not increase NDR1 activity.

Next, we examined whether Fry binds to MOB proteins. In HeLa cells expressed with HA-MOB2, endogenous Fry was

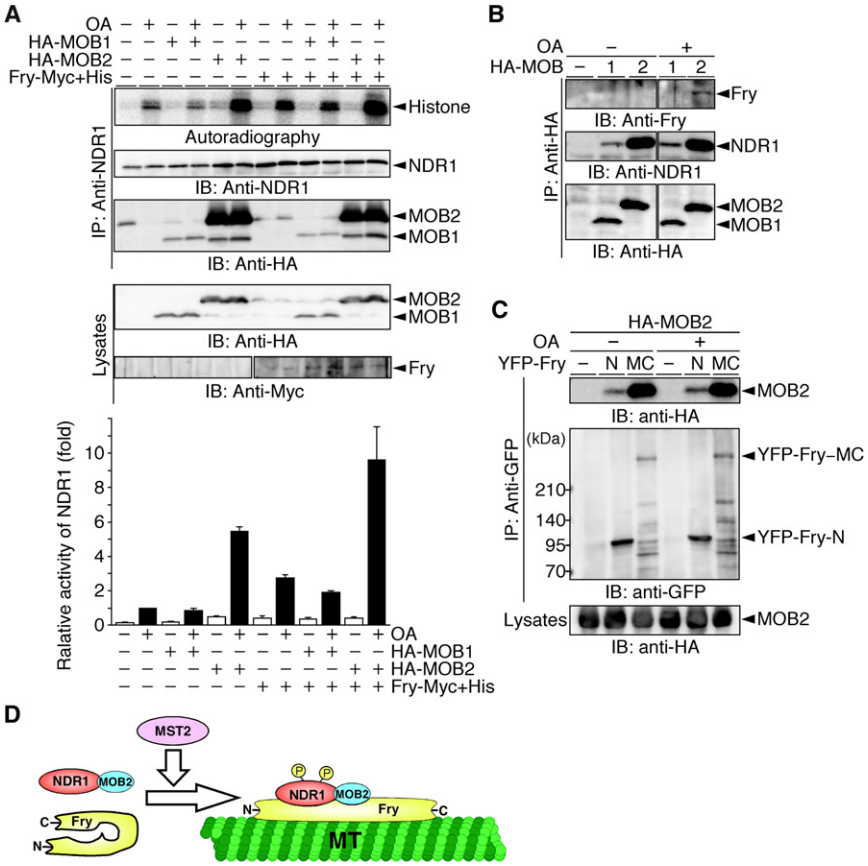

Figure 4. Fry and MOB2 Synergistically Activate NDR1

(A) Fry and MOB2 synergistically activate NDR1. HeLa cells were transfected with HA-MOB1, HA-MOB2, and Fry-(Myc+His), as indicated. Cells were treated with 100 nM OA for 1 hr or untreated. The kinase activity of endogenous NDR1 was determined as in Figure 2D. The relative activity of NDR1 is shown as the means ± SD of triplicate experiments.

(B) OA treatment induces the Fry-MOB2 interaction. HeLa cells transfected with HA-MOB1 or HA-MOB2 were treated with OA or untreated. Lysates were precipitated with anti-HA and analyzed by immunoblotting with anti-Fry, anti-NDR1, and anti-HA.

(C) MOB2 preferentially binds to the C-terminal region of Fry. HeLa cells cotransfected with HA-MOB2 and YFP-Fry fragments were treated with OA or untreated. Lysates were precipitated with anti-GFP and analyzed by immunoblotting with anti-HA and anti-GFP.

(D) A model for control of NDR1 activity.

coprecipitated with HA-MOB2 after OA treatment, but not without OA treatment, whereas NDR1 was coprecipitated with HA-MOB2, irrespective of OA treatment (Figure 4B). These results suggest that the MOB2-Fry interaction is regulated by phosphorylation, whereas the MOB2-NDR1 interaction is independent of phosphorylation. Interaction of Fry with MOB1 was not detected even after OA treatment. When Fry fragments were coexpressed with MOB2 in HeLa cells, the C-terminal fragment (Fry-MC; see Figure S8A) efficiently coprecipitated MOB2 both before and after OA treatment, whereas the N-terminal fragment (Fry-N) did so less efficiently (Figure 4C). Because Fry binds to NDR1 at the N-terminal region (Figure S8), it probably functions as a scaffold for promoting the MOB2-NDR1 interaction and MOB2-mediated NDR1 activation (Figure 4D). Whereas Fry-MC bound to MOB2 constitutively, full-length Fry bound to MOB2 in a manner dependent on OA treatment, which suggests that upstream cues, such as phosphorylation, may induce a conformational change of Fry to an "open" form that is suitable for MOB2 and NDR1 binding (Figure 4D).

Control of the NDR1 Signaling Pathway and Its Physiological Roles

Here, we demonstrated that NDR1 and its regulators MST2 and Fry play crucial roles in aligning chromosomes on the metaphase plate in mammalian cells. This is a novel function for the NDR1 pathway. Because Fry localizes on spindle MTs in mitosis, it may function to localize and activate NDR1 on the spindle for achievement of precise chromosome alignments. It is also possible that Fry itself has a function in regulating the stability of spindle MTs via its multiple MT-binding sites. In *Drosophila*, NDR1 (Trc) and Fry are essential regulators for dendritic branching and tiling of neurons and morphology of wing hairs and bristles [11–16]. It appears that coordinated MT dynamics underlie these morphogenetic processes in *Drosophila*. We showed that Fry is a MT-binding protein and localizes on MTs. Given the important roles of MT dynamics both in chromosome alignment during mitosis and in the morphogenesis of *Drosophila* dendrites and hairs, the Fry-NDR1 pathway may function in these processes in common through regulating MT dynamics. It will be important to identify the physiological substrates of NDR1 kinase and to determine whether these substrates are relevant to the regulation of MT dynamics. MST kinases also activate LATS kinases (the Hippo pathway), which restrain cell proliferation in cooperation with Salvador and MOB proteins [28, 29]. The potential scaffolding proteins, Fry and Salvador, and the MOB family proteins may play important roles in controlling the differential activation of the NDR and LATS signaling pathways by MST kinases.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, eight figures, and three movies and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00807-0.

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