Centrosome-Associated NDR Kinase Regulates Centrosome Duplication

Alexander Hergovich,1 Stefan Lamla,2 Erich A. Nigg,2 and Brian A. Hemmings1,*

1Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland
2Max-Planck-Institute for Biochemistry, Am Klopferspitz 18, D-82152 Martinsried bei München, Germany

*Correspondence: brian.hemmings@fmi.ch
DOI 10.1016/j.molcel.2007.01.020

SUMMARY

Human NDR kinases are upregulated in some cancer types, yet their functions still remain undefined. Here, we report the first known function of a mammalian NDR kinase by demonstrating that human NDR directly contributes to centrosome duplication. A subpopulation of endogenous NDR localizes to centrosomes in a cell-cycle-dependent manner. Overexpression of NDR resulted in centrosome overduplication in a kinase-activity-dependent manner, while expression of kinase-dead NDR or depletion of NDR by small interfering RNA (siRNA) negatively affected centrosome duplication. By targeting NDR to the centrosome, we show that the centrosomal pool of NDR is sufficient to generate supernumerary centrosomes. Furthermore, our data indicate that NDR-driven centrosome duplication requires Cdk2 activity and that Cdk2-induced centrosome amplification is affected upon reduction of NDR activity. Overall, considering that centrosome overduplication is linked to cellular transformation, our observations may also provide a molecular link between mammalian NDR kinases and cancer.

RESULTS

Human NDR Kinase Associates with Centrosomes

The commercially available anti-NDR1 antibody and our recently developed anti-NDR antibodies are not suitable for cell biological studies (Hergovich et al., 2005). Therefore, to explore the subcellular localization and function of endogenous human NDR kinase, we raised a rabbit polyclonal antibody against the C terminus of recombinant human NDR1 (anti-NDRCTD). The antibody recognized human NDR1 and NDR2 equally well (see Figure S1 in the Supplemental Data available with this article online) and immunofluorescence staining of U2-OS cells (that express NDR1 abundantly but not NDR2 [Hergovich et al., 2005]) showed that a portion of human NDR kinase accumulated in the proximity of nuclei (Figure 1A). Similar stainings were seen in NIH 3T3, Cos-7, HeLa, and CHO cells (Figure 1A; data not shown). The anti-T444-P antibody raised against the hydrophobic motif phosphorylation site of human NDR (Hergovich et al., 2005) also detected endogenous NDR in such structures (Figure 1B). Interestingly, these structures turned out to be centrosomes, since anti-NDR immunostainings displayed colocalization with γ-tubulin, a constituent of centrosomes (Figure 1C and Figure S1), and a second centrosome marker (Figure S2). Preincubation of antibodies with antigen abolished centrosome

INTRODUCTION

Centrosomes consist of a pair of centrioles surrounded by pericentriolar material (Bornens, 2002; Nigg, 2004). During S-phase, the single centrosome doubles to yield the two centrosomes that organize the bipolar spindle during mitosis, thus ensuring equal distribution of genetic material between the two daughter cells. The presence of more than two centrosomes in one cell can disturb mitotic progression by the formation of multipolar mitotic spindles, potentially leading to chromosome instability that could promote tumor development or progression. Indeed, centrosome abnormalities are considered as a major factor contributing to chromosome instability in cancer cells, since centrosome amplification occurs frequently in almost all types of cancer (Lingle et al., 2002; Lingle and Salisbury, 1999; Pihan et al., 1998, 2001). However, it remains unclear whether centrosome alterations are a cause or a consequence of tumor development (D’Assoro et al., 2002; Nigg, 2002).

The NDR kinase family represents a subclass of the AGC group of serine/threonine protein kinases and consists of four related kinases (NDR1/2, LATS1/2) in the mammalian genome (Hergovich et al., 2006). Existing literature indicates that LATS1/2 function as tumor suppressors, whereas mammalian NDR1/2 could function as proto-oncogenes (Hergovich et al., 2006). However, despite the detailed description of a molecular activation mechanism in vitro and in tissue culture cells (Bichsel et al., 2004; Hergovich et al., 2005; Millward et al., 1998, 1999; Stegert et al., 2005, 2004; Tamaskovic et al., 2003), we still await the definition of a molecular function of mammalian NDR kinases.
Figure 1. Mammalian NDR Kinase Localizes to Centrosomes

(A and B) U2-OS, NIH 3T3 and Cos-7 cells were processed for immunofluorescence using anti-NDR<sub>CTD</sub> (A) and anti-T444-P antibody (B). Antibody stainings are in green and nuclei are in blue. White arrowheads indicate perinuclear signals. (C) U2-OS cells were stained with anti-T444-P (green) and anti-γ-tubulin (red) antibodies. DNA is stained blue. White arrows highlight magnified areas shown in insets. Other colocalizations are indicated by white arrowheads. (D) Centrosomes extracted from U2-OS cells were separated by centrifugation in a sucrose gradient, before processing for immunoblotting using indicated antibodies. Of note, neither α-tubulin (a mostly cytoplasmic protein) nor Lamin A/C (a nuclear marker) was detected by immunoblotting in these fractions when compared to whole-cell lysates (data not shown). (E) U2-OS cells expressing indicated GFP-NDR1 constructs were fixed and stained with anti-γ-tubulin antibodies. White arrows indicate magnified areas shown in insets. Other centrosomes are highlighted by white arrowheads.
staining (Figure S3), and western blotting confirmed the co-fractionation of human NDR1 species with purified centrosomes (Figure 1D). Furthermore, irrespective of the fixation method, N-terminally GFP- or myc-tagged NDR1 and NDR2 localized to centrosomes as well as C-terminally GFP-tagged NDR1 and NDR2 (Figure S2; data not shown). The analysis of a series of deletion mutants revealed that residues 135 to 165 of NDR1 encompass the centrosome-targeting domain of NDR1 (Figure 1E). NDR kinase was also found on centrosomes of mitotic cells and at the cleavage furrow/tip of the midbody of dividing cells (Figure S4).

A detailed analysis of the distribution of NDR in interphase cells revealed that the labeling with anti-NDR antibodies was restricted to only one of the two centrioles in some cells (Figures S1 and S4). Upon serum starvation, NIH 3T3 cells grow a primary cilium specifically from the older and more mature centriole—the mother centriole (Albrecht-Buehler and Bushnell, 1980). Through visualization of this mother cilium by labeling for acetylated α-tubulin, it became apparent that endogenous NDR associated with the older centriole (Figure S4). The analysis of synchronized U2-OS cells showed that NDR associated only with the mother centrioles during G1- and S-phase, while NDR species were detected on both parental centrioles upon entry into G2-phase (Figure S4). Cells arrested in early S-phase by hydroxyurea treatment maintained the asymmetric distribution of NDR (Figure S4), indicating that cells need to proceed through S-phase to accumulate NDR molecules on both parental centrioles. Overall, our localization data show that endogenous and overexpressed NDR species associate with centrosomes and that the distribution of endogenous NDR species is changing during S/G2 transition and mitotic progression.

**Overexpression of Human NDR Kinase Leads to Centrosome Overduplication**

Considering the intriguing localization patterns of NDR kinase, we examined whether exogenous expression of NDR affects the centrosome cycle (Figure 2). We generated U2-OS cell lines expressing HA-NDR1 wild-type (WT) or kinase-dead (kd) in a tetracycline-inducible manner (Figure 2A). After addition of tetracycline, cells were processed for immunofluorescence at various time points and the number of centrosomes per mononucleated cell was determined (Figure 2B). Overexpression of NDR1(WT) but not NDR1(kd) resulted in centrosome amplification (equal to or more than three centrosomes per mononucleated cell) and centriole duplication (equal to or more than five centrioles per mononucleated cell) with increasing duration of expression (Figures 2C and 2E). Transient expression of NDR1(WT) and NDR2(WT) had a similar effect (Figure S7; data not shown), while expression of inactive NDR kinase [NDR1(kd), (S281A), (T444A), and NDR2(kd)] or LAT51(WT) and LAT52(WT) did not cause centrosome amplification, although all these proteins associated with centrosomes (Figure S5; data not shown).

To discriminate whether this generation of supernumerary centrosomes is a consequence of bona fide centriole overduplication or abortion of cell division, we analyzed NDR1-overexpressing cells with abnormal centrosome number for Cep170 staining (Figure 2F). When the supernumerary centrosomes are a result of overduplication, the majority of cells should contain only one Cep170-positive centriole, while after failure of cell division cells would harbor at least two mature centrioles and hence more than one Cep170-positive centriole (Guarguaglini et al., 2005). As is apparent from Figures 2F and 2G, the vast majority of NDR1-expressing cells with elevated centrosome number contained only one Cep170-positive centriole, indicating that NDR1 most likely causes centrosome amplification by an overduplication mechanism. To confirm this finding, we arrested U2-OS and HeLa cells in S-phase and compared the induction of centrosome amplification to that in untreated exponentially growing cells. In full support of the previous experiment, NDR1(WT) triggered centrosome amplification regardless of the presence or absence of aphidicolin in U2-OS and HeLa cells (Figures 2H and 2I). Considering that centrosomes do not overduplicate spontaneously during prolonged S-phase arrest in HeLa (Meraldi et al., 2002), these findings indicate that excessive NDR kinase activity causes bona fide centrosome overduplication.

**Targeting NDR1 to the Centrosome Is Sufficient to Trigger Centrosome Amplification**

Only a subpopulation of NDR associates with centrosomes (Figures 1 and 2 and Figures S1–S4), while the majority of NDR is cytoplasmic (Hergovich et al., 2005). To test whether centrosome-bound NDR functions in the regulation of centrosome duplication, we fused NDR1(WT) and NDR1(kd) to the PACT (pericentrin-AKAP450 centrosomal targeting) domain of AKAP450—an immobile pericentriolar matrix protein—and generated U2-OS cell lines expressing HA-AKAP-NDR1 (WT) or (kd) in a tetracycline-inducible manner (Figure 3A). Fusion proteins associated almost exclusively with centrosomes after tetracycline addition (Figure 3B). As seen in Figures 3C and 3D, centrosome-tagged NDR1(WT) induced centrosome amplification, whereas NDR1(kd) did not, indicating that the centrosomal pool of active NDR kinase is sufficient to promote centrosome duplication.

However, despite centrosome amplification, no major genomic instability and no increase in the number of multipolar anaphase spindles or multinucleated cells were observed upon NDR overexpression in U2-OS cells (data not shown). This finding was not surprising given that U2-OS cells can cluster excessive centrosomes, thereby allowing the formation of normal mitotic spindles in the presence of excessive centrosomes (Duensing, 2005)—a protective mechanism that has already been noted in other cell lines (Duensing and Munger, 2002; Quintyne et al., 2005; Ring et al., 1982). Nevertheless, these data indicate that overexpression of NDR1(WT) or NDR1(kd) did not
NDR Activity Is Required for Centriole Duplication

**A**

<table>
<thead>
<tr>
<th>tet.:</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-NDR1</td>
<td>wt</td>
<td>kd</td>
</tr>
<tr>
<td>α-HA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-NDR_CTD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-α-tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

wt

kd

α-HA

α-γ-tubulin

merge

**C**

U2-OS Tet-On

≥3 centrosomes (in %)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>wt</th>
<th>kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>48</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>72</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

**D**

α-HA

α-centrin

merge

**E**

U2-OS Tet-On

≥5 centrosomes (in %)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>wt</th>
<th>kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>48</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

**F**

α-Cep170 / α-HA

α-γ-tubulin

merge

**G**

U2-OS

≥3 centrosomes (%)

<table>
<thead>
<tr>
<th>Cep170 positive centriole(s)</th>
<th>1</th>
<th>≥2</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

**H**

U2-OS

≥3 centrosomes (in %)

<table>
<thead>
<tr>
<th>wt</th>
<th>kd</th>
<th>aphidicolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>kd</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

**I**

HeLa

≥3 centrosomes (in %)

<table>
<thead>
<tr>
<th>wt</th>
<th>kd</th>
<th>aphidicolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>kd</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
cause abortion of mitosis, which would have resulted in tetraploidization, thereby confirming our conclusion (see Figure 2) that the generation of excess centrosomes is not a consequence of failure of mitosis.

**NDR Kinase Activity Is Required for Centrosome (Over)Duplication**

Next, we sought evidence for a role of endogenous NDR kinase in centrosome duplication. To this end, U2-OS cells expressing empty vector or NDR1(kd) were arrested in S-phase for a prolonged time and then analyzed by immunofluorescence (Figure 3E). As already reported (Balczon et al., 1995; Meraldi et al., 1999) centrosome overduplication in U2-OS cells upon S-phase arrest. However, centrosome amplification was decreased upon expression of NDR1(kd) or NDR2(kd) (Figure 3F; data not shown). Transient expression of NDR1(kd) in CHO cells also negatively affected the previously reported (Balczon et al., 1995; Matsumoto et al., 1999; Meraldi et al., 1999) overduplication of centrosomes (Figure 3G). Importantly, centrosome amplification was also decreased upon expression of centrosome-tagged NDR1(kd) (Figure 3H), while overexpression of LATS1(kd) or LATS2(kd) had no effect (Figure S5). These results suggest that kinase-dead NDR negatively affects centrosome overduplication during S-phase by functioning as a dominant-negative form on centrosomes.

As a second approach, centrosome overduplication assays were performed in U2-OS cells from which endogenous NDR1 had been depleted (Figure 4). Stable cell lines were generated expressing tetracycline-regulated short hairpin RNA (shRNA) directed against two different specific sequence stretches of human NDR1 (Figure 4A and data not shown). After 3 days of tetracycline treatment, NDR1 protein levels were reduced, and incubation for 6 days resulted in nearly undetectable levels of NDR1, while NDR1 levels increased upon prolonged S-phase arrest in controls (Figure 4A). The antibody signals were also specific in immunofluorescence studies, since centrosomal stainings were diminished in siRNA-expressing cells (Figure 4B and Figure S1). Strikingly, centrosome amplification was decreased in NDR1-depleted cells upon arrest in S-phase (Figures 4B and 4C). Very similar results were obtained when other knockdown cell clones were analyzed (data not shown). To control the specificity of our RNAi experiments, NDR1(WT) and NDR1(kd) cDNAs refractory to siRNA were introduced into U2-OS cells expressing inducible vector-based RNAi (Figure 4D). The expression of siRNA-resistant NDR1(WT) allowed centrosome overduplication upon depletion of endogenous NDR1, while expression of siRNA-resistant NDR1(kd) did not restore centrosome amplification (Figures 4E and 4F). These data indicate that the failure of NDR1-depleted cells to efficiently overduplicate centrosomes is due to the knockdown of endogenous NDR1 levels and further confirm that the kinase activity of NDR is required for centrosome overduplication.

To test whether NDR1 has a role in normal centrosome duplication, we analyzed the number of centrosomes at the end of the centrosome/centriole duplication cycle (Habedanck et al., 2005). Strikingly, while only a small subpopulation of control cells had decreased centriole numbers (less than four centrioles per cell), nearly half of the NDR1-depleted cells had decreased centriole numbers (Figure 4G and 4H; control: 9.1% ± 1.6%; NDR1 knockdown: 44.2% ± 3.5%). NDR1-depleted cells also displayed an increase in cells with reduced centrosome numbers (Figure S6). These findings indicate that endogenous NDR1 contributes to proper progression through the centrosome/centriole duplication cycle in human cells. Overall, the loss-of-function strategy fully confirmed the results obtained with the overexpression of dominant-negative kinase, thereby showing that human NDR1 kinase is required for centrosome (over)duplication in our experimental systems.

**NDR-Driven Centrosome Overduplication Requires Cdk2 Activity**

To address a possible relationship between NDR and Cdk2 (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 2005)
et al., 1999; Meraldi et al., 1999) in centriole/centrosome
duplication, cells expressing NDR1(WT) with either the
Cdk2 inhibitor p27 or catalytically inactive Cdk2(DN)—
which acts as a dominant-negative mutant (Habedank
et al., 2005; Meraldi et al., 1999)—were analyzed by
immunofluorescence, revealing that inhibition of Cdk2

Figure 3. Centrosome-Bound NDR1 Affects Centrosome Duplication
(A–C) U2-OS expressing centrosome-tagged NDR1 wild-type (AKAP-WT) or kinase-dead (AKAP-kd) in a tetracycline-inducible manner were incu-
bated without (lanes 1 and 3) or with (lanes 2 and 4) tetracycline for 24 hr, before processing for immunoblotting (A) or immunofluorescence
(B and C) with indicated antibodies.
(B and C) Arrowheads indicate HA-AKAP-NDR1 species on centrosomes. Insets show enlargements of centrosomes.
(D) Histograms show the percentage of cells with excess centrosomes (≥ 3) at indicated time points.
(E) U2-OS cells expressing empty vector (top) or HA-NDR1(kd) (bottom) were processed for immunofluorescence. Insets show enlargements of cen-
trosomes. DNA is stained blue. A cell expressing NDR1(kd) is indicated by a white arrow.
(F–H) Histograms show the percentage of cells with excess centrosomes (≥ 3) in U2-OS (F and H) and CHO (G) cells. Cells expressing the indicated
NDR1 species were either incubated for 48 hr with 2 mM hydroxyurea (HU; [G]) or 72 hr with 2 μg/ml aphidicolin (F and H). Cumulative data from two
independent experiments. Error bars indicate standard deviations.
Figure 4. Human NDR1 Activity Is Required for Centrosome Duplication

(A and B) U2-OS expressing tetracycline-regulated short-hairpin RNA (shRNA) directed against human NDR1 were incubated without (lanes 1 and 3) or with (lanes 2 and 4) tetracycline for 3 days or 3 days plus 3 days with 2 μg/ml aphidicolin and tetracycline, before processing for immunoblotting (A) or immunofluorescence (B) with indicated antibodies. (B) DNA is stained blue. Arrowheads/arrows indicate the positions of centrosomes. Insets show centrosome enlargements.

(C) Quantification analysis of the experiment shown in (B).

(D and E) U2-OS stably expressing shRNA were infected with empty vector (lanes 1 and 2), HA-NDR1 wild-type (lane 3), or kinase-dead (lane 4) that is refractory to siRNA [WT (6N) or kd (6N)]. After incubation for 72 hr without (−) or with (+) tetracycline and for a further 72 hr with aphidicolin, cells were processed for immunoblotting (D) or immunofluorescence (E) with indicated antibodies.

(F) Quantification analysis of the experiment shown in (E).

(G) U2-OS cells expressing shRNA directed against NDR1 were incubated without (−) or with (+) tetracycline for 96 hr, before processing for immunofluorescence. DNA is stained blue. Insets show enlargements of centrosomes. Monopolar spindles containing two centrioles (middle panels) and bipolar spindles containing only three centrioles (bottom panels) are shown.

(H) Histograms show the percentage of mitotic cells in prophase and prometaphase that displayed the indicated numbers of centrioles with (+ tet) or without (− tet) tetracycline. Data from two experiments with at least 100 cells counted per experiment. Error bars indicate standard deviations.
suppressed NDR-induced centrosome overduplication (Figure S7). Inhibition of Cdk2 by three different small molecule inhibitors resulted in similar findings (Figure S7), while the expression and localization of NDR species was not affected (data not shown). Considering that centrosome overduplication in U2-OS cells is Cdk2 dependent (Meraldi et al., 1999), the data shown in Figure 4 suggest that Cdk2-driven centrosome overduplication is partially impaired when NDR1 activity is reduced. In support of these findings, Cdk2-driven centrosome amplification was impaired upon coexpression of dominant-negative NDR1(kd) in CHO cells, while coexpression of NDR1(WT) had no obvious effect (Figure S7). These data show that Cdk2-driven centrosome duplication can require NDR kinase activity, in addition to NDR-induced centrosome amplification being dependent on Cdk2 activity.

**DISCUSSION**

Our analysis shows that a portion of human NDR kinase is associated with centrosomes (Figure 1) and demonstrates that NDR contributes to centrosome/centriole duplication (Figures 2–4). Given that mainly human NDR1 is expressed in our experimental system, the function of endogenous NDR2 was not addressed intensively. However, overexpression of human NDR2(WT) caused centrosome amplification, while expression of NDR2(kd) negatively interfered with overduplication. Interestingly, in HCT116 cells NDR1 as well as NDR2 must be downregulated together to negatively affect centriole duplication significantly (see Figure S6); hence, it is very likely that endogenous human NDR2 also contributes to centrosome duplication. Taking into account the high degree of sequence conservation between human and mouse NDR species (Devroe et al., 2004; Stegert et al., 2004), further research addressing also the role of mouse NDR1 and NDR2 during centrosome duplication is warranted.

Strikingly, expression of NDR(WT) was sufficient to promote centrosome amplification, while expression of NDR(kd) was not (Figures 2–4), suggesting that this novel function of NDR is kinase activity dependent. Moreover, NDR immobilized on centrosomes still regulated centrosome duplication (Figure 3), indicating that NDR kinase activity localized on centrosomes is sufficient to contribute to centrosome amplification. Therefore, it is very likely that direct substrates of NDR kinase are present on centrosomes. The characterization of such substrates could result in the identification of novel factors that play a role during centrosome duplication. Possibly, NDR kinase will also display a direct relationship to proteins such as Polo-like kinase 4 (PLK4), which was previously implicated in centriole duplication (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). However, the physiological substrates of mammalian NDR kinases remain to be identified (Hergovich et al., 2006). Of note, NDR species associated with centrosomes and membrane structures simultaneously (Figure S2), suggesting that NDR substrates could also be present at plasma membranes as already proposed earlier (Hergovich et al., 2005).

Abnormal centrosome amplification occurs frequently during cellular transformation (Lingle et al., 2002; Lingle and Salisbury, 1999; Pihan et al., 1998, 2001); hence, factors involved in the regulation of centrosome duplication potentially play a role in cancer development (D’Assoro et al., 2002; Nigg, 2002). Considering further that NDR levels are upregulated in some cancer material (Hergovich et al., 2006), one is tempted to speculate that NDR kinases function as oncogenes by causing centrosome overduplication. However, in our experimental settings such a role could not be studied, since U2-OS cells can collect multiple centrosomes into two groups to form a functional bipolar spindle (Duensing, 2005). Consequently, although we report in this study that human NDR kinase plays a role during centrosome overduplication, the involvement of this mechanism in cellular transformation by altering genomic stability is yet to be established by further investigations.

Altogether, the data presented here demonstrate that human NDR kinase plays an important role during centrosome duplication, a function that might contribute to advance our understanding of how centrosome duplication is regulated on the centrosome itself. Further understanding of the mechanism(s) of the action of mammalian NDR kinases may also hold a therapeutic potential for cancer research, besides improving our knowledge of centrosome duplication at the molecular level.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, Chemicals, and FACS Analysis**

U2-OS, HeLa, Cos-7, HCT116, and CHO cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS). NIH 3T3 cells were grown in DMEM containing 5% FCS. Exponentially growing cells were plated at a consistent confluence and transfected the next day using FuGENE 6 (Roche), jetPEI (PolyPlus Transfection), or Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Aphidicolin, hydroxyurea, roscovitine, olomoucine, and Cdk2 inhibitor II were from Sigma and Calbiochem, respectively. For FACS analysis of DNA content, cells were trypsinized, fixed in 70% ice-cold ethanol, then treated with RNase A (10 μg) in propidium iodide (PI) solution (sodium citrate [pH 7.5], 69 μM PI) for 30 min at 37°C and analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

**Generation of Stable Cell Lines**

To generate inducible cell lines, U2-OS T-Rex cells were transfected with pT-Rex-DEST30 vectors encoding for various NDR forms (or pTER constructs expressing shRNA directed against NDR1). Cell clones were selected by growth in the presence of 1 mg/ml G418 (or 400 μg/ml Zeocin) and 50 μg/ml hygromycin B. Stable cell lines were maintained in DMEM supplemented with 0.5 mg/ml G418 (or 200 μg/ml Zeocin) and 50 μg/ml hygromycin B. Further details are available as Supplemental Data.

**Generation and Affinity Purification of Antibodies**

Generation of the phospho-specific antibody raised against phosphorylated Thr444 of NDR1 and anti-NDRNT antibody has been described (Hergovich et al., 2005; Tamaskovic et al., 2003). Anti-NDRCTD protein antibody was raised against the purified, bacterially produced C terminus of human NDR1 (corresponding to amino acids 301 to 465) fused with RNase A (10 μg) in propidium iodide (PI) solution (sodium citrate [pH 7.5], 69 μM PI) for 30 min at 37°C and analyzed using a FACSCalibur flow cytometer (Becton Dickinson).
C-terminally to maltose binding protein (MBP). Rabbit injections and bleed collections were done by Strategic Biosolutions. Anti-protein antibody was purified by first preabsorbing the bleed against 10 mg of immobilized MBP and then binding to 10 mg of GST-NDR1 (301–465) coupled to glutathione sepharose 4B beads (Amersham Biosciences). Antibodies were eluted with 0.2 M glycine (pH 2.5). More details on antibodies are available as Supplemental Data.

**Immunofluorescence Microscopy**

Immunofluorescence microscopy was carried out essentially as described previously (Hergovich et al., 2005). In brief, cells were either fixed in ice-cold methanol for 5 min at −20 °C or 3 % paraformaldehyde for 10 min at 37° C before being permeabilized using 0.2% Triton X-100 in PBS for 2 min at room temperature and incubated with appropriate antibodies.

**Supplemental Data**

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and eight figures and can be found with this article online at http://www.molecule.org/cgi/content/full/25/4/625/DC1/.

**ACKNOWLEDGMENTS**

Thanks to S. Munro (MRC, Cambridge, UK), B. Eddé (CNRS, Montpellier, France), H. Clevers (CBG, Utrecht, The Netherlands), and W. Krek (ETH Zurich, Switzerland) for materials. We also thank J. Liszewski and P. King for critical comments on the manuscript. This work was supported by the Novartis Research Foundation and the Swiss Cancer League.

Received: August 11, 2006

Revised: November 22, 2006

Accepted: January 12, 2007

Published: February 22, 2007

**REFERENCES**


