NUP98 Fusion Oncoproteins Promote Aneuploidy by Attenuating the Mitotic Spindle Checkpoint

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Abstract

NUP98 is a recurrent fusion partner in chromosome translocations that cause acute myelogenous leukemia. NUP98, a nucleoporin, and its interaction partner Rae1, have been implicated in the control of chromosome segregation, but their mechanistic contributions to tumorigenesis have been unclear. Here, we show that expression of NUP98 fusion oncoproteins causes mitotic spindle defects and chromosome missegregation, correlating with the capability of NUP98 fusions to cause premature securin degradation and slippage from an unsatisfied spindle assembly checkpoint (SAC). NUP98 fusions, unlike wild-type NUP98, were found to physically interact with the anaphase promoting complex/cyclosome (APC/C) and to displace the BubR1 SAC component, suggesting a possible mechanistic basis for their interference with SAC function. In addition, NUP98 oncoproteins displayed a prolonged half-life in cells. We found that NUP98 stability is controlled by a PEST sequence, absent in NUP98 oncoproteins, whose deletion reproduced the aberrant SAC-interfering activity of NUP98 oncoproteins. Together, our findings suggest that NUP98 oncoproteins predispose myeloid cells to oncogenic transformation or malignant progression by promoting whole chromosome instability. Cancer Res 74(4): 1079–90. © 2013 AACR.

Introduction

NUP98 represents one of the most "promiscuous" (1) partner genes in chromosomal translocations causing acute myelogenous leukemias (AML), as it is involved in the formation of fusion oncogenes with more than 20 different proteins, among which nine HOX family members (1, 2). Homeodomain-containing transcription factors belonging to the HOX family play crucial roles in embryonic development (reviewed in refs. 3, 4). HOX genes can participate in oncogenesis through their involvement in chromosomal translocations. These lead in some cases to the formation of fusion oncogenes, composed of the N-terminal part of the NUP98 protein, and usually the homeodomain portion of a HOX protein (1, 2, 5). NUP98 codes for a nucleoporin, which is involved in the transport of proteins and RNA across the nuclear membrane (6–9). The NUP98 protein contains two partially characterized functional domains: a GLFG repeat region, which serves as a nuclear transport receptor docking surface (8, 10), and a Gle2 binding sequence (GLEBS) domain, which mediates the interaction with the RAE1 mRNA nuclear export factor (11). Both domains are located within the N-terminal half of NUP98, which is present in essentially all fusion oncogenes (1, 5). For many chromosomal translocations causing cancer, the functional characterization of the components of the oncogenic fusion proteins proved to be crucial to understand the transformation process. NUP98-HOX chimeric proteins, for instance, as they contain a functional DNA-binding domain (DBD), the homeodomain, are held to exert their oncogenic potential via the misregulation of HOX target genes (2, 5, 12, 13). Other reports, however, have challenged this view, showing that the HOX homeodomain may be dispensable (14, 15). These results would suggest that the NUP98 moiety may play an additional role(s) in the oncogenic process, not directly related to transcriptional regulation. A mechanism(s) not exclusively involving transcriptional misregulation would moreover account for oncogenic NUP98 fusion proteins that do not involve transcription factors, yet cause related malignancies (1).

Chromosomal instability due to deregulation of mitotic checkpoint inhibition of the anaphase promoting complex/cyclosome (APC/C) function has been shown to increase the susceptibility to malignant transformation (16–18). Indeed, misregulation of APC/C components has been reported to be associated with human malignancies (19–22). The APC/C regulates mitosis and cell-cycle progression by targeting a series of key substrates for degradation during mitosis. One of these is securin (23), an anaphase inhibitor protein that blocks the action of the cohesin-degrading protease separase
(24). APC/C activity is controlled during mitosis by the spindle assembly checkpoint (SAC), which senses the correct attachment of kinetochores to spindles and blocks APC/C function, preventing sister chromatid separation until all kinetochores are properly attached (reviewed in ref. 25). Despite the established role of the Cdc20-controlled APC/C\(^{Cdc20}\) in securin degradation (26), Nup98 and its heterodimerization partner Rae1 (11) have been proposed to be implicated in APC/C\(^{Cdc20}\)-mediated securin ubiquitination during early mitosis (27). Interestingly, Nup98 shares with the SAC factors and APC/C regulators Bub1 and BubR1 a GLEBS domain, which serves in these proteins as an interaction surface with Bub3, also a regulator of mitosis sharing homology with Rae1 (28, 29).

In this work, we explored whether the expression of NUP98 fusion oncoproteins would affect the process of chromosome segregation. We found that the exogenous expression of three different NUP98 fusions causes mitotic spindle defects and chromosome missegregation, which correlate with an aberrant, untimely degradation of securin, and slippage from an unsatisfied SAC. NUP98 oncoproteins, unlike wild-type NUP98, were found to physically interact with the APC/C\(^{Cdc20}\), thus providing a mechanistic basis for the observed interference with SAC function. We show in addition that NUP98 oncoproteins have an anomalously prolonged intracellular half-life and that the stability of NUP98 depends on a PEST sequence located within the C-terminal portion that is absent in NUP98 oncoproteins. A PEST sequence deletion mutant reproduces the interference with APC/C function displayed by NUP98 oncoproteins. Our results, by establishing the involvement in APC/C misregulation of the common NUP98 N-terminal portion, point to a possible oncogenic mechanism, likely shared by all NUP98 fusions, based on chromosome instability.

Materials and Methods

**Plasmid constructs**

For details on plasmid construction see Supplementary Materials and Methods.

**Cell culture, transfection, and retroviral transduction**

HEK293 (American Type Culture Collection; #CRL-1573) human embryonic kidney cells and human primary fibroblasts (hPF) were cultured in Dulbecco’s Modified Eagle Medium (Celbio ECB750IL-50). The retroviral vector expressing NUP98-HOXD13 (LND13I) and the empty vector (LXI) were used to obtain viral stocks by transient transfection of HEK293 and primary fibroblasts, respectively. The NUP98-HOXD13 vector incorporates the homeodomain of the hematopoietic transcription factor HOXD13, which incorporates the homeodomain of the hematopoietic transcription factor HOXD13, which gives rise to a fusion protein, containing the C-terminal portion of a polypeptide of unknown function, encoded by the LOC348801 gene (33). Finally, NUP98-HHEX originates from a t(10;11)(q23;p15) translocation producing a fusion protein that incorporates the homeodomain of the hematopoietically expressed homeobox gene (HHEX; ref. 34).

**Protein knockdown with siRNAs**

siRNA duplexes targeting the coding sequence of human CDH1 and CDC20, as well as siRNA control duplexes were synthesized by Invitrogen [Stealth siRNA duplexes, 25mer and Stealth RNAi-Negative Control Duplexes (12935–400)]. Two different siRNA duplexes were designed targeting the CDC20 (C0D20HSS101650 and CDC20HSS101651), or the CDH1 (FZRIHSS122071 and FZRIHSS122072) mRNAs. siRNA transient transfection in HEK293 cells was performed using 40 pmol/L of RNAi duplexes per 6 cm wells, using lipofectamine 2000 (Invitrogen; 11668-027) following the manufacturer’s protocol. Protein depletion was detected by Western blot analysis 48 hours after siRNA transfection.

**Antibodies, coimmunoprecipitation, and immunoblotting**

For details on antibodies, coimmunoprecipitation, and immunoblotting see Supplementary Materials and Methods.

**Immunofluorescence staining and mitotic spreads**

Methods for immunofluorescence stainings and mitotic spreads are described in Supplementary Materials and Methods.

**Mitotic checkpoint release in vitro**

Nocodazole-arrested cell extracts preparation from HEK293 cells were obtained as described in (31) with the variation that cells were sonicated 10\(^0\) after lysis. For further details see Supplementary Materials and Methods.

**Results**

**Expression of NUP98-HOXD13 and of two other NUP98 fusion oncoproteins causes mitotic spindle defects and chromosome missegregation**

To verify the effect of NUP98 fusion oncoproteins on chromosome segregation, we exogenously expressed the NUP98-HOXD13, NUP98-LOC348801, and NUP98-HHEX fusions in hPF and HEK293 human embryonic kidney cells. These fusion proteins all originate from AML-associated chromosomal translocations involving the NUP98 gene, located on chromosome 11. They all contain a large portion of the N-terminal part of the NUP98 protein spanning the GLFG and GLEBS motifs (Supplementary Fig. S1A). NUP98-HOXD13 is generated by t(2;11)(q21;p15) translocations, and represents a fusion with the C-terminal homeodomain DNA-binding moiety of the HOXD13 protein (32). The NUP98-LOC348801 fusion (hereafter indicated as NUP98-LOC) is the product of a t(3;11)(q12;p15) translocation, which gives rise to a fusion protein, containing the C-terminal portion of a polypeptide of unknown function, encoded by the LOC348801 gene (33). Finally, NUP98-HHEX originates from a t(10;11)(q23;p15) translocation producing a fusion protein that incorporates the homeodomain of the hematopoietically expressed homeobox gene (HHEX; ref. 34).

We initially tested the effect of NUP98 oncoprotein expression in HEK293 cells, which are transiently transfected with high efficiency. Substantial percentages (80%–85%) of
transfected HEK293 cells were achieved with the expression constructs for the three oncoproteins (not shown). The potential occurrence of abnormal mitoses was investigated by immunofluorescence staining using anti-α-tubulin antibodies. NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX expression caused a considerable increase in the proportion of cells displaying abnormal mitoses, including multispindles and anaphases with lagging chromosomes defined as chromosomes tardily drawn to their spindle poles, which will eventually fail to be included in the reforming nuclei (Fig. 1A; and not shown), up to 24%, 30%, and 20% of total, respectively (Table 1A). Thus, this indicates that the expression of these fusion proteins interferes with the normal process of chromosome segregation. Conversely, expression of wild-type HOXD13 or wild-type NUP98 did not, or only modestly altered the frequency of abnormal mitoses observed in mock-transfected cells, respectively (Table 1A). The results obtained with the NUP98-HOXD13 chimera were also confirmed in primary cells. hPFs were transduced with a retroviral construct expressing NUP98-HOXD13 (ND13; Table 1B), or with the control empty retroviral vector. Also in hPFs, NUP98-HOXD13 expression caused a substantial increase in the percentage of abnormal mitoses (Table 1B and Fig. 1A, bottom).

We next wanted to verify whether the observed increase in abnormal mitoses would result in variations in chromosome numbers. Metaphase spreads of HEK293 cells were prepared after 48 hours from transfection. Cells expressing NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX showed substantially increased frequencies of aneuploid figures, 78%, 74%, and 70%, respectively (Table 1A; Fig. 1B, and not shown). In the same conditions, the expression of wild-type HOXD13 did not significantly alter the frequency of aneuploid cells observed in control mock-transfected cells (Table 1A). Similarly, the expression of wild-type NUP98 did only modestly affect the frequency of aneuploid metaphases (Table 1A). Also in hPFs, NUP98-HOXD13 expression caused a substantial increase in the frequency (up to 85% of expressing cells) of metaphases showing abnormal chromosome numbers (Fig. 1B and Table 1B).

As both NUP98-LOC and NUP98-HHEX were expressed as GFP fusion proteins (GFP-NLOC and GFP-NHEX in Table 1),
we also generated a GFP-NUP98-HOXD13 fusion (GFP-ND13) as a control. GFP-ND13 expression produced essentially the same frequency of aneuploid cells (75%) as NUP98-HOXD13 expression (Table 1), thus excluding a role for the GFP moiety in the observed mitosis and karyotype alterations.

To assess whether the aneuploidies caused by NUP98 fusion oncoproteins are compatible with cell viability, HEK293 cells were transduced with the NUP98-HOXD13 (pCCL-ND13) or with the empty control (pCCL) lentiviral expression vectors, and stably expressing clones were isolated and analyzed for variations in chromosome number. Of note, 50% of the isolated clones expressing NUP98-HOXD13 showed varying degrees of aneuploidy, whereas only 5% of the clones carrying the empty control vector displayed aberrant chromosome numbers (Table 1C). These data show that stable enforced expression of NUP98-HOXD13 significantly raises the frequency of aneuploid cell clones and that the induced aneuploidies can be compatible with cell viability.

We next wanted to verify whether DNA binding was essential to the capability of NUP98-HOXD13 to cause aberrant mitoses and aneuploidy. We thus generated a NUP98-HOXD13 mutant derivative, NUP98-HOXD13IQN, carrying...
mutations within the homeodomain that abolish DNA binding (35). NUP98-HOXD13IQN expression resulted in percentages of abnormal mitoses and aneuploid metaphases superimposable to those caused by NUP98-HOXD13 (Table 1A). Taken together, these results showed that the exogenous expression of three different NUP98 fusion oncoproteins causes substantial perturbation of the chromosome segregation process both in primary as well as in immortalized cells in a DNA-binding–independent manner. These data furthermore suggested a possible interference by NUP98 fusions with the APC/C-controlled activation of chromosome separation.

NUP98-HOXD13, NUP98-LOC, and NUP98-HHEX expression causes untimely securin degradation

We next investigated whether the expression of NUP98-containing fusion oncoproteins would perturb SAC function and APC/C activity. NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX were expressed in HEK293 cells (Supplementary Fig. S1B). Twenty-four hours after transfection, cells were arrested in M phase by treatment with the microtubule-depolymerizing, SAC-activating drug nocodazole. They were subsequently harvested at different time points, to analyze securin protein levels in total cell extracts, as an indication of the capability of the cells to overcome cell-cycle arrest. Immunoblot analysis of total cell extracts showed no variations in securin amounts in HEK293 cells expressing HOXD13, NUP98, or in mock-transfected cells, even 32 hours after nocodazole treatment (Fig. 2A). In cells expressing NUP98-HOXD13, NUP98-LOC, NUP98-HHEX, or NUP98-HOXD13IQN, however, a marked decrease in securin amounts was observed, starting 24 hours after nocodazole treatment (Fig. 2A). In these same cells, lower-level variations in cyclin B1 and geminin amounts were detected only 32 hours after nocodazole treatment (Fig. 2A). To confirm that the observed reductions in securin levels are due to securin degradation, cells were treated in addition with the proteasome inhibitor MG132. In the presence of MG132, no variations in securin amounts were observed in nocodazole-arrested, NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX expressing cells, demonstrating the proteasome-mediated degradation of securin (Supplementary Fig. S2).

Coherently with the reduction in securin levels, nocodazole-arrested cells expressing NUP98-HOXD13, NUP98-LOC, NUP98-HHEX, or NUP98-HOXD13IQN showed a sensible reduction of their mitotic indexes, indicating premature slippage from the unsatisfied SAC (Fig. 2B). The mitotic index of nocodazole-arrested, NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX expressing cells, demonstrating the proteasome-mediated degradation of securin (Supplementary Fig. S2).}

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**Figure 2.** NUP98 fusion oncoproteins cause untimely securin degradation. A, immunoblot analysis showing APC/C substrate levels in nocodazole-treated HEK293. Cells expressing the indicated proteins were treated with nocodazole (0.2 mg/mL) and collected at the indicated time points. Cyclin B1 (CCNB1), geminin (GMN), and securin (SEC) amounts were detected. B, mitotic index, determined using anti-phosphoSer10 antibodies and flow cytometric analysis, of control mock-transfected HEK293 cells and of HEK293 cells transiently expressing the indicated proteins. C and D, hPF, infected with LND13I3N or with the empty vector (LXI3N), were nocodazole-treated 48 hours after infection for the indicated times (C) or starved by serum deprivation, released, and analyzed at the indicated time points (D). Cyclin B1 (CCNB1) and securin (SEC) expression was analyzed by immunoblotting during mitotic block (C) or mitotic entry (D), respectively. Anti-β-actin, loading control.
nocodazole, HEK293 cells exogenously expressing NUP98 or NUP98 oncoproteins in did not show any detectable changes in their mitotic indexes, nor variations in the levels of key cell-cycle proteins (Supplementary Fig. S3B and S3C), indicating that NUP98 oncogene expression per se does not cause an earlier or delayed entry in mitosis.

We then wanted to analyze the effect of NUP98 oncoprotein expression on securin accumulation during the cell cycle in hPFs. NUP98-HOXD13 was exogenously expressed via retroviral gene delivery (LND13; ref. 36), and cells were synchronized alternatively by nocodazole treatment or by serum starvation. Nocodazole-treated NUP98-HOXD13 expressing hPFs (LND13 in Fig. 2C) displayed a substantial decrease in securin amounts, starting 16 hours after nocodazole treatment. No decrease in cyclin B1 amounts were detected in these same cells (Fig. 2C). hPFs were also synchronized without resorting to a drug-mediated activation of the SAC by serum starvation, subsequently released, and analyzed at different time points for variations of securin and cyclin B1 amounts. Unlike hPFs transduced with the control, empty retroviral vector (LXIN), which showed normal cyclin B1 and securin accumulation starting 28 hours after release from starvation, hPFs expressing NUP98-HOXD13, during the same time frame, displayed constant, low levels of cyclin B1, and particularly of securin (Fig. 2D). These results indicate that in hPFs NUP98-HOXD13 interferes with the control of APC/C activity during the cell cycle, altogether preventing physiologic securin and cyclin B1 accumulation. Taken together, our data both in primary, as well as in immortalized cells, show that the exogenous expression of NUP98 fusion oncoproteins causes an attenuation of the mitotic spindle checkpoint, revealed by the aberrant, untimely degradation of securin, pointing to their possible interference with APC/C function.

**NUP98-HOXD13, NUP98-LOC, and NUP98-HHEX coimmunoprecipitate with both the Cdh1 and the Cdc20 APC/C components**

Nup98 was shown to be part, together with its interaction partner Rae1 (37), of the APC/C during mitosis (27); we, therefore, wanted to verify whether NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX were capable of physically interacting with Rae1 and with key components of the APC/C, thus justifying its deregulation. HEK293 cells expressing HOXD13, NUP98, NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX were arrested in mitosis by nocodazole treatment, and total extracts were prepared for immunoprecipitation with specific antibodies against the Rae1, securin, CDH1, and CDC20 proteins. Control HOXD13, as anticipated, did not interact with any of the tested APC/C components (not shown). NUP98 instead was found to coimmunoprecipitate with the CDH1, but not with the CDC20 APC/C activators (Fig. 3). Remarkably, NUP98-HOXD13, NUP98-LOC, and NUP98-HHEX all proved to efficiently coimmunoprecipitate with the RAE1, securin, CDH1, and CDC20 proteins (Fig. 3). Thus, the tested oncoproteins are not only still capable of interacting with RAE1 and with the APC/C, but are in addition aberrantly part of the APC/C, providing a possible mechanistic basis for the observed untimely securin degradation and exit from mitosis in the presence of an unsatisfied SAC.

NUP98-HOXD13, NUP98-LOC, and NUP98-HHEX interfere with APC/C function to induce premature securin degradation

Wild-type NUP98 has been implicated in the control of APC/C function, and NUP98-controlled APC/C activity was claimed to be responsible for securin degradation during metaphase to anaphase transition (27). As NUP98 fusion oncoproteins were found to interact with both APC/C and APC/C, we wanted to verify whether the untimely securin degradation was imputable to misregulation of APC/C, or both. To this end, we used specific siRNAs to knockdown Cdh1 or Cdc20 in HEK293 cells (Supplementary Fig. S4A). Treatment of control or NUP98 oncoprotein–expressing HEK293 cells with siRNAs did not have a detectable effect on the cell cycle in the presence or absence of nocodazole (Supplementary Fig. S4B). To assess the effect of NUP98 oncoproteins on APC/C activity, we used a cell-free system that recapitulates mitotic checkpoint events (31). Extracts prepared from nocodazole-arrested HEK293 cells, exogenously expressing NUP98, NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX, were allowed to be relieved from mitotic block via incubation at 30°C (31). Although degradation of securin was detectable only after 90 minutes in extracts from cells expressing NUP98, or in control untransfected cells (Fig. 4A), in extracts from HEK293 cells expressing NUP98-HOXD13, NUP98-LOC, or NUP98-HHEX securin degradation started already after 30 to 60 minutes (Fig. 4A), confirming also in this experimental setting the earlier aberrant degradation of securin caused by NUP98 fusion oncoproteins. Knocking down of Cdh1 (Fig. 4D), or the use of a control siRNA (Fig. 4B), had no effect on NUP98 oncoprotein-induced premature securin degradation, conversely, knocking down of Cdc20...
completely abolished it, even at 120 minutes after mitotic block relief (Fig. 4C). These results were also confirmed analyzing securin degradation, after Cdh1 or Cdc20 siRNA-mediated knockdown, in intact HEK293 cells (Supplementary Fig. S5).

Our results indicate that, despite the physical interaction by NUP98 oncoproteins with both APC/C<sup>Cdc20</sup> and APC/C<sup>Cdh1</sup>, the induced untimely degradation of securin can be ascribed uniquely to an aberrant interference with the activity of APC/C<sup>Cdc20</sup>.

The C-terminal portion of NUP98 contains a functional PEST sequence

In an attempt to identify possible regulatory domains within the missing C-terminal region of NUP98 that would account for the different functional properties of NUP98 oncoproteins, we analyzed the NUP98 protein sequence in silico searching for novel putative functional motifs. Using the epestfind application of the EMBOSS package (38), we identified a conserved, putative PEST destruction sequence, localized between amino acids 599 and 616 of the NUP98 protein (Fig. 5A). We thus generated a deletion mutant of NUP98 lacking this region (NUP98<sub>D</sub>P; Fig. 5B), and tested its stability within HEK293 cells in comparison with that of wild-type NUP98 and of NUP98 fusions. HEK293 cells expressing NUP98-HOXD13, NUP98-LOC, NUP98-HHEX, NUP98, or HOXD13 were treated with cycloheximide to block protein synthesis, and the amounts of the expressed proteins were monitored at different time points by immunoblotting.
A NUP98 PEST sequence deletion mutant interacts aberrantly with the APC/C\(^{C_{dc20}}\) and causes premature securin degradation and chromosome missegregation

As a deletion of the PEST sequence within NUP98 (NUP98\(\Delta\)P) was sufficient to mimic the protein half-life of NUP98 oncoproteins, we next verified whether the NUP98\(\Delta\)P mutant would recapitulate other aberrant functional properties of NUP98 oncoproteins. We thus tested whether the NUP98\(\Delta\)P mutant would induce premature securin degradation and cause chromosome missegregation in HEK293 cells. Twenty-four hours after transfection with the expression constructs for the NUP98\(\Delta\)P mutant, cells were nocodazole arrested and harvested at different time points. Immunoblot analysis of total cell extracts showed a significant decrease in securin amounts starting 24 hours after nocodazole treatment in cells expressing NUP98\(\Delta\)P (Fig. 6A). In parallel, we verified whether NUP98\(\Delta\)P expression would result in variations in chromosome numbers and in an increase of abnormal mitoses. Metaphase spreads of HEK293 cells were prepared after 48 hours from transfection. Cells expressing NUP98\(\Delta\)P showed substantially increased percentages of aneuploid figures and abnormal mitoses, 71% and 34%, respectively (Fig. 6B). We then verified whether NUP98\(\Delta\)P would aberrantly interact with APC/C components. HEK293 cells expressing NUP98\(\Delta\)P were nocodazole arrested in mitosis, and total extracts were prepared for immunoprecipitation with specific antibodies against the RAE1, securin, CDH1, and CDC20 proteins. NUP98\(\Delta\)P efficiently communoprecipitated with RAE1, securin, CDH1, and in addition, similarly to NUP98 oncoproteins, with CDC20 (Fig. 6C). These results show that a deletion of the conserved PEST sequence within NUP98 not only causes a lengthening of the NUP98 half-life but also seems to be sufficient to recapitulate at least the aberrant functions of NUP98 oncoproteins concerning SAC perturbation.

NUP98 oncoproteins or NUP98\(\Delta\)P expression reduce the amount of BubR1 bound to APC/C\(^{C_{dc20}}\)

Given the presence of a shared structural motif between NUP98 and the APC/C\(^{C_{dc20}}\) regulator BubR1 (both display a functional GLEBS domain; ref. 28), we next wanted to verify whether the aberrant association of NUP98 oncoproteins and NUP98\(\Delta\)P with APC/C\(^{C_{dc20}}\) would result in the premature displacement of BubR1, thus justifying untimely activation of APC/C\(^{C_{dc20}}\) and mitotic slippage.

Using the cell-free system recapitulating mitotic checkpoint events described above (31), we analyzed by immunoprecipitation at different time points extracts from nocodazole-arrested HEK293 cells, exogenously expressing NUP98, NUP98-HOXD13, NUP98-LOC, NUP98-HHEX, or NUP98\(\Delta\)P, allowed to be relieved from the block by incubation. At the onset of incubation (time 0), immunoprecipitation using anti-CDC20 antibodies followed by immunoblotting using anti-NUP98 antibodies (Fig. 6D, left), confirmed the physical interaction of NUP98 oncoproteins and of NUP98\(\Delta\)P, but not of exogenous NUP98 protein (NUP98\(\beta\)), with APC/C\(^{C_{dc20}}\). The amount of BubR1 bound to the APC/C\(^{C_{dc20}}\) at incubation time 0, as revealed by immunoblotting using...
anti-BubR1 antibodies (Fig. 6D, right), was found to be consistently lower in extracts from cells expressing NUP98 oncoproteins or NUP98ΔP with respect to cells expressing NUP98 (Fig. 6D, bottom). After 60 minutes of incubation, whereas only a moderate decrease in NUP98 oncoprotein and NUP98ΔP binding to APC/C^Cdc20 was observed (Fig. 6D, left), BubR1 showed a significant reduction of the amount associated with APC/C^Cdc20 in all samples (Fig. 6D, bottom), in correlation with the onset of full APC/C^Cdc20 activation and substantial securin degradation at this time point (cf. Fig. 4A). These results suggest that the association of NUP98 oncoproteins or NUP98ΔP with APC/C^Cdc20 interferes at least partly with the binding of BubR1, thus likely causing an attenuation of the inhibitory action of BubR1 on APC/C^Cdc20, and consequently slippage from the unsatisfied SAC.
**Discussion**

*NUP98* is one of the most recurrent partner genes in chromosomal translocations involved in AML (1, 2). *NUP98* fusion partners often include a variety of transcription factors, mostly represented by their DBDs. Thus, the majority of the current models, proposed to explain the leukemogenic action of *NUP98* fusion oncoproteins, are centered on transcriptional misregulation (5). *NUP98* fusions, however, also involve proteins not, or not directly implicated with transcriptional control, yet they all cause related hematologic malignancies (reviewed in refs. 1, 5). This leaves the possibility open for additional oncogenic mechanisms, based on a functional domain(s) shared by most, if not all, *NUP98* fusions. The *NUP98* portion, encompassing the N-terminal half of the protein, and containing both a GLEBS domain and FG/GLFG repeats, represents a good candidate for such a functional domain, as it is present in essentially all *NUP98* fusion oncoproteins (1). The capability of *NUP98* to interact via its GLEBS domain with RAE1 and with the APC/C, and its proposed role in the control of chromosome segregation during early mitosis (27), provided an indication as to a possible shared mechanism through which *NUP98* oncoproteins interfere with normal cell division. Indeed, several *NUP98* fusions, including NUP98HOXAC9, NUP98PMX1, and NUP98HOXD13, have been reported to be associated to kinetochores during mitosis, and to display a localization pattern similar to that of APC/C components (39, 40).

Here, we show that three different *NUP98* fusion oncoproteins, two containing DBDs of known homeodomain transcriptional factors, and one containing a polypeptide of unknown function, likely unrelated to transcriptional regulation, if exogenously expressed, substantially perturb chromosome segregation, causing spindle defects and aneuploidy both in primary, as well as in immortalized cells. These results point to a possible interference with APC/C-controlled chromosome separation. We indeed found in cells exogenously expressing *NUP98* fusions, premature exit from mitosis in the presence of an unsatisfied SAC and aberrant securin degradation. In accordance, we found that in hPF, in the absence of drug-mediated SAC activation, *NUP98* oncoprotein expression prevents normal securin accumulation during cell-cycle progression.

SAC attenuation and the consequent chromosome missegregation caused by *NUP98* oncoproteins rest on their anomalous interaction with APC/C<sub>Cdc20</sub>. The structural basis for this aberrant interaction is unclear and will require further investigation. Intriguingly though, we show that the deletion of a PEST sequence (41), which we identified within the C-terminal portion of the *NUP98* protein that is deleted in all known *NUP98* fusion oncoproteins (1), not only has an effect on the overall stability of *NUP98*, but causes the resulting mutant protein (*NUP98ΔP*) to acquire at least some of the aberrant functions of *NUP98* oncoproteins, as *NUP98ΔP* aberrantly interacts with APC/C<sub>Cdc20</sub>, interferes with SAC function, and causes chromosome missegregation. Deletion of the *NUP98* C-terminal portion that includes the PEST sequence, resulting from the formation of *NUP98* oncoproteins, or internal deletion of the PEST sequence, as in *NUP98ΔP*, both lead to an anomalous physical interaction with APC/C<sub>Cdc20</sub>, suggesting that the *NUP98* N-terminal portion undergoes similar structural alterations in both conditions. PEST sequences, especially conditional ones, are held to have an influence on the local or overall structure of the proteins possessing them (41). It is thus tempting to speculate that removal of the PEST sequence triggers conformational changes within the *NUP98* N-terminal portion such as to alter the selectivity of its physical interactions.

As to the means of aberrant APC/C<sub>Cdc20</sub> activation, our results showing a sensible reduction of the BubR1 APC/C regulator upon *NUP98* oncoprotein and *NUP98ΔP* expression point to a mechanism based on direct competition for APC/C<sub>Cdc20</sub> binding. Direct competition that could rest on the structural similarity between the *NUP98* N-terminal portion and BubR1, as both share a functional GLEBS domain (28). Further work, though, will be required to clarify the detailed mechanism of BubR1 displacement via *NUP98* oncoprotein binding to APC/C<sub>Cdc20</sub>.

An additional aberrant functional property of the tested *NUP98* fusion oncoproteins revealed to be their substantially increased intracellular stability. Both the increased intracellular stability and the anomalous interaction of *NUP98* fusion oncoproteins with the APC/C<sub>Cdc20</sub> represent dominant functions with respect to wild-type *NUP98*, which could combine to account for the dominant oncogenic action of *NUP98* fusions in hematologic malignancies (1, 2, 5).

Whole chromosome instability and the ensuing aneuploidy have been since long held to promote tumorigenesis, even if their exact role in malignant transformation has remained elusive (reviewed in ref. 18). Mutant mice models representing gene defects that cause chromosome instability have been shown to be prone to tumorigenesis, indicating that aneuploidy indeed predisposes to oncogenic transformation (18). Aneuploidy is only rarely observed in clinical samples from leukemia patients carrying *NUP98* fusion oncoproteins. We believe that the dramatically aneuploid patterns, observed after 48 hours of *NUP98* fusion oncoprotein expression, represent an initial effect of *NUP98* fusions within cells. This initial genomic instability is most likely subsequently overcome by surviving cells via yet to be defined compensatory mechanisms, which would allow cells to continue proliferating despite the interference of *NUP98* fusions with APC/C function. This notion is indeed sustained by our analysis on single-cell clones stably expressing NUP98-HOXD13, which shows that viable, NUP98HOXD13-expressing clones, displaying varying degrees of aneuploidy, or no obvious chromosomal alterations, can be readily obtained. Chromosome instability and aneuploidy promoted by *NUP98* oncoproteins could therefore increase susceptibility to tumor formation, for instance, by impairing cellular tumor suppressing functions, thus promoting the occurrence of additional mutations (see e.g. refs. 42, 43), such as those reported by Taketani and colleagues (44), and/or by cooperating with...
additional mechanisms implemented by the individual NUP98 translocation partner proteins. Accordingly, the capability of NUP98 oncoproteins to interfere with the SAC is not necessarily to be considered mutually exclusive with other possible, transcriptional or nontranscriptional, oncogenic mechanisms, deployed by the large variety of NUP98 fusions (1). In fact, the oncogenic potential of NUP98 oncoproteins is likely to rest on more than one, coexisting parallel mechanisms (reviewed in refs. 1, 2, 5). It is, however, conceivable that a particular NUP98 fusion may display the prevalence of a partner-specific, for example, transcriptionally based mechanism, as seems to be the case for the NUP98-HOXA9 fusion (45).

In conclusion, our results showing the involvement of NUP98 fusion oncoproteins in APC/C\(^{Cdc20}\) misregulation point to a novel mechanism of action shared potentially by all NUP98 fusions, which could act in combination with transcriptional misregulation. Moreover, it could account for the ever-increasing number of NUP98 oncogenic fusions that cause related hematologic malignancies while involving partner proteins with diverse biochemical functions.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**References**

NUP98 Fusion Oncoproteins Promote Aneuploidy by Attenuating the Mitotic Spindle Checkpoint

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