A mutation linked to retinitis pigmentosa in HPRP31 causes protein instability and impairs its interactions with spliceosomal snRNPs

Martina Huranová†, Jarmila Hnilicová†, Branislav Fleischer, Zuzana Cvačková and David Staněk*

Department of RNA Biology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic

Received January 2, 2009; Revised February 27, 2009; Accepted March 13, 2009

The AD29 mutation in HPRP31 belongs to a series of mutations that were initially linked with the autosomal dominant disorder retinitis pigmentosa (RP) type 11. The HPRP31 gene encodes the hPrp31 protein that specifically associates with spliceosomal small nuclear ribonucleoprotein particles (snRNPs). Despite intensive research, it is still unclear how the AD29 (Ala216Pro) mutation causes RP. In this study, we report that the expression of this mutant protein affects cell proliferation and alters the structure of nuclear Cajal bodies that are connected with snRNP metabolism. Interestingly, these effects can be reversed by the over-expression of the hPrp6 protein, a binding partner of hPrp31. Although Ala216 is not contained within the U4 or U5 snRNP interacting domains, we present several lines of evidence that demonstrate that the association between the AD29 mutant and snRNPs in the cell nucleus is significantly reduced. Finally, we show that the stability of the AD29 mutant is severely affected resulting in its rapid degradation. Taken together, our results indicate that the Ala216Pro mutation destabilizes the hPrp31 protein structure in turn reducing its interaction with snRNP binding partners and leading to its rapid degradation. These findings significantly impact our understanding of the molecular mechanisms underlying RP and suggest that the insufficiency of the functional hPrp31 protein combined with the potential cytotoxicity associated with the expression the AD29 mutant are at least partially causative of the RP phenotype.

INTRODUCTION

Retinitis pigmentosa (RP) is the common name used to describe a group of inherited diseases characterized by the gradual degeneration of retina cells that lead to night blindness and visual field loss. Mutations within a variety of genes have been connected with RP, most of which are specifically expressed in photoreceptor cells. Surprisingly, mutations in three genes directly involved in pre-mRNA splicing have also been linked to autosomal dominant forms of RP. Products of these genes are well-characterized components of the spliceosomal small nuclear ribonucleoproteins (snRNPs) (1–3; reviewed in 4). Of these proteins, hPrp8 is a key protein of the U5 snRNP that lies within the catalytic center of the spliceosome (5,6). hPrp3 and hPrp31 (also known as 61K) are components of the U4/U6 snRNP involved in the formation and stability of U4/U6 and U4/U6•U5 tri-snRNPs (7). In addition, a mutation in a tentative splicing factor PAP-1 has also been linked to RP (8).

Initially, the hPrp31 protein directly binds both the U4 snRNA and the U4 snRNP protein 15.5K to create the core of the U4 snRNP (9,10). This interaction is followed by U4 and U6 snRNAs annealing to form the U4/U6 snRNP. Finally, the key interaction for the formation of the U4/U6•U5 tri-snRNP is the interaction of hPrp31 with the U5 snRNP-specific protein hPrp6 (11). During the splicing reaction, the tri-snRNP undergoes dramatic rearrangements that result in disintegration of the tri-snRNP into individual snRNP components. In a process called the spliceosomal cycle, tri-snRNPs are re-assembled in a step-wise manner similar to de novo formation described above (7). U4/U6 and U4/U6•U5 snRNP formation...
and recycling occur primarily in the nuclear inclusion called the Cajal body that accelerates the snRNP assembly reaction (12–16; reviewed in 17). The Cajal body is a dynamic structure whose integrity depends on transcription/splicing activity as well as snRNP biogenesis (14,16,18–20).

It is largely unknown why mutations in snRNP-specific proteins cause RP symptoms. It was reported that the expression of hPrp31 mutants in retina cells negatively influences splicing of retina-specific genes (21,22). However, most mutations in the HPRP31 gene result in the destabilization of hPrp31 mRNA that leads to a decrease in the synthesis of functional protein (23). Moreover, RP-affected families with large deletions of the HPRP31 gene have been identified (24,25). Together, these observations suggest a haploinsufficiency model that predicts that there is a critical concentration of snRNP protein necessary to support retina cell survival (23,26,27). Therefore, the expression from only one allele may not be sufficient to achieve this critical level of expression in retina cells. This model, however, does not apply to all snRNP mutations, as depletion of one HPRP3 allele does not trigger RP in mouse or zebrafish (28). In addition, it was shown that RP-associated mutations in yeast Prp8 and hPrp3 influence snRNP assembly (29,30) and thus might trigger the death program in sensitive cells.

In this study, we have concentrated on the hPrp31 point mutant, AD29, which contains an Ala216Pro substitution that does not apparently trigger a nonsense-mediated decay. It was recently shown that the expression of the AD29 mutant, while affecting the splicing of mini-gene reporter plasmids, had no apparent negative effect on cell growth and snRNP assembly. Further, we have used co-immunoprecipitation, glycerol gradient ultracentrifugation and Förster resonance energy transfer (FRET) to map the interaction of this mutant protein with other snRNP proteins. Finally, we have probed the stability of the mutant protein and tested whether overexpression of hPrp31s major interacting partner in the tri-snRNP, hPrp6, can reverse phenotypes generated by AD29 expression.

RESULTS

Cells expressing the AD29 mutant exhibit slower proliferation

It has been shown that RP linked mutations in the splicing factor Prp8 cause disruption of U5 snRNP biogenesis in yeast (30). In a human cell line, a mutation in hPrp3 has been shown to have a negative effect on snRNP assembly (29). In this study, we decided to analyze the effects of expression of an hPrp31 mutant on cell growth and snRNP assembly in human cells. To achieve this, we established two stable cell lines that expressed either YFP tagged wild-type hPrp31 (WT31 cells) or mutant AD29 tagged with YFP (AD29 cells) (Fig. 1). Similar to endogenous protein (data not shown; 12,16), the wild-type hPrp31-YFP (WT31-YFP) localized to the cell nucleus and accumulated in Cajal bodies and splicing factor compartments (Fig. 1A). The localization pattern of the AD29-YFP mutant (AD29-YFP) was more diverse (Fig. 1B). In most cells analyzed, AD29-YFP localized to the cell nucleus and the cytoplasm; however, cells displaying a mostly cytoplasmic or nuclear accumulation were also presented. In comparison with the localization of WT31-YFP, AD29-YFP exhibited a more diffuse nuclear staining pattern and weak accumulation in Cajal bodies. A similar localization pattern has previously been observed in cells transiently expressing an AD29-GFP mutant (32). Of note, nucleolar accumulation was observed in cells highly expressing either of the YFP tagged proteins.

In order to test the effects of AD29 mutant expression on cell proliferation, AD29 cells were observed for 24 h and their growth compared with WT31 cells (Fig. 1C). Cells expressing the mutant protein divided 10% slower than cells expressing the wild-type protein. As both stable cell lines expressed the same amounts of endogenous hPrp31 and the expression of WT31-YFP and AD29-YFP was comparable (see below Fig. 3), these data indicate that the AD29 expression has a dominant negative effect on cell proliferation.

During live-cell imaging, we noticed that the localization of AD29-YFP changes during cell cycle. At early G1 phase, AD29-YFP was found mainly in the cytoplasm and only gradually accumulated in the nucleus during interphase. This observation explains the diverse localization of AD29 in an unsynchronized cell population. In contrast, WT31-YFP accumulated in the cell nucleus as soon as the nuclear membrane was formed after mitosis.

The AD29 mutant disrupts formation of Cajal bodies

As described above, our data indicate that the expression of AD29-YFP has a negative effect on cell growth (Fig. 1C). Given that hPrp31 is necessary for tri-snRNP formation, we decided to investigate snRNP metabolism in cells expressing the mutant protein. First, we analyzed cells for the presence of Cajal bodies because these bodies facilitate certain steps in tri-snRNP assembly (17) and their integrity depends on ongoing snRNP biogenesis and/or splicing (18,19). Staining cells with the Cajal body marker coilin revealed that the expression of AD29-YFP altered the structure of Cajal bodies (Fig. 2). Further, a significant fraction of AD29-YFP expressing cells did not have apparent Cajal bodies (Fig. 2C), and if Cajal bodies were present, they were often smaller than in WT31 cells. In the population of AD29 cells that did contained Cajal bodies, we analyzed the localization of snRNP-specific proteins within Cajal bodies. Interestingly, we observed in AD29 cells that accumulation of SART3, a marker of U4/U6 snRNP is reduced while the localization of the U5-specific protein hSnu114 and the U2-specific U2B* were not significantly altered (Fig. 2D). Cajal body integrity is sensitive to snRNP metabolism and splicing. Therefore our data indicate that the expression of the AD29 mutant impacts upon snRNP metabolism and/or splicing.

Interaction between AD29 and snRNPs is significantly reduced

To analyze AD29 association with snRNP-specific proteins, we first employed the technique of FRET to detect protein complexes in situ. Either AD29-CFP or WT31-CFP was co-expressed with the YFP-tagged snRNP-specific protein
SART3, hPrp4 or hPrp6 (Fig. 3A) and FRET was measured by acceptor photobleaching (Fig. 3B). We analyzed these snRNP proteins because they mark different stages of tri-snRNP formation: SART3 is specifically found in the U4/U6 snRNP intermediate, hPrp4 is a component of the U4/U6 snRNP and the tri-snRNP while hPrp6 is the U5-specific protein that interacts with hPrp31 to facilitate U4/U6\textsuperscript{†}U5 tri-snRNP assembly. Cells co-expressing WT31-CFP and SART3-YFP exhibited a high FRET signal specifically within Cajal bodies that is consistent with previously published data that demonstrated that the U4/U6 snRNP is highly concentrated in Cajal bodies (13). A high FRET signal between hPrp6-YFP and WT31-CFP in the nucleoplasm indicates that there is a higher concentration of tri-snRNPs in the nucleoplasm than in Cajal bodies. Finally, the FRET signal between WT31-CFP and hPrp4-YFP was similar in the Cajal body and the nucleoplasm reflecting the localization of both U4/U6 and U4/U6\textsuperscript{†}U5 snRNPs. Notably, the association between the AD29 mutant and U4/U6 snRNP proteins SART3 and hPrp4 (as measured by FRET) was reduced by approximately 40–50%. In contrast, the interaction between AD29 and hPrp6 was lowered only in the nucleoplasm, while this interaction in Cajal bodies was similar to wild-type hPrp31. These data indicate that the mutant protein is still able to partially integrate into snRNPs but its association with snRNPs is compromised.

To further test the interaction between AD29 and snRNPs, immunoprecipitation assays were carried out on WT31 or AD29 cells. As a negative control, the parental cell line was used. WT31 and AD29 cell lines expressed a similar amount of YFP-tagged proteins and their levels were lower than the level of endogenous hPrp31 (Fig. 3C). Co-precipitated proteins were analyzed by western blotting. Both hPrp3 and hPrp4 (proteins found in U4/U6 and U4/U6\textsuperscript{†}U5 snRNPs) along with hSnu114 and hPrp6 (U5 and U4/U6\textsuperscript{†}U5 snRNP-specific proteins) were co-precipitated with WT31-YFP illustrating that WT31-YFP is properly incorporated into U4/U6 and U4/U6\textsuperscript{†}U5 snRNPs. In contrast, AD29-YFP did not pull-down...
any of the tested U4/U6-specific proteins signifying that the single-point mutation is sufficient to disrupt the interaction between AD29 and the U4/U6 snRNP. However, AD29 precipitated with its U5-specific binding partner hPrp6. This finding extends the in vitro binding studies (9,31) and shows in human cells that AD29 interacts with hPrp6. Of interest, we repeatedly detected limited amounts of hSnu114 pulled down by AD29-YFP suggesting that under the immunoprecipitation assay conditions, the AD29 mutant might still be capable of interaction with the U5 snRNP.

To confirm that the AD29 mutant is impaired in its ability to incorporate into snRNPs, we prepared nuclear extracts from AD29 or WT31 stable cell lines. These extracts were subjected to glycerol gradient fractionation and the presence of AD29-YFP or WT31-YFP in individual fractions analyzed (Fig. 3D). Consistent with our previous findings, the AD29 mutant was mainly restricted to the top of the gradient and its sedimentation behavior was similar to bovine serum albumin that has a comparable molecular weight (data not shown). These data further demonstrate that the mutant is not a stable component of snRNP complexes.

Over-expression of hPrp6 is able to rescue some defects in AD29 expressing cells

Although the interaction between AD29 and snRNPs is dramatically reduced, this mutant is still able to instigate a dominant negative effect on both cell growth and Cajal body formation (Figs 1 and 2). It has been shown that AD29 binds strongly to hPrp6 in vitro (31) and our data show that AD29 is able to interact with hPrp6 in vivo (Fig. 3). Thus, it is plausible that AD29 might sequester free U5 snRNP or hPrp6 and that in turn changes the dynamics of tri-snRNP formation. To test this hypothesis, we expressed hPrp6-CFP in AD29 cells and measured the appearance of Cajal bodies (Fig. 4A–C). Expression of hPrp6-CFP but not CFP alone increased the number of cells that contained Cajal bodies to a level comparable to parental HeLa or WT31 cells. This result indicates that the negative effect AD29 has on Cajal body formation can be overcome by the expression of hPrp6.

To further test whether hPrp6 can also rescue the AD29-induced growth phenotype, AD29 cells were transfected with hPrp6-CFP and their proliferation measured as described previously (Fig. 4D). Neighboring AD29 cells that did not express hPrp6-CFP served as an internal negative control. Although the transfection procedure had a general negative effect on cell proliferation (compare Fig. 4D with Fig. 1C), AD29 cells expressing hPrp6-CFP divided faster than their non-transfected neighbors.

The AD29 mutation destabilizes the hPrp31 protein

During live-cell observations, we noticed that the amount of AD29-YFP in individual cells changed significantly during
the 24 h observation period. The observed changes in AD29-YFP accumulation were not connected with any particular cell cycle phase and instead exhibited a rather stochastic behavior indicating that this effect might be a reflection on the dynamics of AD29 expression and degradation. To test the stability of the AD29-YFP protein, cells were treated with the translation inhibitor cycloheximide and YFP fluorescence monitored for 5 h (Fig. 5). While the WT31-YFP fluorescence decreased only marginally, the level of AD29-YFP fluorescence dropped rapidly after only 1 to 2 h incubation in cycloheximide (Fig. 5A–C). The fluorescent signal decreased both in the nucleus and the cytoplasm and no preferential degradation was observed in either of these cell compartments. Similar results were observed when the degradation of the AD29 mutant was analyzed by western blotting (Fig. 5D). While endogenous hPrp31 and WT31-YFP were stably present in cells during the 5 h treatment, AD29-YFP protein disappeared within a couple of hours. Together, these data suggest that the Ala216Pro substitution causes a conformational change that negatively affects the stability of the protein.

**DISCUSSION**

RP is a heritable disease that affects 1 in 4000 people. So far, there have been over 40 loci identified that carry mutations that cause RP. Generally, most of these genes are directly involved in controlling retina cell metabolism. However,
three genes that encode general RNA splicing factors that are ubiquitously expressed have also been implicated in RP. In addition, the gene encoding the putative splicing factor PAP1 is also mutated in RP (4). The mechanism underlying how mutations in splicing factors trigger RP has been extensively investigated during the last couple of years. Such studies has shown that mutations in HPRP3 and HPRP8 can influence snRNP assembly and that many mutations in HPRP31 lead to the degradation of its mRNA by the nonsense-mediated decay pathway, consequently lowering the expression of functional hPrp31 protein.

Here we investigated the hPrp31 point mutation found in the AD29 family that does not apparently activate the mRNA degradation pathway but instead codes for an alanine to proline substitution at residue 216. It has been shown in vitro and by two-hybrid analysis that this amino acid change does not interfere with the hPrp31–hPrp6 interaction (9,31). In addition, a structural analysis of the U4snRNA/15.5K/hPrp31 complex revealed that the Ala216 residue is not directly involved in the interaction between hPrp31 and U4 snRNA or 15.5K; however, the effect of the AD29 mutation on the hPrp31 and U4 snRNP interaction has never been tested (9). Here we have demonstrated that the AD29 mutation dramatically alters the ability of hPrp31 to interact with U4/U6 snRNPs. Analysis of the association of AD29

and U4/U6 snRNPs in situ by FRET revealed that this association was reduced by half. Further, the AD29 mutant precipitated the U5 snRNP, specifically the hPrp6 and hSnu114 proteins but not any of the tested U4/U6 markers. Finally, the AD29 mutant was mainly concentrated at the top of a glycerol gradient indicating that it is not a stable component of snRNPs. These data together indicate that the mutant has a limited ability to associate with U4/U6 snRNPs. In addition, the interaction between AD29 and snRNPs is weak and does not withstand the conditions during snRNP immunoprecipitation and gradient centrifugation. As the mutant protein is partially localized in the cytoplasm, one explanation for reduced interaction with snRNPs could be a defect in nuclear import. However, the Ala216Pro substitution does not affect the nuclear localization signal or interaction with importin b1 and direct measurements of nuclear import did not reveal any differences between wild-type and mutant proteins (32,33). Therefore, we assume that reduced nuclear localization is rather an effect than a cause of reduced AD29 binding to snRNPs.

To date, it is unclear whether the AD29 mutant can bind U4/U6 and U5 snRNPs simultaneously and form the tri-snRNP or whether the mutant binds U4/U6 and U5 snRNPs independently and prevents tri-snRNP formation. Based on the fact that AD29 interacts with hPrp6 in vivo as assayed by both
FRET and immunoprecipitation (Fig. 3) and that overexpression of hPrp6 is able to rescue the AD29 phenotype, we speculate that free AD29 transiently binds via hPrp6 to the U5 snRNP. This interaction prevents the proper association with the U4/U6 snRNP and changes the dynamics of tri-snRNP formation resulting in the observed phenotypes: reduced cellular proliferation (Fig. 1) and disruption of Cajal body formation (Fig. 2). Both phenotypes are likely to be dominant as we did not observe any changes in the expression of endogenous hPrp31 or other tested snRNP proteins (Fig. 3 and data not shown).

Besides the effects of AD29 expression on cell growth and nuclear morphology, we also observed the rapid degradation of the AD29 mutant in HeLa cells. Despite the fact that these results were acquired using cell culture expressing normal levels of endogenous hPrp31, such observation raise two alternative models for RP. The first model predicts that the AD29 mutant is rapidly degraded in all cell types including retina cells and RP symptoms are caused by the insufficiency of functional hPrp31. This hypothesis would be in accordance with previous findings that demonstrated that large deletions of the HPRP31 gene and mutations that destabilize hPrp31 mRNA cause RP (23–25). Additionally, this model would also explain the incomplete penetrance of the AD29 mutation (3).

An alternative hypothetical model would require specific stabilization and/or high expression of the mutant in retina cells. In this case, the mutant would only be able to exert its dominant negative effect on snRNP metabolism and cell viability, when it is present in cells as is the case of our model system. These defects can be reverted by over-expressing the hPrp31 binding partner, hPrp6 whose expression might represent an additional factor that determines whether RP symptoms are manifested in affected individuals. To distinguish between these two models, further investigation is required to determine whether the AD29 mutant is present in targeted retina cells.

**MATERIALS AND METHODS**

**Cells and antibodies**

HeLa cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, penicillin and streptomycin (Gibco BRL). Cells were transfected with Fugene HD (Roche) according to the manufacturer’s protocol. Stable HeLa cell lines were created by transfecting cells with the hPrp31-YFP (13) or AD29-YFP plasmids. To obtain individual YFP positive cell, G418 resistant cells were sorted by

---

**Figure 5.** The AD29 protein is rapidly degraded inside cells. (A–C) To compare the stability of AD29-YFP to WT31-YFP, proteo-synthesis was inhibited by cycloheximide in WT31 or AD29 cell lines and YFP fluorescence measured every 15 min for 5 h. (A and A') AD29 cells at time 0 h and (B and B') 5 h after cycloheximide addition. (A and B) YFP fluorescence (A' and B') bright-field images. Bar represents 10 μm. (C) Graph of relative YFP fluorescence after protein synthesis inhibition. YFP fluorescence did not exhibit any significant decrease before addition of cycloheximide, 15 min post addition of cycloheximide AD29-YFP fluorescence (n = 28 cells) began decreasing indicative of the rapid degradation of the AD29-YFP protein. In contrast, WT31-YFP protein (n = 22 cells) exhibited only a slow decay during the course of the 5 h experiment. (D) Protein synthesis was inhibited as before and proteins detected by western blotting using anti-hPrp31 antibodies that recognize both endogenous and tagged hPrp31 variants. The results show the relative stability of WT31-YFP compared to AD29-YFP that is rapidly degraded after 2 h.
The following antibodies were used: rabbit anti-SART3/p110 antibodies (14), mAb anti-coilin (5P10) (34) kindly provided by M. Carmo-Fonseca, rabbit antibodies against hPrp31 (U4/U6-61K) (11), hPrp4 (U4/U6-60K) (35), hSnu114 (U5-116K) (36) and hPrp3 kindly provided by R. Lührmann. Monoclonal antibodies against U2B" were purchased from Progen and anti-hPrp6 antibodies (H-300) from Santa Cruz Biotechnology.

**Mutation and protein tagging**

SART3-YFP-C3, hPrp4-YFP-C1, hPrp31-CFP-C3 and hPrp31-YFP-C3 were described previously (13). The full-length hPrp6 was amplified by high fidelity PCR (Phusion, Finnzymes) from an EST clone and subsequently sub-cloned into ECFP-C3 or EYFP-C3 vectors derived from EGFP-C3 (Clontech) by using EcoRI/HindIII restriction sites. Correct sequences were confirmed by sequencing. The point G646C mutation within the hPrp31 sequence was introduced by PCR mutagenesis using the ExSite Mutagenesis kit (Stratagene), primers 646-For: 5'-TTCATCCCACCCAACTGTCCATCAT and 646-Rev: 5'-GGACATCCGGGAAGCTCCATCATC and hPrp31-CFP-C3 as a template (13). The correct sequence was verified by sequencing. Subsequently, the AD29 mutant was re-cloned into the EYFP-C3 vector using EcoRI/BamHI restriction sites.

**Indirect immunofluorescence**

Cells were fixed in 4% paraformaldehyde/PIPES (Sigma) for 10 min, permeabilized for 5 min with 0.2% Triton X-100 (Sigma) and incubated with the appropriate primary antibodies. Secondary anti-rabbit antibodies conjugated with FITC or TRITC and anti-mouse antibodies conjugated with TRITC or Cy5 (Jackson ImmunoResearch Laboratories) were used. Images were collected using the DeltaVision microscope system (Applied Precision) coupled with the Olympus IX70 microscope equipped with an oil immersion objective 60x 1.42NA using the same settings for each sample. Stacks of 25 z-sections with 200 nm z-step were collected per sample and subjected to mathematical deconvolution using the measured point spread function (SoftWorx, Applied Precision). Mean intensities in Cajal bodies and nucleoplasm were quantified using SoftWorx as described previously (13). Twenty to 40 cells containing ~40–100 Cajal bodies were analyzed in each experiment.

**Live cell imaging**

Cells were plated on glass bottomed Petri dishes (MatTek) and after 20–24 h imaged using the DeltaVision microscope system coupled with the Olympus IX70 microscope equipped with an oil immersion objective 40x 1.3NA and an environmental chamber controlling CO2 level and temperature. Images were taken every 30 min for 24 h using YFP excitation and emission filters (Applied Precision). To measure the effects of hPrp6-CFP expression on AD29 cell growth, the same microscope set up was used and YFP/CFP filters were used. Eight to 12 positions were imaged simultaneously using the Multi-point visiting function. There were ~30–75 cells per experiment at time 0 h. The number of YFP or YFP/CFP positive cells were counted at each time point and plotted accordingly.

**Western blotting**

Cells were washed with ice-cold PBS, scraped and pelleted at 1000g for 5 min before being resuspended in 30 μl PBS. This was followed by the addition of 30 μl of 2x protein sample buffer and incubation at 95°C for 5 min. Cell extracts were subsequently homogenized by passage through a 22 G needle. Proteins were resolved on a 10% polyacrylamide gel, blotted onto a nitrocellulose membrane and incubated with anti-hPrp31 antibodies followed by incubation with goat anti-rabbit antibodies coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories). The SuperSignal West Pico/Femto Chemiluminiscent Substrate (Pierce) was used to generate luminiscence.

**FRET measurement**

HeLa cells were transfected with constructs encoding fluorescently tagged proteins using Fugene HD, grown for 24–26 h and fixed at room temperature in 4% paraformaldehyde/PIPES (Sigma) for 10 min. After rinsing with Mg-PBS (PBS supplemented with 10 mM Mg2+) and water, cells were embedded in glycerol containing DABCO. FRET was measured by the acceptor photobleaching method as previously described (13) using the Leica SP5 confocal microscope. Intensities of CFP (excited by 405 nm laser set to 5–10% of maximum power) and YFP (excited by 514 nm laser line set to 2% of maximum power) were measured. Following this, YFP was bleached in a region of interest by three to five intensive (30% maximum power) pulses of 514 nm laser line and CFP and YFP fluorescence measured again. Apparent FRET efficiency was calculated according to the equation FRETefficiency (%) = (CFPafter − CFPbefore) × 100/CFPafter. Unbleached regions of the same cell were used as a negative control. Ten cells were measured per each FRET pair.

**Immunoprecipitation**

HeLa, WT31 or AD29 cells were grown on 15 cm Petri dishes, placed on ice, washed three times with ice cold Mg-PBS and harvested into NET-2 buffer (50 mM TRIS–Cl pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40) supplemented with a complete mix of protease inhibitors (Roche) and pulse-sonicated for 90 s on ice. Cell extracts were centrifuged at 13 000 rpm and the supernatant incubated with Protein-G Sepharose beads (GE Healthcare) coated with goat anti-GFP antibodies.
(raised against bacterially expressed full-length EGFP and obtained from David Drechsel, MPI-CBG, Dresden, Germany) for 4 h at 4°C. Captured complexes were extracted by bead incubation in protein sample buffer for 5 min at 95°C and the precipitated proteins were detected by western blotting.

**Glycerol gradient ultracentrifugation**

Nuclear extracts were prepared according to (37), diluted in gradient buffer (20 mM HEPES/KOH pH 8, 150 mM NaCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol) and fractionated in a linear 10–30% glycerol gradient by centrifugation at 32 000 rpm for 17 h using the SW-41 rotor (Beckman). The gradient was divided into 20 fractions (∼620 µl) and proteins from these fractions analyzed by western blotting.

**ACKNOWLEDGEMENTS**

We thank Karla Neugebauer in whose laboratory we performed pilot experiments. We are grateful to Reinhard Luhrmann and Maria Carmo-Fonseca for gifts of antibodies. We also thank Petr Tesina for comments on the manuscript and Alicia Corlett for language proofreading.

**Conflict of Interest statement.** None declared.

**FUNDING**

This work was supported by grants from Max Planck Society (the Partner group program) and from the Academy of Sciences of the Czech Republic (KAN200520801, AV0Z50520514).

**REFERENCES**


