Nuclear deadenylation/polyadenylation factors regulate 3' processing in response to DNA damage

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We previously showed that mRNA 3' end cleavage reaction in cell extracts is strongly but transiently inhibited under DNA-damaging conditions. The cleavage stimulation factor-50 (CstF-50) has a role in this response, providing a link between transcription-coupled RNA processing and DNA repair. In this study, we show that CstF-50 interacts with nuclear poly(A)-specific ribonuclease (PARN) using *in vitro* and in extracts of UV-exposed cells. The CstF-50/PARN complex formation has a role in the inhibition of 3' cleavage and activation of deadenylation upon DNA damage. Extending these results, we found that the tumour suppressor BARD1, which is involved in the UV-induced inhibition of 3' cleavage, strongly activates deadenylation by PARN in the presence of CstF-50, and that CstF-50/BARD1 can revert the cap-binding protein-80 (CBP80)-mediated inhibition of PARN activity. We also provide evidence that PARN along with the CstF/BARD1 complex participates in the regulation of endogenous transcripts under DNA-damaging conditions. We speculate that the interplay between polyadenylation, deadenylation and tumour-suppressor factors might prevent the expression of prematurely terminated messengers, contributing to control of gene expression under different cellular conditions.

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Introduction

The steady-state levels of cellular mRNAs are determined by the balance between their biosynthesis and turnover. The turnover rates of individual mRNAs can vary in response to changes in the cellular environment and the mRNA poly(A) tail is one of the principal structures required for correct regulation of mRNA degradation. The poly(A) tails are also

crucial for regulation of mRNA processing, translation and subcellular localization, such as nuclear export (Colgan and Manley, 1997; Zhao *et al.*, 1999; Mandel *et al.*, 2008). Thus, the poly(A) tail is a fundamental *cis*-acting element that is essential for proper control of gene expression at several different levels in eukaryotes. It is synthesized in the nucleus through a two-step polyadenylation reaction: an initial cleavage step, which specifies the 3' end of the mRNA, followed by synthesis of a 200-adenosine residue tail to the 3' end of the upstream cleavage product (reviewed by Zhao *et al.*, 1999; Shatkin and Manley, 2000). The polyadenylation reaction is by itself a highly regulated event and is used, for example, to regulate tissue or development-specific gene expression and for cell growth control (e.g., Takagaki *et al.*, 1996; Takagaki and Manley, 1998; Chuvpilo *et al.*, 1999). Several examples of cases are known, which link deficiencies in the polyadenylation machinery to disease development, including tumour formation (reviewed by Scorialas, 2002).

The polyadenylation reaction requires the assembly of a rather large number of interacting protein factors that recognize a relatively simple set of *cis*-acting signal sequence elements in the mRNA precursor. Cleavage stimulation factor (CstF) is one of the essential polyadenylation factors. CstF is active most likely as a dimer with each subunit consisting of three protein factors called CstF-77, CstF-64 and CstF-50. CstF-64 interacts directly with the downstream located GU-rich *cis*-acting element. Both CstF-50 and CstF-77 subunits interact specifically with the C-terminal domain (CTD) of the RNA polymerase II largest subunit (RNAP II LS), likely facilitating the RNAP II-mediated activation of 3'-end processing (McCracken *et al.*, 1997; Hirose and Manley, 1998). Furthermore, 3' end processing can be repressed after DNA damage as a result of an interaction between CstF-50 and BRCA1-associated RING domain protein (BARD1; Kleiman and Manley, 1999) and of the proteasome-mediated degradation of RNAP II (Kleiman *et al.*, 2005). We have recently shown that cells with reduced levels of CstF show decreased viability after UV treatment, reduced ability to ubiquitinate RNAP II and defects in repair of DNA damage (Mirkin *et al.*, 2008), supporting the idea that CstF has a direct role in the DNA damage response.

Although most of the polyadenylation factors have been described and the reaction is now relatively well understood, the mechanisms underlying poly(A) removal are much less defined. In mammalian cells, the earliest and rate-limiting step in mRNA decay is the removal of the mRNA poly(A) tail (Wilusz *et al.*, 2001; Chen and Shyu, 2003). PARN is one of the three major poly(A)-specific 3' exoribonucleases identified in mammalian cells and characterized thus far (Mitchell and Tollervey, 2000; Parker and Song, 2004; Wu *et al.*, 2005). It is expressed ubiquitously in all tissues of most eukaryotic organisms (Copeland and Wormington, 2001) and localizes both to the nucleus and the cytoplasm. PARN shows high specificity for single-stranded poly(A) (Korner and Wahle, 1997; Martinez *et al.*, 2001) and its deadenylating activity is

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stimulated by the mRNA 5'-end-located cap structure (Dehlin *et al*, 2000; Gao *et al*, 2000; Martinez *et al*, 2001; Nilsson *et al*, 2007; Wu *et al*, 2009). Although the exact function of PARN in the nucleus is unknown, it has been established that the CBP80 (Balatsos *et al*, 2006) and the poly(A)-binding protein-1 (PABPN1; Gao *et al*, 2001) both inhibit PARN activity. Interestingly, the cap-binding complex (CBC) has also been shown to have a role in polyadenylation by stabilizing the RNA/CstF complex formed in the nucleus and depletion of CBC reduces the mRNA cleavage reaction (Flaherty *et al*, 1997).

In this study, we have found that the polyadenylation factor CstF-50 interacts strongly with the same region (CTD) of PARN as its inhibitor CBP80. Like the previously described CstF/BARD1/BRCA1 complex, CstF/PARN complex formation is stimulated by UV treatment and participates in inhibiting the 3' cleavage reaction of polyadenylation under DNA-damaging conditions. More importantly, here we also show that the CstF-50/PARN interaction activates deadenylation *in vitro* and that UV treatment can activate nuclear PARN deadenylase activity. We also show that the tumour suppressor BARD1 strongly activates deadenylation by PARN in the presence of CstF-50 and that siRNA-mediated knockdown of BARD1 or the BARD1 mutant T734A decreases the UV-induced activation of deadenylation. In addition, our data show that CBP80 and CstF-50 could compete for binding to PARN, providing a mechanism to regulate PARN deadenylase activity under different cellular conditions. Consistent with this, we show that these functional interactions correlate with changes both in expression levels and in the polyadenylation of different mRNA precursors, such as housekeeping genes and some clinically significant genes, upon UV treatment and that reduced expression of PARN or the BARD1 mutant T734A is sufficient to revert the observed changes. On the basis of our study we propose that the CstF/PARN complex has a role in inhibiting the 3' cleavage of polyadenylation and in the activation of deadenylation in the nucleus under DNA-damaging conditions, suggesting the existence of alternative mechanisms to regulate gene expression under different cellular conditions.

Results

The polyadenylation factor CstF-50 binds to the deadenylation factor PARN under DNA-damaging conditions and this is accompanied with an increase in PARN expression levels

To identify proteins that interact with the polyadenylation factor CstF-50, we performed some time ago an yeast two-hybrid screen and identified the BRCA1-associated BARD1 as a prominent interactor (Kleiman and Manley, 1999). We have recently revisited the primary data of this screen and realized that one of the most abundant interactors of CstF-50 corresponded to the C-terminal fragment of PARN. PARN was not recognized at the time when the screen was performed as the amino-acid sequence of PARN was not known until later. Nevertheless, identification of PARN as an abundant yeast two-hybrid interactor of CstF-50 suggests a functional interaction between the two components as well as between the deadenylation and polyadenylation machineries. To further investigate this possibility, we performed 'pull-down' assays using GST-tagged full-length CstF-50 (GST-CstF-50) and full-

length PARN (His-PARN), the C-terminal fragment of PARN (amino acids 443–639, His-CTD-PARN) and the N-terminal fragment of PARN (amino acids 1–470, His-NTD-PARN). The results showed that both His-PARN (Figure 1A, lane 2; and Figure 1B, left panel, lane 4) and His-CTD-PARN (Figure 1B, lane 6) interacted directly and strongly *in vitro* with GST-CstF-50. However, the derivative encompassing the N-terminal region of PARN did not bind either to GST-CstF-50 (Figure 1B, lane 5) or to GST alone (lane 7). As samples were treated with either RNase A (upper panel) or micrococcal nuclease (lower panel), the observed CstF/PARN interaction was probably not due to an RNA tethering effect. All together, these results indicate that the CTD of PARN, which has been described to interact with CBP80 (Balatsos *et al*, 2006), constitutes the PARN/CstF-50 interaction domain.

To examine the status of the CstF-50/PARN complex in nuclear extracts (NEs), we analysed extracts from HeLa cells by co-immunoprecipitation assays. For these studies, we used an antibody directed against either PARN or the CstF subunit CstF-64, to ensure that any detected interactions were between PARN and intact CstF, and samples were treated with RNase A. Figure 1C (left panel) shows that only a very small fraction of nuclear PARN co-precipitated with CstF-64. Similar results were obtained in the reciprocal co-immunoprecipitation analysis (Figure 1C). As the results of the co-immunoprecipitation assays did not reflect the strong interaction observed in the GST 'pull-down' assays, we decided to analyse the complex formation under conditions of DNA damage as earlier studies had shown a link between CstF and UV-induced DNA damage response (Kleiman and Manley, 2001). Thus, the co-immunoprecipitation assays were repeated using NEs of cells exposed or not to UVC light (20 J m^{-2}) and allowed to recover for 2 h as described before (Kleiman and Manley, 2001). Surprisingly, we observed in this case a significant increase in the detected amount of CstF/PARN complexes as well as an increase in PARN expression (Figure 1C). Extraneous antibodies did not immunoprecipitate either protein (lanes 3 and 4), nor did either antibody cross-react with other proteins (not shown). The increased amounts of PARN in the extracts of UV-irradiated cells cannot solely explain the increased association between the two proteins. Although similar amounts of PARN and CstF are immunoprecipitated by their own antibody in samples exposed or not to UV, a complex formation with CstF is detected only in the UV-treated samples, even in darker exposures of the western blot analysis (not shown).

To further investigate the increased expression levels of PARN, NEs of HeLa cells treated with UV irradiation and allowed to recover for the time periods indicated in Figure 1D were analysed. As described before (Kleiman and Manley, 2001), western blots showed no significant changes in either components of CstF (CstF-64) and CPSF (CPSF-160, not shown) or BRCA1/BARD1 (not shown) in response to UV. No changes were detected in the topoisomerase II (Topo II) levels as well. However, a transient increase in the expression of PARN was observed from 2 to 10 h after UV treatment, but normal levels were restored after 15 h, reaching the levels in untreated cells (not shown).

As UV treatment was known to induce CstF/BARD1/BRCA1 complex formation (Kleiman and Manley, 2001), we decided to test whether the tumour suppressor BARD1 might

Figure 1 CstF-50 interacts with PARN upon DNA damage and this interaction is accompanied with increase in PARN levels. **(A)** Interaction of GST-CstF-50 and His-PARN. Immobilized GST-CstF-50 or GST on glutathione beads was incubated with 1 μ g of His-PARN. Bound proteins were eluted, resolved by SDS-PAGE and detected with anti-PARN antibodies. 5% of PARN used in the reaction is shown as input. Recombinant proteins were treated with either RNase A (upper panel) or micrococcal nuclease (lower panel). **(B)** Requirement of PARN CTD for CstF-50 interaction. GST and GST-CstF-50 were used in 'pull-down' assays with the indicated His-PARN derivatives. The samples analysed in the left panel were analysed as in panel A. Coomassie blue staining of the purified recombinant proteins after SDS-PAGE is shown in the right panel. The positions of size markers are indicated. **(C)** CstF, BARD1 and PARN co-immunoprecipitate from the NEs of HeLa cells treated with UV irradiation. The NEs were immunoprecipitated with anti-CstF-64, anti-PARN, anti-H2A or preimmune antibodies. Equivalent amounts of the pellets (PD) and supernatants (SN) were resolved by SDS-PAGE and proteins were detected by immunoblotting using antibodies against PARN and CstF-64. Antibodies against Topo II were used as control for specificity. The positions of Topo II, CstF-64 and PARN are indicated. 20% of the NE used in the immunoprecipitation reaction is shown as input. **(D)** CstF-64, Topo II and PARN protein levels in the NEs from UV-treated HeLa cells were monitored by western blotting. The relative density of each band was determined using the Image J program. **(E)** BARD1 does not interact strongly with PARN. Left panel: Immobilized His-PARN on nickel beads was incubated with 1 μ g of GST-CstF-50, GST-BARD1 or GST. Right panels: Immobilized GST-CstF-50 or GST-BARD1 on glutathione beads was incubated with 1 μ g of His-PARN. Bound proteins were eluted, resolved by SDS-PAGE and detected using anti-PARN, anti-BARD1 and anti-GST antibodies. 5% of the proteins used in the reaction is shown as input.

also interact with PARN. Interestingly, a significant amount of BARD1 co-precipitated with PARN in NEs from UV-treated cells (Figure 1C). Similarly, we could detect PARN in the reciprocal co-immunoprecipitation experiment where we used antibodies directed against BARD1 (data not shown). Although these results do not show how many complexes CstF can form with PARN and BARD1, they clearly show that UV treatment induced the interaction between those factors. Interestingly, appearance of this/these complex(es) coincided with the observed inhibition of 3' cleavage under DNA-damaging conditions (Kleiman and Manley, 2001).

To test whether BARD1 might also interact directly with PARN, we performed 'pull-down' assays using recombinant His-PARN, GST-CstF-50 and GST-BARD1 (Kleiman and Manley, 1999) polypeptides (Figure 1E). In the panel on the left, the 'pull downs' were performed using nickel beads. Whereas only a small amount of BARD1 is pulled down by

His-PARN (lane 3), this amount increases in the presence of CstF-50 (lane 4). In the panel on the right, the 'pull downs' were performed using glutathione beads and only GST-CstF-50 associated with His-PARN. These results indicate that the BARD1/CstF/PARN complex could be formed in the presence of CstF-50, suggesting that CstF-50 acts as a scaffold to bring PARN and BARD1 into the same complex. Taken together, these sets of experiments showed several lines of evidence that PARN, CstF-50 and BARD1 interact with each other after UV treatment.

PARN is necessary for UV-induced inhibition of the 3' cleavage reaction

To study the significance of the interaction between PARN and CstF-50, we performed siRNA-mediated knock-down of PARN in HeLa cells. Figure 2A shows that a 24 h siRNA treatment resulted in substantial depletion of PARN

Figure 2 PARN is necessary for the UV-induced inhibition of 3' cleavage. **(A)** NEs from cells treated with PARN/control siRNA and UV irradiation, and allowed to recover for 2 h, were analysed for pre-mRNA 3' cleavage. NEs were incubated in a reaction mix containing L3 pre-mRNA. The positions of pre-mRNA and the 5' cleavage product are indicated. The protein levels of RNAP II, BRCA1, Topo II, BARD1, PARN and CstF-64 were analysed by western blotting. **(B)** NEs from cells treated with PARN/control siRNA and different UV irradiation recovery times were analysed as described in panel A. **(C)** NEs from cells treated with PARN siRNA and UV irradiation, and allowed to recover for 2 h, were incubated in a reaction mixture containing L3 pre-mRNA and increasing amounts of His-PARN, His-CTD-PARN, His-NTD-PARN and GST proteins.

($\approx 90\%$) in NEs, independently of the UV treatment. We next investigated the effect of siRNA-mediated knockdown of PARN on the UV-induced inhibition of 3' cleavage described earlier (Kleiman and Manley, 2001). Surprisingly, depletion of PARN abolished the UV-induced inhibition of mRNA 3' end cleavage (Figure 2A, compare lanes 2 and 4), indicating that PARN has an inhibitory effect on mRNA 3' cleavage under DNA-damaging conditions. In consistence with previous work in our laboratory, NEs from control siRNA-treated cells showed UV-induced inhibition of 3' processing. As observed before (Kleiman and Manley, 2001; Kleiman *et al*, 2005), no significant changes were detected in CstF, BRCA1, Topo II or BARD1 levels in response to UV, whereas the levels of RNAP II were reduced after UV treatment.

Extending these results, we followed during a time-course experiment the 3' cleavage activity after UV treatment in the presence or absence of siRNA targeting PARN (Figure 2B). From this analysis it is evident that absence of PARN abolished the UV-induced inhibition of 3' cleavage. To further document the involvement of PARN in the response, we added back recombinant His-PARN (Figure 2C, upper left), His-CTD-PARN (lower left), His-NTD-PARN (lower right) and GST protein as control (upper right) into 3' cleavage reactions performed using PARN siRNA knockdown and UV-treated extracts. Only increasing concentrations of His-PARN and His-CTD-PARN recovered the inhibition of 3' cleavage reaction observed after UV treatment. Altogether, these results indicate that PARN has an inhibitory effect on mRNA 3' cleavage under DNA-damaging conditions. Initially, this inhibition was ascribed to CstF/BARD1/BRCA1 complex formation (Kleiman and Manley, 2001) and proteasome-mediated degradation of RNAP II (Kleiman *et al*, 2005). However, these results indicated that other factors, such as PARN, might also be involved in the response. Although our data do not uncover the mechanism involved in this cellular

response, it is possible that the UV-induced inhibition of 3' cleavage by PARN could be the result of several alternative mechanisms, such as a direct interaction between PARN and the essential polyadenylation factor CstF-50, by a destabilization effect of the essential CstF/RNA complex, and/or by the formation of any other inhibitory complex.

CstF-50 has a role in the UV-induced activation of nuclear PARN deadenylase activity

The results presented above showed that PARN was induced upon UV treatment and that complex formation between PARN and CstF-50 paralleled the inhibitory effect of UV treatment on the 3' end cleavage reaction. PARN has previously been shown to be present both in the nucleus as well as in the cytoplasm (Korner *et al*, 1998). As CstF-50 is primarily located in the nucleus (Zhao *et al*, 1999; Shatkin and Manley, 2000), we asked what happened to nuclear PARN deadenylation activity during the response to DNA damage. HeLa cells were first exposed to UV light and then allowed to recover for 2 h before NEs were prepared, and assayed for the presence of deadenylation activity of a radio-labelled L3(A₃₀) RNA substrate. Figure 3A (upper panel) shows that the deadenylation activity detected in the NEs of cells unexposed to UV treatment was very weak and this activity increased significantly after UV treatment. A clear increase in deadenylation was also observed in a time-course experiment (upper panel). siRNA-mediated knockdown of PARN showed an effect not only in the UV-induced inhibition of 3' cleavage (Figure 3B, lower panel) but also in the UV-induced activation of deadenylation (upper panel, compare lanes 2 and 4). Western blot analysis confirmed the knockdown of PARN (not shown). Besides, these results confirm that PARN is one of the major deadenylases under regulation in this DNA-damage response. Supporting these results, the UV-induced activation of deadenylation in NEs was enhanced

Figure 3 DNA damage induces nuclear PARN deadenylase activity. (A) Upper panel: Effect of UV irradiation on L3(A₃₀) deadenylation. NEs from cells treated with UV (20 J m⁻²) irradiation and allowed to recover for 2 h were analysed for deadenylation. The incubation times for the deadenylation assay are indicated in the figure. Lower panel: The UV-induced activation of PARN deadenylase activity is stimulated by the 5' cap structure. NEs from cells treated with different UV doses were allowed to recover for 2 h. Those NEs were tested for deadenylation assays using an m⁷-GpppG-capped L3(A₃₀) or a non-capped L3(A₃₀) substrate. The deadenylation reaction was incubated for 90 min. (B) The UV-induced activation of L3(A₃₀) deadenylation is PARN-dependent. NEs from cells treated with UV irradiation and treated with control/PARN siRNA were allowed to recover for 2 h and were analysed for deadenylation as well as 3' cleavage reactions as described in panel A and Figure 2A, respectively. The deadenylation assays were incubated for 90 min. The protein levels of RNAP II, BRCA1, Topo II, BARD1 and PARN were analysed by western blotting (data not shown). (C) Effect of different UV doses on the activation of deadenylation and inhibition of 3' end cleavage. NEs from cells treated with UV irradiation (20 and 40 J m⁻²) and allowed to recover for the time periods indicated in the figure were analysed for deadenylation and 3' end cleavage as described in panel B. (D) Increase of CstF and PARN co-immunoprecipitation from the NEs of HeLa cells treated with UV irradiation and allowed to recover for 1, 2 and 24 h. The NEs were immunoprecipitated using anti-CstF-64 antibodies. Samples were analysed as in Figure 1C. The NEs used in the immunoprecipitation assays were also tested in deadenylation assays as in panel B. (E) UV treatment activates the poly(A)-specific 3'-exonuclease activity of PARN. The deadenylation assays using NEs from cells treated with UV irradiation were performed in the presence of L3(A₃₀) and ML43(G₁₅) RNA substrates radioactively labelled in their RNA body. The reactions were analysed by electrophoresis on 10% polyacrylamide/7 M urea gels.

by the presence of an m⁷-guanosine cap on the substrate L3(A₃₀) RNA (Figure 3A, lower panel). Previous observations have shown that PARN activity is stimulated by a 5' cap (Dehlin *et al*, 2000; Gao *et al*, 2000; Martinez *et al*, 2001).

Further analysis showed that activation of deadenylation in NEs was dependent on UV dose and was transient, increasing between 2 and 5 h after UV treatment and disappearing after 10 h (Figure 3C, upper panel). Interestingly, this transient pattern of deadenylation activation was reflected by concomitant inhibition of the 3' end cleavage reaction (lower panel). Thus, UV treatment not only induced the association of CstF-50, PARN and BARD1 (Figures 1A–C and E, and 3D) but also activated PARN-dependent deadenylation activity in NEs (Figure 3A–D), suggesting that CstF-50 could participate in the UV-induced activation of PARN-mediated deadenylation. Extending these studies, we determined the RNA substrate specificity of the deadenylating activity present in the NEs of UV-treated cells using an ML43(G₁₅) RNA substrate, which was radioactively labelled

in its RNA body. The ML43(G₁₅) RNA substrate was unaffected by the addition of NEs from UV-treated cells to the deadenylation reaction (Figure 3E). As shown in Figure 3A–D, the RNA substrate L3(A₃₀) was efficiently deadenylated to an RNA product of the L3 size under those conditions.

To directly test if CstF-50 could influence PARN activity we performed *in vitro* reconstituted deadenylation reactions, where we monitored the deadenylation of L3(A₃₀) RNA substrate in a reaction using a limiting amount of His-PARN and in the absence or presence of increasing amounts of GST-CstF-50. Addition of GST-CstF-50 enhanced the deadenylation activity of PARN up to 10-folds (Figure 4A, compare lanes 4 and 9), suggesting that CstF-50 is an activator of PARN activity. Importantly, we did not detect any deadenylation activity when using CstF-50 alone (lanes 10–11) or in combination with the deadenylase-deficient PARN fragment (CTD-PARN; Figure 4B), which interacts strongly with CstF-50 (Figure 1B). Interestingly, CstF-50 failed to increase the deadenylase activity of the NTD-PARN derivative, which lacks the

Figure 4 CstF50 can activate PARN deadenylase activity in the absence of any other factors. (A) CstF-50 activates PARN deadenylase activity *in vitro*. Deadenylation assays using different concentrations of His-PARN were performed in the presence of a radioactively labelled capped L3(A₃₀) RNA substrate. Increasing amounts of recombinant GST-CstF-50 were added to the reaction. The reactions were analysed by electrophoresis on 10% polyacrylamide/7 M urea gels. The positions of the polyadenylated mRNA L3(A₃₀) and the L3 deadenylated product are indicated. (B) CstF-50 was not able to activate the C-terminal derivative of PARN (His-CTD-PARN), which is inactive in deadenylation. Deadenylation reactions were analysed as in panel A. The deadenylated L3 substrate is shown in lane 1 as control. (C) The CTD of PARN is required for CstF-50 activation. Catalytically active His-NTD-PARN was analysed for deadenylase activity as in panel A. (D) Left panel: CstF-50 activates the poly(A)-specific 3'-exonuclease activity of PARN. Deadenylation assays using His-PARN and GST-CstF-50 were performed in the presence of L3(A₃₀) and ML43(G₁₅) RNA substrates radioactively labelled in their RNA body. The reactions were analysed by electrophoresis on 10% polyacrylamide/7 M urea gels. Right panel: CstF-50 activates PARN deadenylase activity and not endonucleolytic cleavage. Deadenylation assays with His-PARN and GST-CstF-50 were performed in the presence of capped L3(A₃₀) and ML43(G₁₅) RNA substrates radioactively labelled with [α -³²P]ATP. The reactions were analysed by one-dimensional TLC. Ori and P indicate the location of origin of separation and mononucleotide product, respectively.

CstF-50-interacting domain (Figure 4C), indicating that the CstF-50/PARN interaction is necessary for the activation of PARN activity. Extending these studies, the RNA substrate specificity of the PARN deadenylating activity was determined using the ML43(G₁₅) RNA substrate, which was radioactively labelled in its RNA body. Consistent with previous studies (Aström *et al*, 1991; Martinez *et al*, 2000), the ML43(G₁₅) RNA substrate was unaffected by addition of recombinant PARN (Figure 4D, left panel). These results did not change after addition of a mix of recombinant PARN and CstF-50 to the deadenylation reaction. As shown in Figures 3 and 4A–C, the RNA substrate L3(A₃₀) was efficiently deadenylated to an RNA product of the L3 size under those conditions. Then, we analysed the products formed by the deadenylation reaction of the A-tail labelled L3(A₃₀) and ML43(G₁₅) RNA substrates by one-dimensional thin-layer chromatography (TLC). As shown in Figure 4D (right panel), only release of 5'AMP mononucleotides was detected after incubation of the L3(A₃₀) RNA substrate with either recombinant PARN alone or with a mix of recombinant PARN and CstF-50, suggesting that the observed activity is a 3'

exonuclease and not endonucleolytic cleavage. Release of 5'AMP mononucleotides was not detected when the ML43(G₁₅) RNA substrate was used in the reaction.

Taken together, we conclude that CstF-50 activates PARN deadenylation activity in the absence of any other factors, suggesting that the CstF-50/PARN complex that we have identified could have a role both in the UV-induced inhibition of the 3' end cleavage of the polyadenylation reaction as well as in the concomitant activation of nuclear deadenylation.

The CstF-50/BARD1 complex can rescue PARN deadenylase activity from the CBP80-induced inhibition

The UV-induced association of the CstF, PARN and BARD1 (Figure 1C and E) raises the possibility that BARD1 has a role not only in the UV-induced inhibition of polyadenylation but also in the UV-induced activation of deadenylation in the presence of CstF-50. To investigate this possibility, we monitored the deadenylation of the L3(A₃₀) RNA substrate in a reaction mix using a limiting amount of His-PARN, a limiting concentration of GST-CstF-50 and in the absence or presence of increasing amounts of GST-BARD1 (Figure 5A). GST-

Figure 5 BARD1 increases the CstF-50-induced activation of PARN deadenylase activity. **(A)** CstF-50/BARD1 strongly activates PARN deadenylase activity *in vitro*. Deadenylation assays using different concentrations of His-PARN were performed in the presence of a radioactively labelled capped L3(A₃₀) RNA substrate. Recombinant GST-CstF-50 and increasing amounts of GST-BARD1 were added to the reaction containing limiting amounts of PARN. The reactions were analysed by electrophoresis on 10% polyacrylamide/7 M urea gels. The positions of the polyadenylated mRNA L3(A₃₀) and the L3 deadenylated product are indicated. **(B)** siRNA knockdown of both BARD1/BRCA1 expression decreases the UV-induced activation of deadenylation. NEs from cells treated with control/BARD1-BRCA1 siRNA and allowed to recover for 2 h after UV irradiation were analysed for deadenylation. The protein levels of BRCA1, Topo II, BARD1 and PARN were analysed by western blotting. **(C)** U2OS cells stably transformed with BARD1 T734A mutant or wild-type BARD1 were untreated or treated with UV (20 J m⁻²). Two hours after UV treatment, NEs were prepared, treated with micrococcal nuclease and immunoprecipitated with anti-FLAG (panel on the left) or anti-CstF-64 (panel on the right) antibodies. Supernatants and pellets were analysed by western blotting using antibodies against Topo II, PARN, CstF-64 and FLAG. **(D)** NEs from U2OS cells stably transformed with BARD1 T734A mutant or wild-type BARD1 were also analysed for deadenylation.

BARD1 significantly enhanced deadenylation by PARN only in the presence of CstF-50, implying that the CstF-50/BARD1 complex is a stronger activator of PARN deadenylase activity than CstF-50 alone. Using a limited amount of PARN and the lowest concentration of CstF-50 tested (Figure 4A, lane 5), complete deadenylation was observed after addition of BARD1 (Figure 5A, lane 7), reaching deadenylation levels that are similar to those observed using 20 times more PARN (lane 4). Importantly, we did not detect any increase in deadenylation activity when using either the CstF-50/BARD1 complex alone (Figure 5A, lane 8) or using limiting amounts of PARN and increasing amounts of BARD1 (lanes 9–10).

To further characterize the role of BARD1 in the activation of deadenylation under DNA-damaging conditions, we performed siRNA-mediated knockdown of BARD1/BRCA1 in HeLa cells as described before (Kleiman *et al*, 2005). As BRCA1 and BARD1 stabilize each other (Hashizume *et al*, 2001), treating the cells with both BRCA1 and BARD1 siRNA simultaneously was necessary to obtain substantial depletion of BARD1 ($\approx 90\%$, Figure 5B, lower panel) in NEs. Interestingly, samples from BARD1/BRCA1 siRNA-treated cells showed a decrease of almost 50% in the UV-induced

activation of deadenylation (Figure 5B), supporting the idea that BARD1 induces the CstF-50-mediated activation of PARN after UV treatment. Thus, our results indicate that in the presence of CstF-50, tumour suppressor BARD1, which is involved in the UV-induced inhibition of polyadenylation, activates PARN-mediated deadenylation both *in vitro* and in samples from UV-exposed cells.

We further analysed the role of BARD1 in the UV-induced activation of deadenylation by using stable cell lines expressing the FLAG-tagged BARD1 mutant T734A. Previously, we have shown that this mutant of BARD1 is defective not only in the UV-induced phosphorylation of BARD1 but also in the DNA-damage functions of BARD1, such as inhibition of mRNA 3' cleavage and degradation of RNAP II (Kim *et al*, 2006). Those studies showed that UV-induced phosphorylation of BARD1 has an important role in the BARD1/CstF interaction (Kim *et al*, 2006). Extending those studies, here we show that the BARD1 mutant T734A did not associate with the CstF/PARN complex after UV treatment (Figure 5C, compare lanes 2 and 4). However, NEs from cells expressing either the WT or the BARD1 mutant T734A showed the formation of the CstF/PARN complex after UV treatment (compare lanes 14 and 16), supporting the idea that BARD1

It has been described that the CBC complex, through its CBP80 subunit, binds to the CTD of PARN and inhibits PARN deadenylase activity (Balatsos *et al*, 2006) and also enhances the stability of the RNA/CstF complex and thereby activates the 3' end cleavage reaction (Flaherty *et al*, 1997). CBP80 inhibits PARN activity without any need for the cap structure, indicating that inhibition occurs through a direct interaction between the factors (Balatsos *et al*, 2006). Interestingly, our results showed that CstF-50 (Figure 1B), like CBP80, binds to

To characterize the interaction of CstF-50 and CBP80 to the same region of PARN, competition assays were performed by incubating His-PARN immobilized on nickel beads with limiting amounts of either GST-CBP80 or GST-CstF-50, and with increasing amounts of either GST-CstF-50 or GST-CBP80, respectively. Protein samples were treated with RNase A before the binding assays. Increasing amounts of CstF-50

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significantly diminished the binding of CBP80 to immobilized PARN (Figure 6B, lanes 4–7), indicating that CstF-50 and CBP80 compete for binding to the same region of PARN. Similar conclusions were reached when increasing amounts of CBP80 were used in the 'pull-down' assay (lanes 8–11).

To examine the possible regulatory relationship between the two different PARN-associated complexes, we performed *in vitro* deadenylation assays with PARN in the presence and/or absence of recombinant CstF-50 and CBP80. As shown in Figure 6C, addition of CstF-50 could partially suppress the CBP80-induced inhibition of PARN activity (compare lanes 4 to 5–6), and thereby activate PARN deadenylase activity. Release of the CBP80-induced inhibition of PARN activity was even more pronounced when BARD1 was also included into the reactions (compare lane 9–11). Taken together, these results indicate that CBP80 and CstF-50 could compete for binding to PARN under different cellular conditions, inhibiting deadenylase activity through the PARN/CBP80 complex under normal conditions and activating deadenylase activity under DNA-damaging conditions through CstF-50/PARN complex formation.

PARN is involved in the degradation of different endogenous transcripts under different cellular conditions

The data presented above provided evidence that DNA damage induces the activation of PARN-mediated mRNA deadenylation in the nucleus. To further investigate this, we determined the expression levels of different endogenous mRNAs in cells treated by UV irradiation and with siRNAs targeting PARN. Briefly, 24 h after transfection with the indicated siRNAs, cells were exposed to UV light and total nuclear RNA was purified at different time points after UV treatment. Gene expression was analysed by qRT-PCR. Random or oligo-(dT) primers were used for the RT reaction and qPCR reactions were performed using commercially available primers.

First, we analysed the expression levels of two housekeeping genes, GAPDH and β -actin, under different cellular conditions. Our qRT-PCR (Figure 7A) analysis showed that the mRNA levels of these genes decreased under DNA-damaging conditions in cells treated with control siRNA. These data are consistent with our earlier studies (Mirkin *et al*, 2008) and others previous observations (Kartasova *et al*, 1987; Dheda *et al*, 2004; Akeo *et al*, 2007; Maccoux *et al*, 2007), which showed that GAPDH RNA expression can change significantly in different biological systems and under different conditions, and that these variations can lead to experimental error between analysed samples when GAPDH is used as control. Interestingly, the UV-induced decrease in the mRNA levels of endogenous housekeeping genes was lost when we treated the cells with siRNAs targeting PARN (Figure 7A). A similar pattern of changes in the mRNA levels of housekeeping genes was observed using the stable cell line expressing the BARD1 mutant T734A (Figure 7A), which did not show BARD1/CstF/PARN complex formation and activation of deadenylation after UV treatment (Figure 5C and D). These results indicate that BARD1/CstF/PARN complex formation has an important role to decrease the mRNA levels of housekeeping genes under DNA-damaging conditions, and thereby might contribute to the UV-

induced decrease in the cellular levels of total mRNA. As it has also been shown that PARN can promote the deadenylation of AU-rich element (ARE)-containing mRNAs (Lai *et al*, 2003; Moraes *et al*, 2006), we also analysed two ARE-containing mRNAs, that is, c-fos and c-myc, by qRT-PCR. Both mRNAs increased transiently under DNA-damaging conditions in cells treated with control siRNA and in a sarcoma cell line expressing WT BARD1 (Figure 7A), in keeping with earlier studies that indicate that ARE elements within the 3'UTR can control mRNA levels under different cellular conditions. For example, ARE elements can decrease mRNA stability under non-stress conditions and can increase mRNA stability after UV treatment in mammalian cells (Blattner *et al*, 2000; Wang *et al*, 2000; Bollig *et al*, 2002; Gowrishankar *et al*, 2005). Supporting our results, Blattner *et al* (2000) showed that c-fos mRNA expression increased 45 min to 1 h after UV treatment and then dramatically decreased 2 h after UV treatment. Interestingly, PARN knock-down cells and cells expressing the T734A BARD1 mutant showed increase in the mRNA levels of both c-fos and c-myc in samples from cells not treated with UV (Figure 7A). These results suggest that the BARD1/CstF/PARN complex has a role in decreasing the levels of short-lived mRNAs involved in the control of cell growth and differentiation, keeping their expression levels low under non-stress conditions. Both reduced expression of PARN and lack of BARD1/CstF/PARN complex formation have a slight effect on the UV-induced increase in the expression levels of these genes, suggesting that other mechanism(s) might be involved in determining the mRNA levels of these genes under DNA-damage conditions.

Finally, we tested for enrichment of different mRNAs in the poly(A)⁺ RNA population after UV treatment of cells treated with PARN siRNA and in cells expressing the T734A BARD1 mutant (Figure 7B). As described under Materials and methods, only a slight difference in the amount of total poly(A)⁺ RNA and total RNA was detected in samples purified under different conditions. Quantification of qRT-PCR results shows that the studied mRNAs, c-fos, c-myc, actin and GAPDH, showed a slight decrease in the enrichment in the poly(A)⁺ preparation over the total RNA fraction after UV treatment of samples from control siRNA-treated cells and cells expressing WT BARD1. This is consistent with our initial finding that UV treatment inhibits 3' processing (Kleiman and Manley, 2001). Interestingly, our results showed that those mRNAs were enriched ~2–4-fold in the poly(A)⁺ preparation over the total RNA fraction in samples from PARN depleted cells and from cells expressing the T734A BARD1 mutant. Interestingly, such enrichment in the poly(A)⁺ preparation showed a stronger increase in samples from UV-treated cells. These results indicate that the BARD1/CstF/PARN complex is involved in the UV-induced decrease of polyadenylated mRNAs either by inhibition of 3' cleavage or by activation of deadenylation.

Taken together, these results provide evidence that the BARD1/CstF/PARN complex has important roles in the regulation of the levels of different endogenous mRNAs under different cellular conditions. As we proposed before, it is possible that the competition between CBP80 and CstF-50 for binding to PARN under different cellular conditions could have a role in regulating the PARN activity and, therefore, the mRNA levels of different genes.

Figure 7 Effect of PARN expression on endogenous gene expression after UV treatment. **(A)** Real-time PCR analysis of GAPDH, β -actin, c-fos and c-myc expression after UV-treatment using RNA samples from cells treated with control/PARN siRNA and from cells expressing WT/T734A BARD1 mutant. As the RT products of GAPDH from cells treated with control siRNA and not treated with UV were used as endogenous control, the log value corresponding to this sample was zero. The values shown in the figure have been adjusted to avoid the presentation of negative values. The data shown are the mean $\pm$ s.e.m. from three independent experiments. **(B)** Real-time PCR analysis of GAPDH, β -actin, c-fos and c-myc mRNAs polyadenylation. Total and poly(A)⁺ RNA were prepared after UV treatment from HeLa cells treated with control/PARN siRNA and from cells expressing the WT/T734A BARD1 mutant. RNA purification and RT-PCR reactions were performed as described under Materials and methods. Equal volumes of both total and poly(A)⁺ RNA samples were used as template in the RT reactions. Equal amounts of cDNAs were used in qRT-PCR reactions using primers specific for GAPDH, β -actin, c-fos and c-myc mRNAs. Relative quantification was performed using standard curves of known amounts of total cDNA. The results shown are the average of four PCRs from two different RNA extractions.

Discussion

During the DNA repair process, control of gene expression either by transcription or by RNA processing is important to allow the access of the repair enzymes and to prevent the formation of deleterious proteins. After UV irradiation, the cellular levels of mRNA are transiently decreased (Hanawalt,

1994; Ljungman *et al*, 1999). The cellular mechanisms involved in this response are unknown but imply a functional interaction of the DNA repair, transcription and RNA processing machineries. Supporting this idea, it has been described that polyadenylation is transiently inhibited upon UV treatment (Kleiman and Manley, 2001; Kleiman *et al*, 2005; Mirkin *et al*, 2008). As mRNA poly(A) tails are important for

regulation of mRNA stability (reviewed by Zhao *et al*, 1999; Shatkin and Manley, 2000; Mandel *et al*, 2008), changes in the polyadenylation levels either by activation/inhibition of the reaction or by controlling the balance between polyadenylation and deadenylation could account for some of the changes in mRNA levels after UV treatment.

We have proposed in a previous work that the polyadenylation factor CstF-50 has a coordinating role in the nuclear response to UV-induced DNA damage through its interaction with different factors in different cellular environments (Kleiman and Manley, 1999, 2001; Kleiman *et al*, 2005; Mirkin *et al*, 2008). In this work, we have discovered that CstF-50 interacts with the deadenylation factor PARN (Figure 1) and that this interaction has a role in inhibiting the 3' cleavage of the polyadenylation reaction (Figures 2 and 7) and activation of deadenylation upon DNA damage treatment (Figures 3–7). Here we also found that BARD1 is not only involved in the UV-induced inhibition of 3' cleavage (Kleiman and Manley, 1999, 2001) but also in the UV-induced activation of deadenylation in the presence of CstF-50 (Figure 5A–D). Furthermore, we found that the previously identified nuclear CBP80/PARN deadenylation inhibitory complex decreased significantly in abundance under DNA-damaging conditions, whereas the complex containing CstF-50/PARN increased in abundance and that CstF-50 competes with CBP80 for binding to the same region of PARN (Figure 6A and B). In fact, addition of CstF-50 and BARD1 to *in vitro* deadenylation reactions reverted the inhibitory effect that CBP80 had on PARN activity (Figure 6C). Finally, we determined that both PARN knockdown and abolishment of BARD1/CstF/PARN complex formation had an effect on the expression levels and on the polyadenylation of different genes under different cellular conditions (Figure 7A and B). Taken together, our results suggest that interplay between these factors might control gene expression under DNA-damaging conditions by regulating polyadenylation/deadenylation.

On the basis of our studies we propose the following regulatory scenario (summarized in Figure 8). In the absence of DNA damage treatment, CBP80 binds to the CTD of nuclear PARN and inhibits its hydrolytic activity to ensure that PARN does not degrade the mRNA. The cap structure is not a prerequisite for CBP80-mediated inhibition of PARN (Balatsos *et al*, 2006). In this situation, CBC is also known to enhance the polyadenylation of pre-mRNAs by increasing the stability of the RNA/CstF complex (Flaherty *et al*, 1997). As a result of these functional interactions, polyadenylation takes place and normal levels of total mRNA are observed. After DNA damage the BRCA1/BARD1-containing complex is recruited to sites of DNA repair to inhibit mRNA processing by RNAP II ubiquitination followed by degradation of the RNAP II LS, or by covalent modification of other element/s of the complex. This facilitates DNA repair and/or prevents the polyadenylation of aborted nascent mRNAs. If UV-induced inhibition of mRNA 3' cleavage is bypassed, the CstF/PARN interaction may provide a fall-back mechanism to ensure that erroneously polyadenylated mRNAs are eliminated by activation of deadenylation in a cap-dependent manner. In this situation we propose that CBP80 dissociates from PARN, allowing PARN to interact with the CstF-50/BARD1 complex. This reorganization will result in activation of deadenylation and contribute to inhibition of polyadenylation. The final outcome will therefore constitute inhibition of polyadenylation and activation of deadenylation, contributing to the observed decrease in the levels of total mRNA under DNA-damaging conditions. A similar mechanism for control of gene expression has been described by others. For instance, Kim and Richter (2006) have shown that the length of the cytoplasmic poly(A) tail is regulated by polyadenylation/deadenylation under different cellular conditions by direct interaction of PARN with the polyadenylation factor CPEB. Moreover, Mauxion *et al* (2008) have shown that the tumour suppressor BTG2 is a general activator of the cytoplasmic deadenylases Pop2/Caf1 and Ccr4, and that overexpression of

Figure 8 A model of poly(A) tail dynamics after DNA damage. In the absence of DNA damage treatment, CBC binds to nuclear PARN, inhibiting its deadenylase activity. Under those conditions, CBC also enhances the polyadenylation of pre-mRNAs by increasing the stability of the RNA/CstF complex. As a result of these functional interactions, polyadenylation takes place and normal levels of total mRNA are observed. After exposure to UV treatment, the elongating RNAP II–CstF holoenzyme complex stalls at the sites of damage. A BRCA1/BARD1-containing complex is recruited to sites of repair, inhibiting RNAP II and the associated polyadenylation machinery by ubiquitination followed by degradation of the RNAP II. Under those conditions, the CBP80 protein dissociates from PARN, allowing binding of PARN to the CstF-50/BARD1 complex. As a result of these functional interactions, polyadenylation is inhibited and a 5'-cap-dependent deadenylation decay pathway is activated, generating the observed decrease in the levels of total mRNA. Given that CstF-50 can functionally interact with all the elements of this model, we propose an important role for this protein in the transcription-coupled DNA damage response.

BTG2 accelerates the deadenylation of several reporters and endogenous transcripts, such as GAPDH and β -actin.

It has been shown that PARN co-purifies with essential nonsense-mediated decay factors (NMD; Lejeune *et al*, 2003; Maquat, 2004) and that siRNA-mediated downregulation of PARN abrogates NMD (Lejeune *et al*, 2003). Although those reports focused on cytoplasmic PARN, it is possible that activation of deadenylation by CstF/PARN/BARD1 complex formation in the nucleus might signal the degradation of those erroneously polyadenylated, prematurely terminated mRNAs, providing a mechanism of nuclear mRNA decay. Consistent with this our previous work showed that prematurely terminated polyadenylated mRNA transcripts can be detected *in vivo* after DNA damage, especially under conditions when the CstF/BARD1/BRCA1 checkpoint is not activated (Mirkin *et al*, 2008).

Control of polyadenylation/deadenylation in the nucleus could represent a mechanism to regulate gene expression, which could be important to allow a rapid response during development or after stress treatment. For example, UV treatment induces a decrease in the cellular levels of total mRNA to avoid the expression of deleterious proteins that may be harmful to the cell (Hanawalt, 1994; Ljungman *et al*, 1999). UV treatment also induces the stabilization of ARE-containing mRNAs, such as c-fos, kin17, c-jun, I κ B and c-myc (Blattner *et al*, 2000), to induce the expression of some proteins involved in DNA repair and cell cycle. Our results indicate that the BARD1/CstF/PARN complex can decrease the mRNA levels of housekeeping genes under DNA-damaging conditions and of ARE-containing genes under non-stress conditions (Figure 6A and B). Consistent with our results, it has been shown that ARE-dependent deadenylation has an important role in the mRNA decay of several oncogenes involved in the regulation of cell growth and differentiation (Blattner *et al*, 2000; Lai *et al*, 2003; Moraes *et al*, 2006). Here we propose that expression of different genes, such as housekeeping and ARE-containing genes, might be regulated in the nucleus by a functional interaction between CstF/BARD1, CBP80 and PARN under different cellular conditions. As the tumour suppressor BARD1 is involved in this response, it is possible that malignant cells show altered levels of polyadenylation of certain mRNAs. Supporting this idea, enhanced polyadenylation has been detected in certain tumour cells (Kumar *et al*, 1995; Scorilas, 2002), polyadenylation is inactivated in the M-phase (Colgan *et al*, 1996, 1998) and expression levels of poly(A) polymerase can interfere with cell growth (Zhao and Manley, 1998). Moreover, anti-proliferative transcription factors, such as BTG2 and TOB, have been shown to enhance deadenylation and subsequent mRNA decay (Ezzeddine *et al*, 2007; Mauxion *et al*, 2008). Furthermore, the expression levels of certain ARE-containing genes, such as c-jun and c-fos, increase significantly in cancer cells (Zajchowski *et al*, 2001; Andersen *et al*, 2002; Milde-Langosch, 2005). Interestingly, microRNAs, which have been either directly involved in human cancers or described as oncogenes or tumour suppressors, can direct rapid deadenylation of mRNAs and subsequent decay (Wu *et al*, 2006; Zhang *et al*, 2007).

Taken together, it can be concluded that regulation of the levels of 3' end polyadenylation is an important event in controlling cell growth and in the response to certain stresses, such as UV treatment, and that polyadenylation/deadenyla-

tion process may represent a new mode of global regulation of gene expression.

Materials and methods

Tissue culture methods and DNA-damaging agents

HeLa cells were cultured in Dulbecco's modified Eagles medium (DMEM)– fetal bovine serum (FBS; 10%). U2OS cells stably transfected with different BARD1 expression constructs were cultured as described by Kim *et al* (2006). Cultures at 90% confluence were exposed to UV and harvested at the indicated time points. UV doses (20 or 40 J m⁻²) were delivered in two pulses using a stratlinker (Stratgene). Before pulsing, the medium was removed and replaced immediately after treatment.

NE preparation and immunoblot analysis

After UV treatment, NEs were prepared from harvested cells essentially as described (Kleiman and Manley, 2001). The NEs were quickly frozen and stored at -80°C . A 60- μg weight of each NE was analysed by immunoblotting using the indicated antibodies.

Knockdown expression of PARN and BARD1/BRCA1 in HeLa cells using siRNA

An siRNA specific for human PARN, BARD1/BRCA1 and a control siRNA used as non-silencing were obtained from Dharmacon. The knockdowns and NE preparation were performed as described by Kleiman *et al* (2005). A fraction of the cells were exposed to UV and harvested after the indicated time periods.

Immunoprecipitation analysis

A 100- μg weight of total protein from each NE was pre-cleared with 50 μl of protein-A-Sepharose and immunoprecipitated with an anti-CstF-64 mAb (generously provided by Dr Manley, Columbia University), an anti-PARN pAb (generously provided by Dr Wormington, University of Virginia; Korner *et al*, 1998), an anti- α -H2A pAb (Millipore), anti-Flag M2 (Sigma) or preimmune sera bound to protein-A-agarose beads as described before (Kleiman and Manley, 1999). The beads were recovered by centrifugation and treated at 4°C with 50 μg of RNase A per ml for 10 min or with 0.033 U μl^{-1} of micrococcal nuclease (Sigma). Aliquots of pellets and supernatants were analysed by SDS-PAGE and immunoblotting.

Purification of recombinant proteins

cDNAs encoding the full-length CstF-50 and BARD1 were inserted into pGEX-2TK and expressed in *Escherichia coli*, and GST-fusion proteins were purified by binding to and elution from glutathione-agarose beads as described by Kleiman and Manley (1999). A plasmid encoding His-PARN and its derivatives were transformed into BL21 cells; His-fusion proteins were expressed and purified by binding to and elution from an Ni-agarose column (Qiagen) as described by Nilsson and Virtanen (2006). Protein samples were treated at 4°C with 50 μg of RNase A per ml for 10 min during the binding assays.

Protein–protein interaction assays

Interaction assays using GST-fusion proteins were performed as described by Kleiman and Manley (2001). Samples were treated at 4°C with 50 μg of RNase A per ml for 10 min or with 0.033 U μl^{-1} of micrococcal nuclease (Sigma). Equivalent amounts of pellets and supernatants were analysed by immunoblotting. Competition assays using His-PARN were performed as described above except for the binding buffer (20 mM HEPES (pH 7.9), 0.5 M KCl, 0.5% NP-40, 10% glycerol, 2 mM β -mercaptoethanol and 2.5 mM imidazole).

3' Cleavage assays

³²P-labelled L3 pre-mRNA substrates were prepared as described by Kleiman and Manley (1999). Protein concentrations of the extracts were equalized by Bradford assays (Bio-Rad) before use in processing reactions. Cleavage assays using equivalent amounts of total protein were performed as described by Kleiman and Manley (1999).

Deadenylation assays

³²P-labelled L3(A₃₀) or ML43(G₁₅) substrates were prepared as described by Aström *et al* (1991) and Martinez *et al* (2000). Deadenylation assays using His-PARN, PARN derivatives and using different concentrations of GST-CstF-50, GST-BARD1 and GST-CBP80 were performed as described by Martinez *et al* (2001). Incubations were performed at 30°C for different time periods; the reactions were terminated and analysed by either electrophoresis on 10% polyacrylamide/7 M urea gels or by one-dimensional TLC. TLC on PEI-cellulose-F plates (Merck) was performed as described by Martinez *et al* (2000). The liberated 5'AMP product was analysed by TLC using 0.75 M KH₂PO₄ (pH 3.5) (H₃PO₄) as solvent.

Analysis of endogenous mRNAs by qRT-PCR

Total nuclear RNA was purified from HeLa and U2OS cells using the RNeasy kit (Qiagen). The RNA concentration of the total RNA samples obtained under different conditions was equalized. Equivalent amounts (2 µg) of purified RNA were used as a template to synthesize cDNA using random hexamer primers or oligo-d(T) primers and MMLV reverse transcriptase (Promega) according to the manufacturer's protocol. Commercially available primers were used in the qRT-PCR reactions (Applied Biosystems). For the experiment where the poly(A)⁺/total mRNA ratio was determined (Figure 7B), poly(A)⁺ was prepared by treating total nuclear RNA samples (198 µg) with Oligotex resins (Qiagen), yielding 6–8 µg of poly(A)⁺ RNA. RT reactions were performed using random primers and an

equal volume of both total RNA and poly(A)⁺ RNA samples. Equal amounts of total or poly(A)⁺ cDNAs were used in the qRT-PCR reactions performed using specific primers to observe the poly(A) enrichment as described by Gomes *et al* (2006). Relative quantification of both cDNA samples was performed by using standard curves of known amounts of total cDNA.

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Conflict of interest

The authors declare that they have no conflict of interest.

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