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A role for hnRNP C1/C2 and Unr in internal initiation of translation during mitosis

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The upstream of N-Ras (Unr) protein is involved in translational regulation of specific genes. For example, the Unr protein contributes to translation mediated by several viral and cellular internal ribosome entry sites (IRESs), including the PITSLRE IRES, which is activated at mitosis. Previously, we have shown that translation of the Unr mRNA itself can be initiated through an IRES. Here, we show that UNR mRNA translation and UNR IRES activity are significantly increased during mitosis. Functional analysis identified hnRNP C1/C2 proteins as UNR IRES stimulatory factors, whereas both polypyrimidine tract-binding protein (PTB) and Unr were found to function as inhibitors of UNR IRES-mediated translation. The increased UNR IRES activity during mitosis results from enhanced binding of the stimulatory hnRNP C1/C2 proteins and concomitant dissociation of PTB and Unr from the UNR IRES RNA. Our data suggest the existence of an IRES-dependent cascade in mitosis comprising hnRNP C1/C2 proteins that stimulate Unr expression, and Unr, in turn, contributes to PITSLRE IRES activity. The observation that RNA interference-mediated knockdown of hnRNP C1/C2 and Unr, respectively, abrogates and retards mitosis points out that regulation of IRES-mediated translation by hnRNP C1/C2 and Unr might be important in mitosis.

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Introduction

During mitosis, protein synthesis is rapidly and severely repressed (−75%). Inhibition of translation during mitosis results mainly from inhibition at the initiation step of global cap-dependent translation (Scharff and Robbins, 1966; Fan and Penman, 1970). In general, translation is initiated by the recruitment of the small ribosomal subunit to the cap structure, a process that is mediated by several canonical eukaryotic initiation factors: eIF3 (binds the 40S small ribosomal subunit), eIF4E (binds the cap) and eIF4G (binds both eIF4E and eIF3) (Gingras *et al*, 1999). During mitosis, hypophosphorylation of both eIF4E (cap-binding protein) and its interacting protein 4E-BP1 abrogates this recruitment (Bonneau and Sonenberg, 1987; Pyronnet *et al*, 2001). However, it is well known that progression through mitosis depends on the expression of critical proteins, implying that the corresponding mRNAs can escape the global inhibition of cap-dependent translation during mitosis (Sheets *et al*, 1994; Groisman *et al*, 2000). Indeed, few genes have been described to be translated by an alternative cap-independent mechanism that can over-ride the global protein synthesis inhibition. This mechanism is driven by an internal ribosome entry site (IRES), which allows the expression of the respective gene in specific conditions when general cap-dependent protein synthesis is impaired. These conditions include mitosis as well as hypoxia, apoptosis, viral infection and amino-acid starvation (Holcik, 2004). IRESs, originally discovered in picornaviruses, are *cis*-acting elements, located mainly at the 5′-UTR of the mRNA. They allow initiation of translation by recruitment of the small ribosomal subunit to its secondary RNA structure close to the initiator AUG, independent of the 5′ cap (Jang *et al*, 1988; Pelletier and Sonenberg, 1988). IRESs that are active mainly during the G2/M stage of the cell cycle include cellular IRESs, as well as viral IRESs, such as hepatitis C and HIV type I (Cornelis *et al*, 2000; Honda *et al*, 2000; Brasey *et al*, 2003). The importance of IRES-mediated translation during G2/M is exemplified by the CDK11/PITSLRE^{p58} kinase. This kinase isoform, which is exclusively translated during G2/M via an IRES, is required for centrosome maturation and bipolar mitotic spindle formation during mitosis (Cornelis *et al*, 2000; Petretti *et al*, 2006). Although the exact molecular mechanism of IRES-mediated internal initiation of translation is still unclear, it is understood that most known IRESs depend on the binding of several IRES *trans*-acting factors for efficient initiation of translation (Borovjagin *et al*, 1994; Pilipenko *et al*, 2000). We have shown previously that the 'upstream of N-Ras protein' (Unr) contributes to PITSLRE IRES-mediated translation, generating CDK11/PITSLRE^{p58} during mitosis (Tinton *et al*, 2005). Interestingly, the enhanced activity of the PITSLRE IRES during mitosis corresponds with the binding of Unr to the PITSLRE IRES, owing to enhanced Unr protein expression. It was shown that the UNR mRNA itself can be translated by an IRES (Cornelis *et al*, 2005). In the present study, we investigated UNR IRES-mediated translation during the G2/M phase of the cell cycle and its respective requirement for *trans*-acting factors. In addition to the formerly identified polypyrimidine tract-binding protein (PTB)

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(Cornelis *et al*, 2005), we here identify hnRNP C1/C2 and Unr protein as factors that interact with the UNR IRES. Functional analysis indicated that hnRNP C1/C2 is an UNR IRES *trans*-stimulating factor, whereas Unr is an inhibitor of UNR IRES-mediated translation. In correspondence with these observations, we found that the binding of hnRNP C1/C2 is remarkably enhanced at the G2/M phase of the cell cycle, whereas the binding of Unr and PTB is repressed. The functional relevance of hnRNP C1/C2 and Unr proteins in mitosis is underscored by the observation that RNA interference (RNAi)-mediated knockdown of hnRNP C1/C2 and Unr, respectively, abrogates and retards mitosis. Altogether, we provide evidence for the existence of an IRES-regulated signaling cascade that might be important for proper transition through mitosis.

Results

UNR IRES activity is induced during the late G2/M phase of the cell cycle

Previously, we provided evidence that translation of the UNR mRNA can be initiated by an internal initiation mechanism. We also observed that Unr protein expression is enhanced at G2/M, most likely because of its role in the regulation of cell-cycle-dependent IRESs, such as the cellular PITSLRE IRES and the viral HCV IRES, during G2/M stage of the cell cycle (Lu *et al*, 2004; Tinton *et al*, 2005). These observations led us to investigate whether the UNR IRES contributes to the upregulation of Unr protein expression at G2/M. UNR IRES activity was examined using bicistronic reporter plasmids in which either the UNR IRES (Di-pRF-UNR) or no sequence (Di-pRF) was cloned between the coding sequences of *Renilla* (first cistron) and firefly luciferase (second cistron). These plasmids were transfected in HEK293 T cells and transfectants were grown asynchronously or blocked with thymidine in the G1/S phase. When the thymidine block was released, the cells synchronously progressed through the S and G2 phase and reached mitosis after 12 h, characterized by cell swelling and DNA condensation (data not shown). Western blot (WB) analysis of cyclin B1 expression confirmed that the cell population was enriched in cells in the G2/M stage of the cell cycle (Figure 1A). In correspondence with our previous observations, enhanced Unr protein expression was detected in mitotic cells. Analysis of the UNR IRES activity in the corresponding Di-pRF-UNR transfectants revealed that UNR IRES-driven translation was 2.5-fold higher in mitotic cells than in cells arrested in the G1/S phase (Figure 1B). In contrast, the ratio between firefly and *Renilla* luciferase activity remained unchanged in Di-pRF-transfected cells irrespective of the treatment. These data indicate that UNR IRES-mediated translation is enhanced in mitosis and might contribute to the enhanced expression of Unr proteins.

The importance of UNR IRES-mediated translation during mitosis was further underscored in an analysis of the distribution of the UNR mRNA between subpolysomes and polysomes during cell cycle progression when compared to a cap-dependent β-actin mRNA. We used Ba/F3 cells that were synchronized in G1 by IL-3 depletion for 14 h. At the indicated time points after restimulation with IL-3, Ba/F3 cells were harvested and samples were prepared for quantitative PCR analysis, WB analysis and mitotic index determination. Cyclin B1 expression and mitotic index indicate that

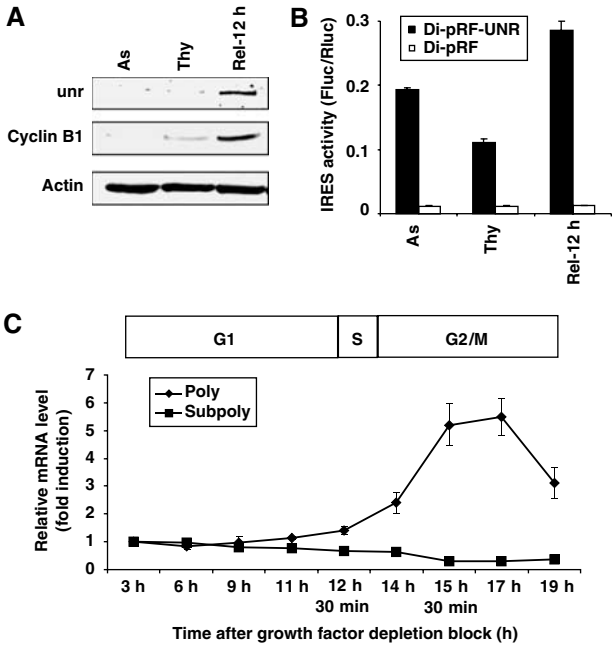

Figure 1 UNR IRES activity is enhanced during mitosis. HEK293 T cells were transfected with the bicistronic reporter vectors containing either the UNR IRES (Di-pRF-UNR) or no intercistronic sequence (Di-pRF) between the *Renilla* and firefly luciferase coding sequences. Subsequently, part of the transfectants were either grown asynchronously (As) or arrested at the S phase by thymidine (Thy) or collected at mitosis 12 h after thymidine block release (Rel-12 h). (**A**) WB analysis of cell extracts from asynchronously growing (As), S-phase arrested (Thy) and mitotic cells (Rel-12 h) was performed using the indicated antibodies. (**B**) UNR IRES activity was calculated as the ratio between firefly and *Renilla* luciferase activities in cell extracts. The bars represent the averages ($n = 3$) ± s.d. (**C**) Distribution of the UNR mRNA between subpolysomes and polysomes during cell cycle progression using real-time quantitative-PCR. Ba/F3 were synchronized in G1 by IL-3 depletion for 14 h. Polysomal and subpolysomal RNA samples were prepared at indicated time points after restimulation with IL-3. The RT-PCR was performed on the corresponding cDNA synthesized from each sample. Data were analyzed using the $^{2-\Delta\Delta C_T}$ method.

the amount of G2/M-specific cells start to peak at time point 14 h until time point 17 h after growth factor deprivation block (Figure 1 and Supplementary data, Sup_4.pdf).

Relative changes in the distribution of the UNR mRNA between subpolysomes and polysomes during cell cycle progression were monitored by real-time quantitative PCR. Polysomal and subpolysomal RNA samples were prepared at the indicated time points. Real-time-PCR was performed on the corresponding cDNA synthesized from each sample.

Using the $^{2-\Delta\Delta C_T}$ method, the data are presented as the fold change in gene expression (Livak and Schmittgen, 2001). They are normalized to the endogenous β-actin reference gene and are relative to the amount of transcript expressed at time point 3 h (Figure 1C). This analysis shows that the unr mRNA remains associated with the polysomes in mitotic cells suggesting that the UNR IRES does indeed function efficiently in M phase.

HnRNP C1/C2 binds to the UNR IRES during the G2/M phase of the cell cycle

In order to elucidate the molecular mechanism driving UNR IRES-mediated translation during mitosis, we aimed at

identifying UNR IRES *trans*-acting factors that bind to the IRES during the G2/M transition of the cell cycle. In UV crosslinking assays, radiolabeled UNR IRES RNA was incubated with cytoplasmic cell extracts derived from untreated asynchronously proliferating HEK293 T cells or from nocodazole-treated HEK293 T cells. Nocodazole inhibits microtubule polymerization and arrests the cells at the G2/M transition of the cell cycle. Comparison of the two UV crosslinking profiles reveals that the crosslinking of a 41–43 kDa protein doublet to radiolabeled UNR IRES RNA is strongly enhanced during mitosis (Figure 2A). The molecular size of the protein doublet corresponds to that of the hnRNP C1/C2 proteins, which are known to be involved in c-myc, XIAP and c-sis IRES-mediated translation (Sella *et al*, 1999; Holcik *et al*, 2003; Kim *et al*, 2003). RNA affinity chromatography was performed to confirm the identity of the 41–43 kDa protein doublet. Biotinylated UNR IRES RNA, immobilized on streptavidin-agarose beads, was mixed with cell extracts from asynchronously growing HEK293 T cells or from nocodazole-arrested HEK293 T cells. After intensive washing, UNR IRES-bound proteins were eluted from the beads and analyzed by WB with anti-hnRNP C1/C2 antibodies. As shown in Figure 2B, the UNR IRES can indeed efficiently precipitate hnRNP C1/C2 proteins from cytoplasmic cell extracts derived from G2/M-arrested cells. Therefore, the 41–43 kDa protein doublet detected in Figure 2A most likely corresponds to endogenous hnRNP C1/C2 proteins. The interaction of UNR mRNA with hnRNP C1/C2 proteins was further confirmed by the observation that endogenous UNR mRNA was specifically copurified with immunoprecipitated endogenous hnRNP C1/C2 proteins as detected by RT-PCR (data not shown).

In order to identify the regions within the UNR IRES RNA that are involved in the binding of hnRNP C1/C2, several deletion mutants were generated (Figure 2C). HnRNP C1 is known to have a high affinity for U-rich stretches (Gorlach *et al*, 1994). The UNR IRES RNA contains two of these oligo(U) stretches: a discontinuous stretch located at position 259–269 and a continuous stretch located at position 348–355. To map the hnRNP C1/C2 binding pocket, binding of Flag-tagged hnRNP C1 to different UNR IRES fragments was examined by RNA affinity chromatography. Figure 2D illustrates that Flag-tagged hnRNP C1 can be precipitated by the UNR IRES and the UNR (1–390) IRES fragment, which contain both oligo(U) stretches (Figure 2D). However, binding of hnRNP C1 was strongly reduced with the fragments lacking the continuous oligo(U) stretch (UNR (1–334) and

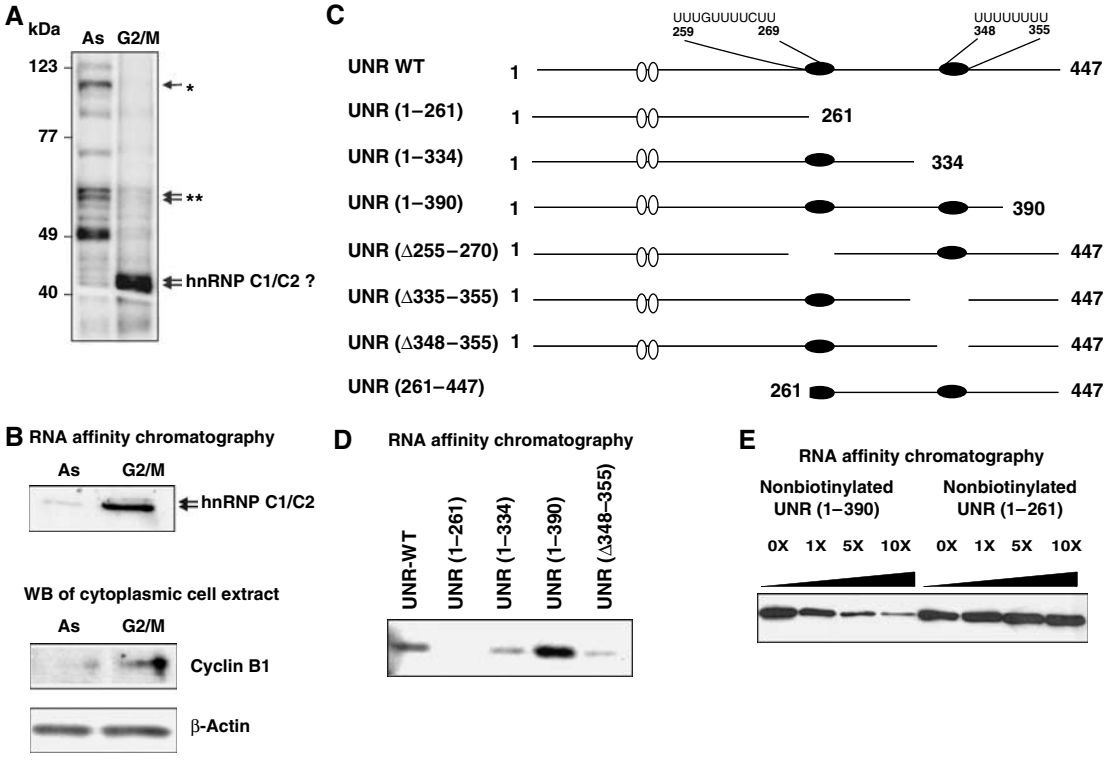

Figure 2 HnRNP C1/C2 binds to the UNR IRES during mitosis. (**A**) UV crosslinking assays were performed by incubating a ^{32}P-labeled RNA probe corresponding to the UNR IRES in the presence of cytoplasmic cell extracts from either asynchronously growing HEK293 T cells (As) or HEK293 T cells arrested at the G2/M transition by nocodazole (G2/M). The arrows depict the expected position of endogenous hnRNP C1/C2 proteins. The asterisks indicate bands that might correspond to crosslinked Unr (*) and PTB (**) proteins. (**B**) UNR IRES RNA affinity chromatography was performed with cytoplasmic extracts from either asynchronously growing HEK293 T (As) cells or HEK293 T cells arrested by nocodazole (G2/M). UNR IRES-bound proteins were analyzed by WB using anti-hnRNP C1/C2 antibodies (upper panel). WB analysis of the cytoplasmic cell extracts used for RNA affinity chromatography was performed with the indicated antibodies. (**C**) Schematic representation of the UNR IRES deletion mutants used in this paper. The black ellipses indicate the two oligo(U) stretches. The open ellipses indicate a purine stretch. (**D**) The oligo(U) stretch located between nucleotides 348 and355 is needed for efficient binding of hnRNP C1/C2 to the UNR IRES. RNA affinity chromatography was performed with biotinylated RNA corresponding to the indicated UNR IRES fragments using cytoplasmic extracts from hnRNP C1-Flag-expressing HEK293 T cells. (**E**) The binding of hnRNP C1 to the UNR IRES is a specific interaction. UNR IRES RNA affinity chromatography with cytoplasmic extracts from hnRNP C1-Flag-transfected HEK293 T cells was performed in the absence or presence of 1–10 molar excess of non-biotinylated RNA corresponding to either the UNR (1–390) or the UNR (1–261) IRES mutant.

UNR (Δ348–355)) and completely abrogated in the absence of both oligo(U) stretches (UNR (1–261)). In agreement with these results, overexpressed hnRNP C1 and endogenous hnRNP C1/C2 proteins could be UV crosslinked to radiolabeled wild-type (WT) UNR IRES and UNR (1–390) IRES RNA, whereas these proteins could not be detected in the reactions with UNR (1–261) IRES RNA (data not shown). Altogether, these observations indicate that the continuous (348–355) oligo(U) stretch is most important for the binding of hnRNP C1/C2, although the discontinuous oligo(U) stretch might display some affinity for hnRNP C1.

The specificity of the interaction between the UNR IRES and hnRNP C1/C2 was further examined in a UNR IRES RNA affinity chromatography competition assay, in which the cytoplasmic extracts of hnRNP C1-Flag-overexpressing cells were preincubated with a molar excess of either the non-biotinylated UNR IRES (1–390) fragment, which can bind hnRNP C1,or the non-biotinylated UNR IRES (1–261) fragment, which cannot. Figure 2E shows that a molar excess of UNR IRES (1–390) RNA can compete with WT UNR IRES RNA for binding to hnRNP C1, whereas a molar excess of UNR IRES (1–261) RNA does not. This further confirms the specific interaction between hnRNP C1/C2 and the oligo(U) stretch in the UNR IRES.

HnRNP C1 stimulates UNR IRES-mediated translation

To determine if the binding of hnRNP C1 to the UNR IRES affects UNR IRES-mediated translation, we examined the effect of overexpression of hnRNP C1 on UNR IRES-mediated translation. Figure 3A shows that overexpression of hnRNP C1 stimulates the activity of the WT UNR IRES and also of the UNR IRES deletion mutants that still contain the (348–355) oligo(U) stretch involved in the binding of hnRNP C1 (UNR (1–390); UNR (Δ255–270)). In contrast, overexpression of hnRNP C1 did not significantly affect the translation mediated by UNR IRES deletion mutants that lack this oligo(U) stretch (UNR (1–261); UNR (1–334); UNR (Δ335–355)). These observations indicate that the binding of hnRNP C1 to the UNR IRES at the continuous oligo(U) stretch enhances its activity. In accordance with the binding experiment shown in Figure 2D, these observations indicate that the hnRNP C1 stimulating role on UNR IRES-mediated translation is mainly dependent on the presence of the continuous (348–355) oligo(U) stretch.

To exclude that translation of the second cistron could be initiated from an aberrant mRNA species generated through putative promoter activity or alternative splicing mediated by the cloned IRES sequence, we analyzed the effect of siRNAs directed against either the first or second cistron on the expression of both cistrons. siRNA directed against the first or second cistron should impede translation of both cistrons to the same extent only if translation of the first and second cistron is initiated from an intact bicistronic mRNA. As a positive control for a well-established IRES, we used Di-pRF-cmyc. To knock down the first or the second cistron, cells were transfected with either Di-pFR-UNR or Di-pRF-UNR, each in combination with either an empty vector or an hnRNP C1 expression vector in the presence of siRNA directed against the coding sequence of firefly luciferase (si Fluc). Di-pFR-UNR is identical to Di-pRF-UNR with the exception that *Renilla* luciferase was replaced by the firefly luciferase coding sequence and 'vice versa'. Supplementary Figure 2A and B illustrates that knockdown of either the first or the second cistron results in abrogation of the expression of both Fluc and Rluc from the respective dicistronic mRNAs (Sup_4.pdf). These results clearly indicate that translation of both cistrons is initiated from an intact bicistronic mRNA, and that the observed hnRNP C1-stimulated translation of the second cistron does not result from the generation of aberrant cryptic monocistronic mRNAs after hnRNP C1 overexpression.

The impact of hnRNP C1/C2 binding on the activity of the UNR IRES was also investigated by RNAi-mediated knockdown of endogenous hnRNP C1/C2 protein expression. We transfected several bicistronic reporter plasmids in HEK293 T cells in combination with either hnRNP C1/C2-specific siRNA (si hnRNP C1/C2) or nonspecific siRNA (si none). Transfection of si hnRNP C1/C2 into HEK293 T cells resulted in a large reduction of endogenous hnRNP C1/C2 protein expression as compared to transfection of nonspecific siRNA, but did not affect the expression of unrelated β-actin (Figure 3B). Knockdown of hnRNP C1/C2 results in a two-fold decrease in the activity of the UNR IRES and of the UNR IRES (1–390) deletion mutant that is still capable of binding hnRNP C1/C2 (Figure 3B). In contrast, hnRNP C1/C2 knockdown did not significantly affect the activity of the UNR IRES (1–261) deletion mutant that cannot bind hnRNP C1/C2.

To test the physiological significance of hnRNP C1/C2 in Unr protein expression, we investigated the effect of siRNA-mediated hnRNP C1/C2 knockdown on Unr protein levels. Figure 3C demonstrates that knockdown of hnRNP C1/C2 in HEK293 T cells is associated with reduced levels of Unr protein expression. A similar effect of hnRNP C1/C2 knockdown on Unr protein expression was also observed in HeLa cells (data not shown). Altogether, these data demonstrate that hnRNP C1/C2 is a relevant positive regulator of UNR IRES-mediated translation and Unr protein expression.

Binding of hnRNP C1/C2 during G2/M is involved in the induction of UNR-IRES activity

Next, we investigated whether deletion of the UNR IRES oligo(U) stretch, needed for the binding of hnRNP C1/C2, impedes the induction of UNR IRES-mediated translation during mitosis. HEK293 T cells were transfected with a bicistronic reporter construct containing either the WT UNR IRES or the UNR (Δ335–355) IRES deletion mutant as intercistronic sequence. After arresting these transfectants in the G1/S phase of the cell cycle by thymidine treatment, the cells were released from the thymidine block by refreshing the medium. After 12 h, chromosomal condensation and cellular swelling indicated that the cells were in mitosis. Samples were taken from cells arrested in G1/S and from dividing cells. As expected, the activity of the WT IRES was clearly higher during mitosis than during G1/S arrest (Figure 3D). In contrast, we could not observe a noteworthy difference in the activity of the UNR (Δ335–355) IRES deletion mutant between cells in the G1/S phase and mitotic cells. These data indicate that the UNR IRES oligo(U) stretch, which is needed for interaction with hnRNP C1/C2, is essential for the induction of UNR IRES activity during mitosis, indicating a crucial role for hnRNP C1/C2.

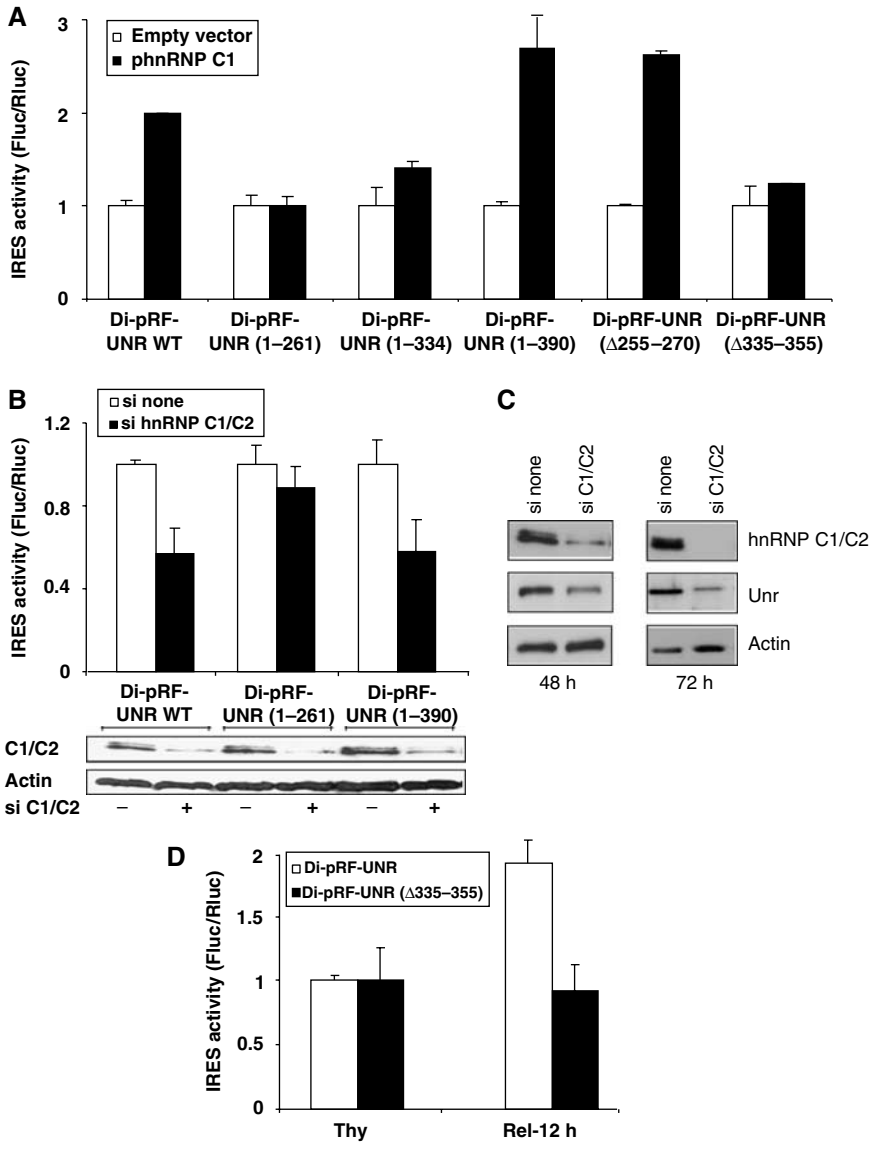

Figure 3 HnRNP C1 is a positive modulator of UNR IRES activity. (**A**) Overexpression of hnRNP C1 stimulates UNR IRES-mediated translation. The indicated bicistronic reporter vectors were transfected into HEK293 T cells in combination with either an empty expression vector or 25 ng of an expression vector containing the hnRNP C1 coding sequence. The IRES activity was calculated as the ratio between firefly and *Renilla* luciferase. The white bars represent the average ($n=3$) ± s.d. IRES activity of the different UNR IRES mutants in cells not overexpressing hnRNP C1; this value was set as one. The black bars represent the average ($n=3$) ± s.d. relative IRES activity of the different UNR IRES mutants in cells overexpressing hnRNP C1. (**B**) Knockdown of hnRNP C1/C2 expression inhibits UNR IRES-mediated translation. The indicated dicistronic plasmids were transfected into HEK293 T cells in combination with siRNAs directed against either no known mRNA (si none) or against hnRNP C1/C2 (si hnRNP C1/C2). The white bars represent the average ($n=3$) ± s.d. IRES activity (Fluc/Rluc) of the different UNR IRES mutants in cells with normal hnRNP C1/C2 expression; this value was set as one. The black bars represent the relative average ($n=3$) ± s.d. IRES activity of the different UNR IRES mutants in cells with downregulated hnRNP C1/C2 expression. The lysates used for luciferase measurements were subjected to WB analysis using anti-hnRNP C1/C2 (upper panel) or anti-β-actin (lower panel) antibodies. (**C**) Knockdown of hnRNP C1/C2 is associated with reduced Unr protein expression. HEK293 T cells were transfected with either si none or si hnRNP C1/C2 (indicated as si C1/C2). Twenty-four hours later, the cells were seeded in six-well plates and grown for an additional 24 or 48 h before total cell extracts were analyzed by WB using anti-hnNRP C1/C2, anti-Unr and anti-β-actin antibodies. (**D**) HEK293 T cells were transfected with the indicated bicistronic vectors and subsequently arrested in G1/S by thymidine or collected at mitosis 12 h after thymidine block release. The bars represent the average ($n=3$) ± s.d. of the IRES activities (Fluc/Rluc). The IRES activity measured in the asynchronously grown transfectants was set as one.

The Unr protein itself is a potential UNR IRES trans-inhibiting factor

Unr is a member of the cold-shock domain protein superfamily. Interestingly, several of these proteins, both eukaryotic and prokaryotic, are known to regulate their own expression at the level of translation (Graumann and Marahiel, 1998; Fukuda *et al*, 2004). We questioned whether the Unr protein itself is a UNR IRES *trans*-acting factor. Therefore, we investigated the binding of the Unr protein to the UNR IRES and analyzed the impact of this potential binding on UNR IRES-driven translation.

In the above-described UV crosslinking reactions with the UNR IRES probe in the presence of HEK293 T cytoplasmic cell extracts, one of the most intensive bands migrates as a

100 kDa protein, which is approximately the size of the Unr protein (Figure 2A). RNA affinity chromatography revealed that both the WT UNR IRES and the UNR (1–261) IRES deletion mutant can efficiently bind to endogenous Unr proteins, whereas the UNR (261–447) mutant cannot (Figure 4A). Similar results were obtained by UV crosslinking assays (data not shown). The specificity of this interaction was further confirmed in a competition experiment (Figure 4B). Altogether, these results indicate that the Unr protein binds to the 5′-half of the UNR IRES. This region indeed contains several purine-rich nucleotide tracts (Figure 2C), which are known to be the favorable binding sequences for Unr (Triqueneaux *et al*, 1999).

Next, we examined whether binding of the Unr proteins impacts UNR IRES-driven translation. Bicistronic reporter assays indicated that overexpression of Unr inhibits the activity of the WT UNR IRES and the UNR (1–264) IRES deletion mutant that can bind the Unr protein (Figure 4C). In contrast, translation mediated by the UNR (261–447) IRES deletion mutant, which cannot interact with the Unr protein, was not considerably affected by overexpression of UNR.

To exclude that the impact of Unr overexpression on UNR IRES activity is due to the generation of cryptic monocistronic mRNAs, we analyzed the effect of siRNAs directed against either the second or first cistron on the expression of both cistrons. Overexpression of Unr did not affect the impact of firefly luciferase knockdown on the expression of both cistrons in Di-pRF-UNR- or Di-pFR-UNR-transfected cells (Supplementary Figure 3: Sup_4.pdf). These results demonstrate that translation of both cistrons is indeed initiated from an intact bicistronic mRNA, and that Unr-inhibited translation of this second cistron does not result from the synthesis of aberrant cryptic monocistronic mRNAs. Altogether, these data suggest that the Unr protein is a negative regulator of UNR IRES-mediated translation

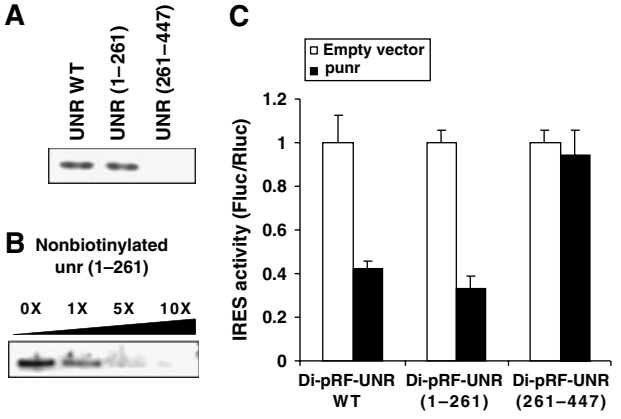

Figure 4 The Unr protein is a negative regulator of UNR IRES-driven translation. (**A**) The Unr protein binds to the UNR IRES. Biotinylated RNA corresponding to the indicated UNR IRES fragments bound to streptavidin beads was incubated with cytoplasmic extracts from HEK293 T cells. Binding of endogenous Unr was analyzed by WB using anti-unr antibodies. (**B**) The binding of Unr to the UNR IRES is a specific interaction. UNR IRES affinity chromatography was performed with cytoplasmic extracts of HEK293 T cells in the absence or presence of 1–10 molar excess of non-biotinylated RNA corresponding to the UNR (1–261) IRES mutant. UNR IRES-bound proteins were analyzed by WB using anti-unr antibodies. (**C**) Overexpression of Unr inhibits UNR IRES-driven translation. The indicated bicistronic reporter vectors were transfected into HEK293 T cells in combination with either an empty expression vector or 25 ng of an expression vector containing the Unr coding sequence. The white bars represent the average ($n=3$) ± s.d. of the IRES activity (Fluc/Rluc) of the different UNR IRES mutants in cells not overexpressing Unr; this value was set as one. The black bars represent the relative average ($n=3$) ± s.d. of the IRES activity of the different UNR IRES mutants in cells overexpressing Unr.

Interplay between hnRNP C1/C2, PTB and Unr during G2/M

So far, we have identified three *trans*-regulating factors for the UNR IRES. HnRNP C1/C2 proteins are stimulating factors, whereas PTB (Cornelis *et al*, 2005) and Unr are negative regulators. Next, we investigated a potential role for these factors in the activation of UNR IRES activity during G2/M. We first analyzed the binding of these proteins to the UNR IRES at the G2/M transition of the cell cycle. Figure 2A shows that next to enhanced binding of hnRNP C1/C2 proteins to the UNR IRES, the pre-mitotic phase is also associated with a reduced binding of a 56–58 kDa protein doublet and of a 100 kDa protein, most likely corresponding to PTB and Unr, respectively. These observations were further confirmed by UNR IRES RNA affinity chromatography with cytoplasmic extracts of cells either grown asynchronously or arrested at the G2/M transition. WB analysis of the proteins that bound to the biotinylated UNR IRES RNA revealed that, in contrast to hnRNP C1/C2, the interactions with both PTB and Unr were considerably reduced in the presence of cytoplasmic extracts from nocodazole-treated cells (Figure 5A, left panel). These results indicate that the observed decreased binding of the inhibitory proteins, Unr and PTB, to the UNR IRES at the start of mitosis might contribute to enhanced UNR IRES-mediated translation during cell division. To evaluate whether differential binding of the *trans*-regulating factors (Unr, PTB and hnRNP C1) to the UNR IRES RNA in G2/M is related to their expression levels in the cytoplasm, we measured their cytoplasmic levels in normal and nocodazole-treated cells. Both hnRNP C1/C2 and PTB are localized mainly in the nucleus and migrate to the cytoplasm only under certain conditions. So the enhanced binding of hnRNP C1/C2 to the UNR IRES might be a consequence of the translocation of hnRNP C1/C2 proteins from the nucleus to the cytoplasm. Indeed, WB analysis of the cytoplasmic cell extracts used for UNR IRES RNA affinity chromatography showed that hnRNP C1/C2 proteins accumulate in the cytoplasm of cells arrested at G2/M transition (Figure 5A, right panel). The reduced binding of PTB and Unr proteins to the UNR IRES in cells arrested at the G2/M transition does not correspond with the unchanged low expression levels of cytoplasmic PTB and the enhanced cytoplasmic Unr expression levels, respectively. Possibly, hnRNP C1/C2 proteins compete with the inhibitory PTB and Unr proteins for interaction with the UNR IRES during mitosis. To evaluate this possibility, we performed UNR IRES RNA affinity chromatography using cytoplasmic extracts of either mock-transfected HEK293 T cells, HEK293 T cells overexpressing hnRNP C1 or mixtures of both cytoplasmic extracts. Indeed, binding of endogenous PTB and Unr proteins to the biotinylated UNR IRES RNA is significantly reduced in the presence of increasing amounts of hnRNP C1 (Figure 5B, left panel). Similarly, overexpression of hnRNP C1 negatively affects crosslinking of radiolabeled UNR IRES RNA to both endogenous PTB

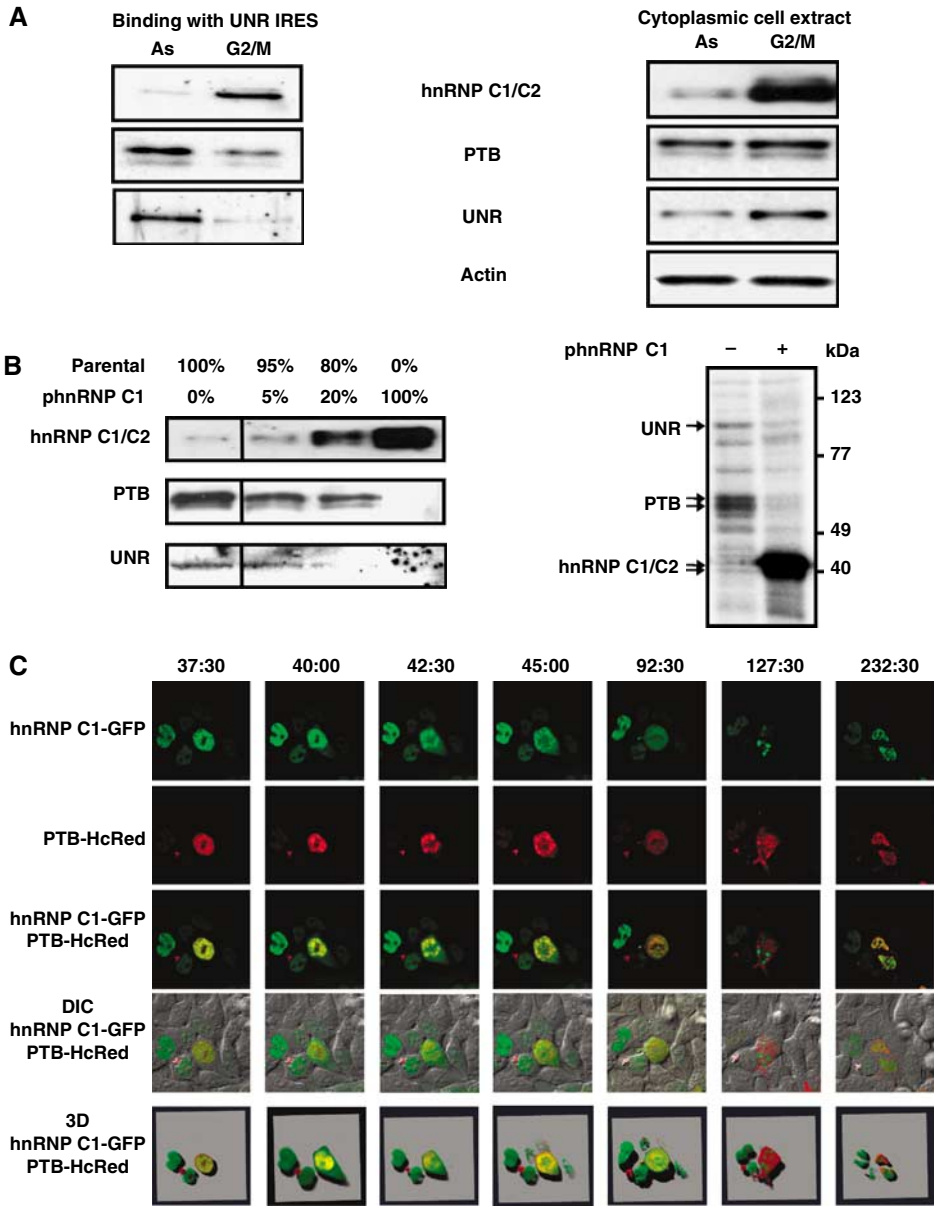

Figure 5 Binding of UNR IRES *trans*-acting factors at the onset of mitosis. (**A**) Mitosis is associated with diminished binding of Unr and PTB. Left: UNR IRES RNA affinity chromatography was performed with cytoplasmic extracts from either asynchronously (As) growing HEK293 T cells or HEK293 T cells arrested by nocodazole (G2/M). UNR IRES-bound proteins were analyzed by WB using the indicated antibodies. Right: WB analysis of the cytoplasmic cell extracts used for RNA affinity chromatography with the indicated antibodies. (**B**) Binding of hnRNP C1/C2 to the UNR IRES impairs the binding of PTB and Unr. UNR IRES RNA affinity chromatography was performed with cytoplasmic extracts derived from either parental HEK293 T cells or hnRNP C1-overexpressing HEK293 T cells or mixtures (95/5, 80/20) of both cell extracts. UNR IRES-bound proteins were analyzed by WB using the indicated antibodies. (**C**) Translocation of hnRNP C1 from the nucleus to the cytoplasm occurs at an earlier time point than translocation of PTB. pPTB-HcRED and pEGFP-hnRNP C1 were cotransfected into HEK293 T cells. After 24 h, the transfectants were placed into a fluorescence time-lapse microscope at 37°C and 5% CO_2. Pictures of phase contrast, green fluorescence and red fluorescence were taken every 2.5 min during 16 h. 3D rendering based on series of z-stack images was performed. The data set is representative for three independent experiments, each following the mitosis of one individual cell.

(56–58 kDa protein doublet) and endogenous Unr (97 kDa protein) (Figure 5B, right panel). These data indicate that binding of hnRNP C1/C2 to the UNR IRES negatively affects the interaction of UNR and PTB proteins with the IRES, either by direct competition for proximal binding sites or by altering the structural features of the IRES.

Together, these data led us to hypothesize that in non-mitotic cells Unr protein expression is kept low by the binding of PTB and Unr to the UNR IRES. However, at the onset of mitosis, hnRNP C1/C2 proteins migrate from the nucleus to the cytoplasm and bind to the UNR IRES RNA, relieving the suppression of UNR IRES-mediated translation (Figure 8).

To confirm that at the onset of mitosis hnRNP C1/C2 migrates from the nucleus to the cytoplasm before PTB, live cell imaging was performed. HEK293 T cells were cotransfected with expression plasmids encoding hnRNP C1 fused to GFP and PTB fused to HcRed. Twenty-four hours after transfection, the expression of hnRNP C1-GFP and PTB-HcRed in living cells was examined by microscopy. As expected, both hnRNP C1-GFP and PTB-HcRed could only be seen in the

nucleus. Subsequently, the localization of hnRNP C1-GFP and PTB-HcRed in cells expressing both fluorescent proteins was monitored every 2.5 min for 16 h using a fluorescence time-lapse microscope. At 37.30 min after initiation of recording in one of the selected cells, both hnRNP C1-GFP and PTB-HcRed were still only detectable in the nucleus (Figure 5C, 37:30). This cell started to detach from the culture matrix 2.5 and 5 min later (40:00 and 42:30 min), indicating the prophase of mitosis. At this time point, hnRNP C1-GFP proteins also localized in the cytoplasm, whereas the expression of PTB-HcRed was still restricted to the nucleus. At time point 45:00, also PTB-HcRed proteins could be detected in the cytoplasm, probably indicating the start of nuclear envelope breakdown. Later, the localization of hnRNP C1-GFP and PTB-HcRed proteins completely overlapped again. Movies illustrating the represented time-lapse experiment and 3D configurations at time point 37:30 and 40:00 are included as Supplementary data (Sup_1_time lapse.mpg, Sup_2_3Da.mpg and Sup_3_3Db.mpg). The full-length movie is available at: http://www.dmbr.ugent.be/cm/images/movies/BS/Schepens_et_al.avi. These data together indicate that the translocation of hnRNP C1/C2 from the nucleus to the cytoplasm occurs at an earlier stage of mitosis than the translocation of PTB.

A role for hnRNP C1/C2 and Unr in progression through the cell cycle

To verify the biological relevance of the above-described molecular interactions, we investigated their significance for proper execution of the cell cycle. Unr and hnRNP C1/C2 protein expression levels were downregulated individually by transfection of siRNA directed against their corresponding mRNAs. Examination of cell proliferation revealed that knockdown of hnRNP C1/C2 protein expression is associated with an extensive inhibition of HEK293 T-cell proliferation

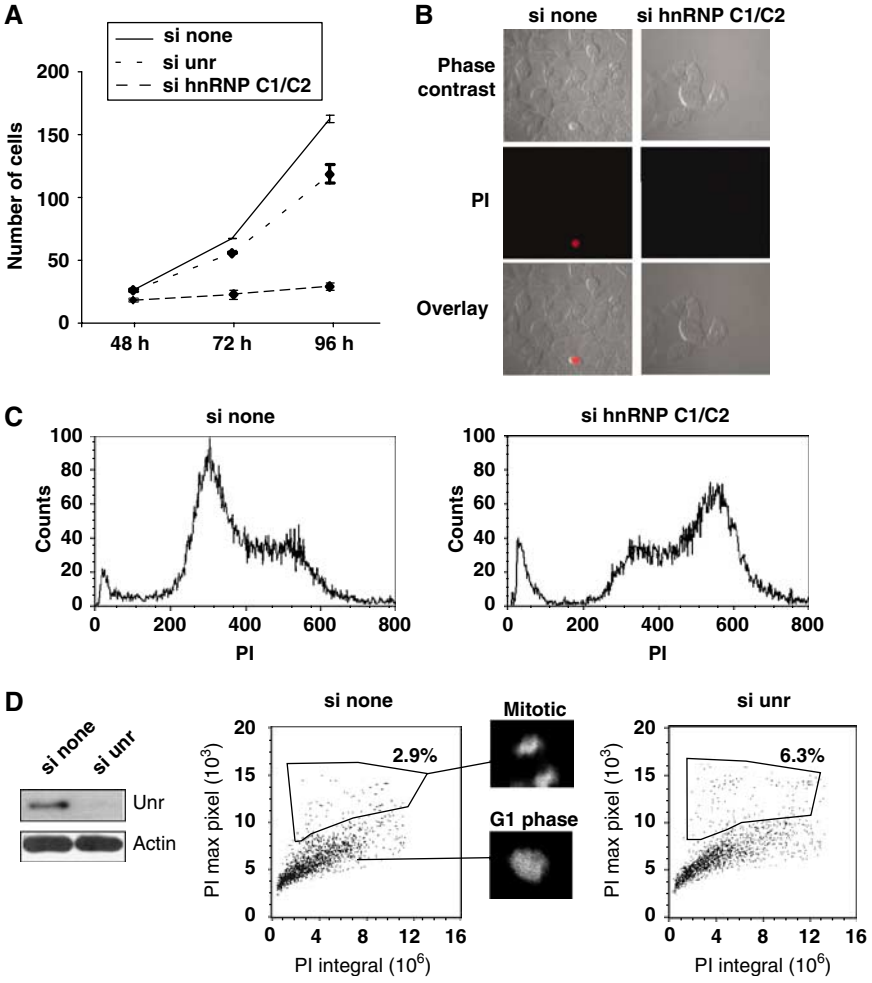

Figure 6 hnRNP C1/C2 and Unr are needed for proper progression through mitosis. (**A**) Knockdown of hnRNP C1/C2 blocks cell growth. HEK293 T cells were transfected with either si none, si hnRNP C1/C2 or si unr. Twenty-four hours later, the cells were seeded in six-well plates and grown for an additional 24, 48 or 72 h before they were counted. (**B**) Knockdown of hnRNP C1/C2 is not associated with enhanced cell death. Ninety-six hours after transfection, PI was added to the cells transfected with either si none or si hnRNP C1/C2. Subsequently phase contrast and red fluorescence pictures were taken. (**C**) Knockdown of hnRNP C1/C2 is associated with an accumulation of cells in the G2/M phase of the cell cycle. Seventy-two hours after transfection, DNA content of the si none and si hnRNP C1/C2 transfectants was assayed by flow cytometric analysis of PI fluorescence. (**D**) Knockdown of Unr is associated with a delay in mitosis. Seventy-two hours after si none (left) or si unr (right) transfection, DNA content (PI integral) and maximal pixel intensity were assayed by laser scanning microscopy. The population of mitotic cells within the PI integral/PI maximal pixel intensity scatter plots was isolated by setting boarders based on retracing cells. An example of PI fluorescence of retraced cells in both the mitotic and intherphase population is shown. Knockdown of Unr protein expression was confirmed by WB analysis of cell extracts of the transfectants using the indicated antibodies.

(Figure 6A) and HeLa cell proliferation (data not shown). Analysis of cell death by measuring propidium iodide (PI) uptake (Figure 6B), lactate dehydrogenase leakage (data not shown) or caspase activity (data not shown) indicates that the defect in the proliferation of hnRNP C1/C2-depleted cells is not the result of enhanced cell death, but rather a consequence of a defect in cell cycle progression. Therefore, we wanted to investigate whether the defect in cell proliferation is associated with an arrest or delay in specific phases of the cell cycle. Because G1 cells are characterized by a diploid ($2n$) DNA content and G2/M cells by a tetraploid ($4n$) DNA content, cell cycle analysis was performed by assessment of cellular DNA content via flow cytometry of PI-stained nuclei. As expected, 72 h after transfection, most (54.6 ± 2.7) of the cells transfected with control siRNA (si none) displayed a diploid DNA content, with only a minority (23.9 ± 0.7) displaying a tetraploid DNA content (Figure 6C, left panel). In contrast, only a minority (21.4 ± 1.1) of hnRNP C1/C2-deprived cells displayed a diploid DNA content, whereas the majority (57.3 ± 1.5) displayed a tetraploid DNA content (Figure 6C, right panel). These data show that knockdown of hnRNP C1/C2 is associated with a shift from the G1 phase to the G2 phase, indicating a defect in G2/M transition.

In comparison to the severe effect of hnRNP C1/C2 knockdown on cell proliferation, knockdown of Unr protein expression is associated with a modest reduction in cell proliferation (Figure 6A). A more detailed analysis of the si unr-treated cell population was performed by determining the mitotic index either visually by fluorescence microscopy (data not shown) or by laser scanning cytometry after PI staining (Figure 6D). Both approaches showed approximately a two-fold increase ($180 \pm 33\%$) in mitotic cells in the si unr-treated cell population as compared to a control cell population treated with si none. Altogether, these data indicate that both hnRNP C1/C2 and Unr are needed for proper progression through the G2/M phase.

An IRES-dependent cascade contributes to PITSLRE IRES-mediated translation

Because also the PITSLRE IRES is active during the G2/M phase of the cell cycle, we investigated whether hnRNP C1/C2 proteins might also be involved in PITSLRE IRES-mediated translation. In order to investigate if hnRNP C1/C2 proteins can interact with the PITSLRE IRES, we performed PITSLRE IRES RNA affinity chromatography using hnRNP C1-overexpressing cells. Previously, we demonstrated that the PITSLRE IRES can efficiently interact with Unr proteins. For the purpose of a positive control, the cells were cotransfected with E-tagged Unr. The UNR IRES, which efficiently interacts with both the hnRNP C1 and Unr proteins, was used as an additional positive control for hnRNP C1 interaction. As expected, both overexpressed Unr and hnRNP C1 proteins were precipitated by the UNR IRES. In contrast, we could not observe binding of hnRNP C1 proteins to the PITSLRE IRES (Figure 7A). In line with these results, there was no detectable binding of hnRNP C1 proteins to the PITSLRE IRES in UV crosslinking assays (data not shown).

Previously, we reported that enhanced expression of Unr during G2/M transition stimulates PITSLRE IRES-mediated translation (Tinton *et al*, 2005). So, the herein described role of hnRNP C1/C2 proteins in UNR IRES-mediated translation suggests a molecular cascade in which translocation of

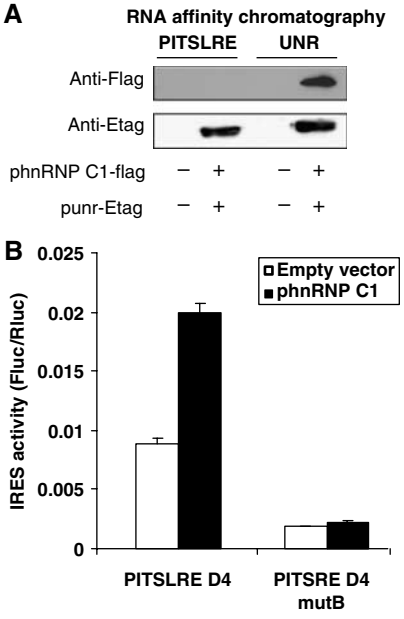

Figure 7 HnRNP C1/C2 stimulates PITSLRE IRES-mediated translation via Unr. (**A**) hnRNP C1/C2 do not interact with the PITSLRE IRES. Biotinylated RNAs corresponding to the PITSLRE or UNR IRES were immobilized on streptavidin beads and incubated with cytoplasmic cell extracts from HEK293 T cells overexpressing Flag-tagged hnRNP C1 or E-tagged Unr. IRES-bound proteins were analyzed by WB using anti-Flag (upper panel) and anti-Etag (lower panel) antibodies. (**B**) Overexpression of hnRNP C1 stimulates PITSLRE IRES-mediated translation. The dicistronic reporter plasmids Di-4 and Di-4 mutB were transfected in HEK293 T cells together with either an empty or a phnRNP C1 expression vector. Thirty-two hours after transfection, the cells were lysed and luciferase activities were measured. The bars represent the average ($n = 2$) $\pm$ s.d. IRES activity (Fluc/Rluc).

hnRNP C1/C2 proteins during G2/M stimulates IRES-mediated translation of Unr proteins, and Unr proteins, in turn, contribute to PITSLRE IRES-mediated translation. To test this hypothesis, we analyzed whether hnRNP C1 proteins can indirectly stimulate the PITSLRE IRES. The PITSLRE IRES mutant Di4-mutB, lacking the Unr-binding site was used as a negative control. Dicistronic reporter plasmids, harboring either the full-length PITSLRE IRES or the mutB PITSLRE IRES as intercistronic sequence, were cotransfected in HEK293 T cells with an empty expression vector or an hnRNP C1 expression vector. Figure 7B illustrates that hnRNP C1 proteins can indeed stimulate PITSLRE IRES-dependent translation. However, hnRNP C1 proteins were not able to stimulate the activity of PITSLRE mutant B IRES, which is defective for Unr binding. These data are in line with a molecular cascade comprising hnRNP C1/C2 proteins that stimulate IRES-mediated translation of the Unr proteins, and Unr, in turn, contributes to PITSLRE IRES-mediated translation.

Discussion

Previously, we have shown that translation of the Unr mRNA can be initiated through an IRES (Cornelis *et al*, 2005). Here, we show that this IRES can be used to escape the global inhibition of cap-dependent translation during mitosis. Indeed, compared to asynchronously growing cells or cells

arrested in the S phase, UNR IRES-mediated translation is significantly higher in G2/M cells. In order to investigate the molecular mechanism of UNR IRES-mediated translation during mitosis, UNR IRES *trans*-acting factors were identified by RNA affinity chromatography and UV crosslinking experiments. These revealed that not only hnRNP C1/C2, PTB but also the Unr protein itself can specifically interact with the UNR IRES. Functional analysis identified hnRNP C1/C2 proteins as UNR IRES stimulatory factors, whereas both PTB and Unr were found to function as UNR IRES inhibitory factors.

Interestingly, the binding of hnRNP C1/C2 to the UNR IRES was greatly enhanced at G2/M transition. At interphase, hnRNP C1/C2 proteins reside in the nucleus, but at the onset of mitosis, probably before nuclear envelope breakdown, these proteins migrate to the cytoplasm where they can bind to the UNR IRES RNA and possibly to other IRESs. However, as we observed that endogenous nuclear hnRNP C1/C2 proteins can interact with endogenous nuclear UNR mRNA (data not shown), hnRNP C1/C2 proteins might translocate to the cytoplasm as preformed complexes with the UNR IRES, at the onset of mitosis. It is not clear how the translocation of the hnRNP C1/C2 proteins from the nucleus to the cytoplasm is regulated in pre-mitotic cells.

In contrast to the enhanced binding of hnRNP C1/C2 proteins to the UNR IRES during mitosis, binding of PTB and Unr proteins is reduced. This is not due to a reduction of cytoplasmic expression of either of these proteins. Possibly, secondary modifications might alter the RNA affinity of these proteins. Alternatively, binding of other *trans*-acting factors during mitosis, such as hnRNP C1/C2 proteins, can interfere with the binding of Unr and PTB proteins. Indeed, we could show that binding of the hnRNP C1/C2 proteins to the UNR IRES negatively interferes with the binding of PTB and Unr. Because the PTB-binding site is located in direct proximity of the binding site for hnRNP C1/C2, diminished binding of PTB in the presence of hnRNP C1/C2 is most likely the result of direct competition. In contrast, the Unr-binding site is further away from the hnRNP C1/C2-binding site. Therefore, reduced binding of Unr proteins to the UNR IRES is more likely to result from hnRNP C1/C2-induced conformational changes in the IRES.

Our combined observations indicate that during interphase the binding of the inhibitory factors PTB and Unr suppresses UNR IRES-mediated translation. However, at the onset of mitosis, hnRNP C1/C2 proteins migrate to the cytoplasm and bind to the UNR IRES, relieving the inhibitory interactions and stimulating UNR IRES-mediated translation (Figure 8). This model is supported by the observation that a UNR IRES mutant defective in hnRNP C1/C2 binding cannot be stimulated anymore during mitosis, indicating that the binding of hnRNP C1/C2 proteins is needed for the stimulation of UNR IRES-mediated translation during mitosis.

To evaluate the functional relevance of the identified upstream IRES-dependent cascade involved in CDK11/PITSLRE^{p58} expression during mitosis, we examined the respective roles of hnRNP C1/C2 and Unr proteins in mitosis by knockdown studies. A cell population depleted of hnRNP C1/C2 displayed a cell cycle arrest in the G2/M phase of the cell cycle, indicating a crucial role for hnRNP C1/C2 proteins during G2/M transition or mitosis. One function of hnRNP C1/C2 might be a stimulatory role in UNR IRES-dependent translation and subsequent increased Unr and CDK11/PITSLRE^{p58} protein expression during mitosis. The latter might be functionally significant, as it was reported that CDK11/PITSLRE^{p58} is essential for centrosome maturation and bipolar mitotic spindle formation during mitosis (Petretti *et al*, 2006). In addition, depletion of Unr is associated with a delay in mitosis, again indicating a role in mitosis. This is in line with the observed correlation between Unr expression levels and mitosis: Unr protein expression is maximal at mitosis, whereas its expression is low in quiescent non-dividing cells (Ferrer *et al*, 1999; Tinton *et al*, 2005). Our reported data indicate that enhanced expression of Unr during mitosis might be needed for the regulation of the expression of CDK11/PITSLRE^{p58} or other proteins involved in the progression through mitosis.

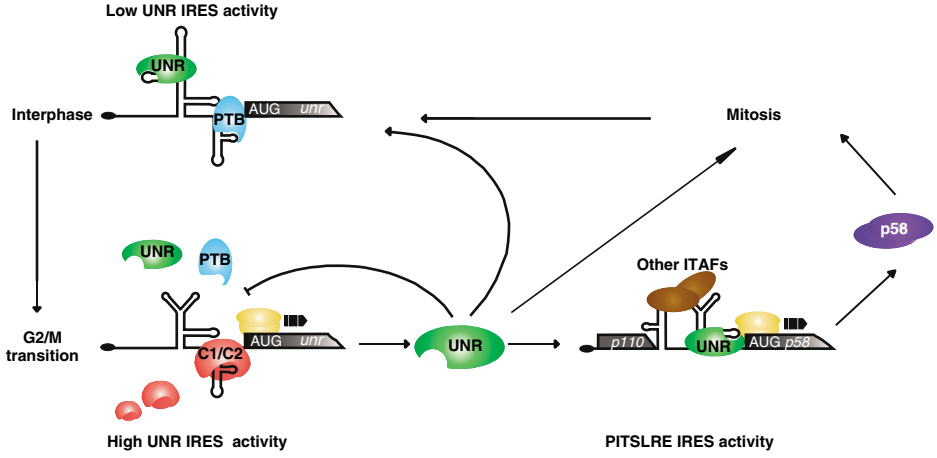

Figure 8 Model of UNR and PITSLRE IRES-mediated translation during cell cycle progression. During interphase, UNR IRES activity is kept low through the binding of PTB and Unr proteins. Early in prophase, before nuclear envelope breakdown, hnRNP C1/C2 proteins translocate from the nucleus to the cytoplasm where they can compete with PTB and Unr for binding to the UNR IRES RNA. The interaction with hnRNP C1/C2 proteins stimulates UNR IRES-mediated translation resulting in enhanced Unr protein expression levels. Accumulating Unr proteins, in turn, contribute to PITSLRE IRES-mediated translation, the latter being needed for the maturation of the mitotic spindle and progression through mitosis. Possibly, high Unr expression levels at the end of mitosis might contribute to the downregulation of UNR IRES-mediated translation at the entry of G1.

Materials and methods

Detailed information concerning plasmid construction, cell culture, transient transfection, reporter gene assays, WB procedures, used antibodies, cell cycle analysis and time-lapse imaging microscopy is included as Supplementary data as Sup_4. pdf.

Polysome and subpolysome fractionation and quantitative PCR

The protocol for polysome and subpolysome fractionation was adapted from De Jong *et al* (2006). Before harvesting, cycloheximide was added to the medium at a concentration of 0.1 mg/ml for 5 min at 37°C. Approximately 5×10^7 cells were harvested and placed on ice. Cells were washed twice with ice-cold PBS containing 0.1 mg/ml cycloheximide and incubated in extraction buffer (0.2 M Tris–HCl, 0.1 M KCl, 70 mM Mg-acetate and 10 mM EGTA, 0.25 M sucrose) supplemented with 1% Triton X-100, 50 U/ml SUPERase (Ambion, Austin, TX, USA), 10 mM dithiothreitol (DTT) and 0.1 mg/ml cycloheximide for 3 min on ice with occasional mixing. The nuclei and debris were removed via successive centrifugation steps: a first centrifugation at 500 *g* for 5 min; the cleared supernatant was centrifuged again at 12 000 *g* for 5 min. The supernatant was recovered and layered onto a discontinuous sucrose gradient in SW41 Ultra-Clear tubes (Beckman Coulter). The gradient consists of 0.75 ml 1.65 M sucrose and 1.25 ml 1.00 M sucrose buffered with 0.2 M Tris–HCl, 0.1 M KCl, 70 mM Mg-acetate and 10 mM EGTA. The gradients were centrifuged for 4 h at 150 000 *g* to pellet the polysomes. The polysomal pellets are resuspended in extraction buffer. The subpolysomal fraction was recovered from the gradient at the 1.65 and 1.00 M sucrose interface. RNA in the resuspended polysomal pellets and in the subpolysomal fraction was isolated with an Rneasy kit (Qiagen) according to the manufacturer's instructions.

cDNA was synthesized from the polysomal and subpolysomal fractions using SuperScript II reverse transcriptase (Invitrogen). Cycle thresholds (C_T) were determined per transcript in triplicate using the LightCycler®480 System (Roche, Mannheim, Germany) detection system using SYBR Green I as reporter dye (LightCycler®480 SYBR Green I Master, Roche).

UV crosslinking assays and immunoprecipitation

For UV crosslinking assays, DNA templates for synthesis of the RNA probes were generated by linearizing pUC19T7 plasmids containing the UNR IRES with the appropriate restriction enzyme. Internally labeled RNA probes were synthesized by *in vitro* transcription with T7 polymerase (Ambion) in the presence of 50 μCi [α-^{32}P]UTP (Amersham Pharmacia Biotech).

HEK293 T cells were used to prepare cytoplasmic extracts for the UV crosslinking assays. The cells were washed with cold PBS and recovered by centrifugation at 2500 *g* for 5 min at 4°C. The pellets were dissolved in 100 μl lysis buffer A (10 mM Hepes–KOH (pH 7.4), 3 mM $MgCl_2$, 40 mM KCl, 5% (v/v) glycerol, 1 mM DTT, 0.3% (v/v) Nonidet P-40, 200 U/ml aprotinin, 0.1 mM PMSF, 10 μg/ml leupeptin). The lysates were centrifuged at 20 000 *g* for 10 min at 4°C, the supernatants were recovered, the protein concentration was adjusted to 10 mg/ml and the samples were stored at −80°C. UV crosslinking assays were performed as described by Tinton *et al* (2005). ^{32}P-labeled RNA probes ($\pm 1 \times 10^6$ c.p.m.) were incubated with 10 μl cytoplasmic extract (100 μg proteins) for 20 min at 30°C in a 25 μl reaction mixture containing 10 mM Hepes–KOH (pH 7.4), 3 mM $MgCl_2$, 5% (v/v) glycerol, 1 mM DTT, 100 mM KCl, 40 U RNasin (Promega) and 6 μg tRNA. After RNA binding, the reaction mixtures were irradiated with UV light on ice for 30 min using a 'GS gene pulser UV chamber' (Bio-Rad). The samples were then incubated with RNase A and RNase T1 for 60 min at 37°C. The RNA–protein complexes were resolved on 10% SDS–PAGE, the gels were dried and the results were visualized with a Phosphorimager (Molecular Dynamics).

RNA affinity chromatography

Biotinylated RNA probes were synthesized from linearized pUC19 T7 plasmids by *in vitro* transcription with T7 polymerase (MEGAshortscript T7 RNA polymerase kit; Ambion) in the presence of biotinylated CTP (ratio 4:1, CTP:bioCTP) (Pierce). Biotinylated RNA (50 pmol) was incubated with 25 μl streptavidin beads (Pierce) in 200 μl binding buffer: 20 mM Hepes–KOH (pH 7.4), 50 mM KCl, 5% (v/v) glycerol, 1 mM DTT, 0.5 mM EDTA, 200 U/ml aprotinin, 0.1 mM PMSF, 10 μg/ml leupeptin and 25 μg/ml tRNA. After 1 h at 4°C on a rotary mixer, beads were washed twice with binding buffer and the cytoplasmic cell extract (200 μg; prepared as described above) was added to a total volume of 500 μl; incubation was continued for 2 h. After washing the beads three times with binding buffer and twice with binding buffer in which KCl concentration was adjusted to 100 mM, RNA-bound proteins were eluted by addition of 200 μl Laemmli sample buffer and analyzed by WB analysis.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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