IMP-3 protects the mRNAs of cyclins D1 and D3 from GW182/AGO2-dependent translational repression

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Abstract. IGF-2 mRNA binding protein 3 (IGF2BP3, IMP-3) is a well-known post-transcriptional regulatory factor of gene expression, mainly involved in embryonic development and oncogenesis. We have previously demonstrated that a subset of IMP-3 targets, such as the mRNAs of cyclins D1, D3 and G1, are positively regulated by IMP-3, and that this regulation depends on nuclear localization of IMP-3. In the present study, we show that as a first step following a knock-down of IMP-3, the protein levels of the cyclins rapidly decrease, while their mRNAs remain stable and associated with the polyribosomes, though not translated. We have elucidated the molecular mechanisms of this regulation, demonstrating that IMP-3 and its protein partners ILF3/NF90 and PTBP1 bind to the 3'UTRs of the cyclin mRNAs and protect them from the translational repression induced by miRNA-dependent recruitment of AGO2/GW182 complex in human cancer cells.

Introduction

Post-transcriptional regulation of gene expression plays a very important role at all stages of normal development, maintenance and/or pathology of cells, tissues and organs. Post-transcriptional regulation depends on various RNA-binding proteins (RBPs) that determine the translational status of mRNAs, their structure, localizaton and degradation rate. Regulatory RBPs can be recruited to their target mRNAs via specific recognition sequences (such as AU-rich sequences for HuR protein, or CPE sequence for CPEB family) (1,2). Alternatively, RBPs have been shown to bind to specifically structured elements within target RNAs, regardless of their nucleotide sequence. The IMPs/IGF2BPs (IGF-2 mRNA-binding proteins 1-3) are likely to belong to this latter group. IMP target sequences within RNAs vary to a large extent in different experimental models (3-6), and this complexity in target recognition might be due to the presence of numerous RNA binding domains in the IMPs, as well as by the variety of RNP complexes where they have been shown to participate (7). A large number of RNA-binding regulatory proteins are also recruited to their target mRNAs via short non-coding RNAs, such as miRNAs (reviewed in ref. 8). Cooperation or competition between different RNA-binding protein complexes determines the rate of protein expression from a large number of mRNAs (9).

Post-transcriptional regulation of gene expression is particularly important in the case of proteins characterized by a short half-life, which are often critical for the control of cell cycle, signal transduction pathways, circadian rhythm, antigen processing and other processes (10). We have shown that IMP-3/IGF2BP3 protein binds to and positively regulates the expression of cyclins D1, D3 and G1 in a number of human cancer cell lines (11). This regulatory mechanism controls the cell cycle and proliferation. In the present study, we explore the molecular mechanisms underlying the regulation of the expression of cyclins by IMP-3 in human rhabdomyosarcoma cells.

Materials and methods

Cell culture, miRNAs, plasmids and constructs. RD embryonic rhabdomyosarcoma (eRMS) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured as indicated on the ATCC website. miRNA precursors were purchased from Life Technologies (Carlsbad, CA, USA) and transfected at the final concentration of 50 nM using Lipofectamine RNAiMAX (Invitrogen Carlsbad, CA, USA), according to the manufacturer's instructions. FLAG-HA-IMP3 expression construct and stable cell line was previously described (11). psiCHECK-2 reporter constuct was purchased from Promega (Madison, WI, USA) miRNA target site blockers were designed by Exiqon, and the sequences were: TSB-CCND1-let-7g: TGAGGTAAGCGTGAGC; TSB-CCND3-miR-15: CATGAGGTATTGTGAAAC and the negative control A. TSBs were transfected at final concentration of 20 nM 24 h after the transfection of relevant miRNAs.

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RNA interference. Transient transfection of siRNA was performed using Lipofectamine RNAiMAX. Cells ($5x10^5$) were plated in 6-well plates, siRNA duplexes were transfected at 20 nM final concentration for 48 h. The efficiency of the siRNA-mediated knockdown of gene expression was evaluated by quantitative reverse transcription PCR (qRT-PCR) and/or western blotting. Unless otherwise indicated, all experiments were performed 48 h after siRNA transfection.

Transient transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells ($2x10^5$) were plated in 6-well plates; 24 h later the cells were transfected with 1 μg of plasmid for 48 h. Efficiency of transfection was evaluated by immunofluorescence (IF) and/or western blotting.

siRNA target sequences were: IMP 3 ggauucuccuaguagcauucau; IMP 3_2 auggaucauuccucaugua; GW182/TNRC6A gaaaugcucugguccgcuauu; GW182/TNRC6A_2 gcagccucca gcacaaccucuu; TNRC6B caucugggacaaggugauuguagacg; TNRC6C ggaauggagacacugugaacucagc; AGO1 ggaguuacuuu cauagcauuu; AGO2 gcacggaaguccaucugaa; PTBP1 aacaauga caagagccgugac; ILF3 ccccagaggacgacaguaaa; ILF3/NF110 gc ggauccgacuacaacuacg; ILF3/NF90 cuuccuagagcgucuaaaagu; HuR gaggcuccagucaaaaacca; HNRNPA2B1 ccaggggcucaugu aacugu; Control siRNA uagcaaugacgaaugcgua.

Luciferase reporter assays. The 3'UTR of human CCND1 or CCND3 mRNA were cloned into a psiCHECK2-reporter vector (Promega) downstream of the reporter gene (*Renilla* luciferase). HeLa cells were seeded at 20,000 cells/well in a 96-well plate. Twenty-four hours later, 10 ng of psiCHECK2-E2F5-3'UTR was co-transfected with 50 nM of miRNA mimic and/or 20 nM siRNA. Co-transfection was performed with Lipofectamine 2000 (Life Technologies). Forty-eight hours after transfection, the relative levels of *Renilla* vs. firefly luciferase activity (control of transfection efficiency) were measured with Dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. The luminescence signal was quantified on a Mithras LB 940 multilabel reader and analyzed with MikroWin software (Berthold Technologies).

Antibodies. The following antibodies were used for western blotting. IMP-3 (N-19) sc-47893, CCND1 (DCS-6) sc-20044, CCND3 (D-7) sc-6283, PTBP1/HNRNPI sc-16547, ILF3 sc-136197 and HuR sc-5261 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GW182 rabbit polyclonal (A302-329A) was obtained from Bethyl Laboratories (Montgomery, TX, USA). Rat anti-AGO1 (SAB4200084), AGO2 (SAB4200085), actin (A5441, clone AC-15), anti-goat IgG (I9140) anti-rabbit IgG (A0545) and anti-mouse IgG (I8765) were purchased from Sigma-Aldrich.

Quantitative real-time RT-PCR. The qRT-PCR primers for the detection of IMP-3, CCND1, CCND3 and Cyclo A were as previously described (11,12). qRT-PCR was performed using a LightCycler (Roche).

Cell lysis and sucrose gradients. RIPA lysis for western blotting. The cell pellets were incubated in 5 volumes of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitors] for 15 min on ice followed by sonication for 7.5 min (30 sec on, 1 min off) at high intensity. After centrifugation at 16,000 x g for 15 min at 4˚C, the supernatant was recovered for western blotting.

Total lysate from RD cells ($3x10^7$ cells) was prepared as previously described (13), treated or not with 10 mM puromycin for 15 min at 37˚C, and applied to a 21-47% sucrose gradient in 20 mM Tris-HCl (pH 8.0); 140 mM KCl; 5 mM $MgCl_2$. Centrifugation was carried out at 40,000 rpm for 2 h and 15 min using a Beckman SW 41 rotor. Fractions (0.8 ml) were collected, adsorbance at 260 nm was measured, and each fraction was ethanol-precipitated, treated with DNAse (Promega), and RNA was extracted with phenol-chloroform and used for qRT-PCR analysis.

Immunoprecipitation of IMP-3 complexes. Protein complexes were immunoprecipitated from whole cell extracts [lysis buffer: 10% glycerol, 20 mM Tris-HCl, pH 8, 0.2 mM EDTA, 0.1% NP-40, 0.5 M KCl, protease inhibitors (cOmplete, Roche)], pre-cleared for 1 h with protein A/G-agarose (Thermo Fisher Scientific), treated or not with 1 mg/ml protease-free RNAse A (Roche), using IMP-3 antibody (sc-47893; Santa Cruz Biotechnology) or goat IgG (Sigma-Aldrich) and protein A/G-agarose. Complexes were separated on a 4-12% polyacrylamide gel (Invitrogen), and stained using the SilverQuest kit from Invitrogen, according to the manufacturer's instructions. Mass spectrometry identification of proteins was carried out by Dr R. Tomaino, Harvard Medical School.

Drug treatment

DRB treatment. RD cells ($4x10^5$) were plated in 6-well plates and transfected with control or IMP-3 siRNAs; DRB (Sigma-Aldrich D1916) was added to a final concentration of 100 μM and the cells were collected at indicated time-points.

Cycloheximide treatment. RD cells ($4x10^5$) were plated in 6-well plates and transfected with control or IMP-3 siRNAs; 48 h later, cycloheximide (Sigma-Aldrich C 19881G) was added to a final concentration of 20 μg/ml for 2 h.

In situ hybridization. Probes used were: CCND1 sense, TAATACGACTCACTATAGGGAGACCCTCGGTGTCCT ACTTCAA and CCND1 antisense, ATTTAGGTGACACT ATAGGGGATGGTCTCCTTCATCT; CCND3 sense, TAAT ACGACTCACTATAGGGAGATGGATGCTGGAGGTATG TGA and CCND3 antisense, ATTTAGGTGACACTATAGA ATGAAGGCCAGGAAATCA.

Cells were seeded on poly-Lysine-coated glass cover slides and transfected as previously described. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at RT, washed with PBS, incubated with 0.3% H_2O_2 dissolved in methanol for 30 min at RT and washed with PBS. Afterwards, cells were incubated with prehybridization solution (50% formamide, 2X SSC, 10 mM Na_2HPO_4) for 90˚C at RT in a humidified chamber followed by incubation in hybridization solution (1.5 μl salmon sperm DNA, 1.5 μl tRNA and 200-500 ng of DIG-labelled mRNA probes pre-heated for 2 min at 90˚C and placed on ice for 2 min, then mixed with 15 μl of 60% formamide, 10 mM Na_2HPO_4 and 15 μl of 20%

dextran sulphate, 4X SSC, 0.4% BSA) in a humidified chamber at 37˚C overnight. Cells were washed twice with 2 ml of prehybridization solution at 37˚C for 30 min, then twice with 2X SSC, 0.1% Triton at 37-50˚C for 5 min, twice with 1X SSC, 0.1% Triton at 37˚C for 5 min, and 5 times with PBS-Tween at 37˚C for 5 min. Cells were incubated for 1 h in blocking buffer (2% sheep serum, 2 mg/ml BSA, in PBS-Tween) at RT followed by incubation with anti-DIG-biotin antibody (Roche) (diluted 1:500 in blocking buffer) overnight in a humidified chamber at 4˚C. To visualize the CCND1 and D3 mRNAs, the TSA Biotin Systems (Perkin-Elmer) and the Enhanced Liquid Substrate system (Sigma-Aldrich 3,3-Diaminobenzidine) were used according to the manufacturer's instructions.

Protein pull-down by biotinylated RNA. RT reactions were performed using the SuperScript® III First-Strand Synthesis system (Life Technologies) and total RNA from RD cells. Phusion™ High-Fidelity DNA Polymerase kit for PCR (Thermo Fisher Scientific) was used for PCR amplification of desired fragments. All forward primers had a T7 promoter sequence: TAATACGACTCACTATAGGGAGA. The primers are listed in Table I.

MEGAscript® T7 Transcription kit (Life Technologies) was used for RNA *in vitro* transcription and RNA 3' End Biotinylation kit (Pierce) for biotinylation of RNA fragments, all of the above according to the manufacturer's instructions.

Biotin pull-down assay was performed as follows: the cell pellet was lysed in buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol + protease inhibitor (cOmplete, Roche) for 15 min on ice, and supernanant was collected. Biotinylated RNA probes were incubated with cell lysate in TENT buffer [50 mM Tris-Cl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 1% Triton X-100] for 30 min at RT, immobilized on streptavidin-agarose beads, washed three times with TENT buffer and analysed by western blot analysis to reveal the proteins bound to RNA probes.

Table I. Primers used in the present study.

Name	Region	Sequence	Location
CCND1: NM_053056.2			
1 D1 F	3'UTR	ggacgtggacatctgagggc	1082
1 D1 R	3'UTR	ctcccccaccgctcagggtt	1362
2 D1 F	3'UTR	taaccctgagcggtgggga	1342
2 D1 R	3'UTR	gctttatcaggaaaagcaca	1622
3 D1 F	3'UTR	ttgtgcttttcctgataaag	1602
3 D1 R	3'UTR	tgctacgctggctggtgccc	1882
4 D1 F	3'UTR	cgggcaccagccagcgtagc	1862
4 D1 R	3'UTR	tatttcctacacctattgga	2142
5 D1 F	3'UTR	ccaataggtgtaggaaatag	2122
5 D1 R	3'UTR	actttcaaacaccagttggc	2402
6 D1 F	3'UTR	tgccaactggtgtttgaaag	2382
6 D1 R	3'UTR	aaaataaactgtattaaatc	2662
7 D1 F	3'UTR	agatttaatacagtttattt	2642
7 D1 R	3'UTR	cacttcctaaataaaaatta	2922
8 D1 F	3'UTR	gtaatttttatttaggaagt	2902
8 D1 R	3'UTR	acatggcagtatatgacaca	3182
9 D1 F	3'UTR	caatgtcatatactgccatg	3162
9 D1 R	3'UTR	tctctggggacaccggcgcg	3457
10 D1 F	3'UTR	ccgcgccggtgtccccagag	3437
10 D1 R	3'UTR	aaacagaacactagtacata	3732
11 D1 F	3'UTR	ttatgtactagtgttctgtt	3712
11 D1 R	3'UTR	tggttcagacagacgccgca	4007
12 D1 F	3'UTR	tgcggcgtctgtctgaacca	3987
12 D1 R	3'UTR	ttaccagttttatttctaga	4282
CCND3: NM_001760.3			
6 D3 F	3'UTR	gccctctggagtggccacta	1055
6 D3 R	3'UTR	tcccatcagcctggcccacc	1320
7 D3 F	3'UTR	gccaggctgatgggacagaa	1305
7 D3 R	3'UTR	tctaggagcagctgtcagca	1570
3 D3 F	3'UTR	acagctgctcctagagggag	1555
3 D3 R	3'UTR	tatagcagctccttggccac	1820
4 D3 F	3'UTR	caaggagctgctatagcctg	1805
4 D3 R	3'UTR	tttttccaagaagccaaagc	2055

Results and Discussion

The expression of CCND1 and D3 is regulated on post-transcriptional level in IMP-3 KD cells. To elucidate the molecular mechanisms of the regulation of expression of the cyclins by IMP-3, we started by comparing the levels of CCND1 and D3 mRNA and protein in IMP-3 KD cells, compared to control. The short half-life of CCND1 and D3 proteins (30-40 min) allows to perform these studies on endogenous proteins. We have not included CCNG1 in the present study, because although its expression is regulated by IMP-3, this atypical cyclin is very stable (with a half-life of over 48 h). A clear decrease of the levels of CCND1 and D3 proteins becomes visible as early as 24-32 h post-transfection of IMP-3 siRNA, whereas the corresponding mRNAs do not vary significantly at these time-points (Fig. 1A-C). These results suggest that the expression of the cyclins is initially downregulated by a post-transcriptional mechanism which does not depend on the mRNA levels. Next, we have addressed the question of the mRNA stability of cyclins by blocking the transcription in IMP-3 KD vs. control cells with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB). As shown in Fig. 1D, the stability of mRNAs of CCND1 and D3 does not depend on the presence of IMP-3. Moreover, the subcellular localization of the cyclin mRNAs did not appear to change when IMP-3 was decreased. As we have previously shown (11), both in control and IMP-3 KD cells, the CCN mRNAs are efficiently exported to the cytoplasm, even though in IMP-3 KD cells they had a more perinuclear localization.

The mRNAs of CCND1 and D3 are associated with polyribosomes in IMP-3 KD cells, but their translation is repressed. To address the translational status of the cyclin mRNAs in IMP-3 KD cells, we performed sucrose gradient separation of polyribosomes, followed by RNA isolation from each fraction. Subsequently, we used RT-qPCR to evaluate the relative amounts of the mRNAs of CCND1 and D3 in polyribosomal vs. monosomal fractions in IMP-3 KD and control cells. The association of the mRNAs of CCND1

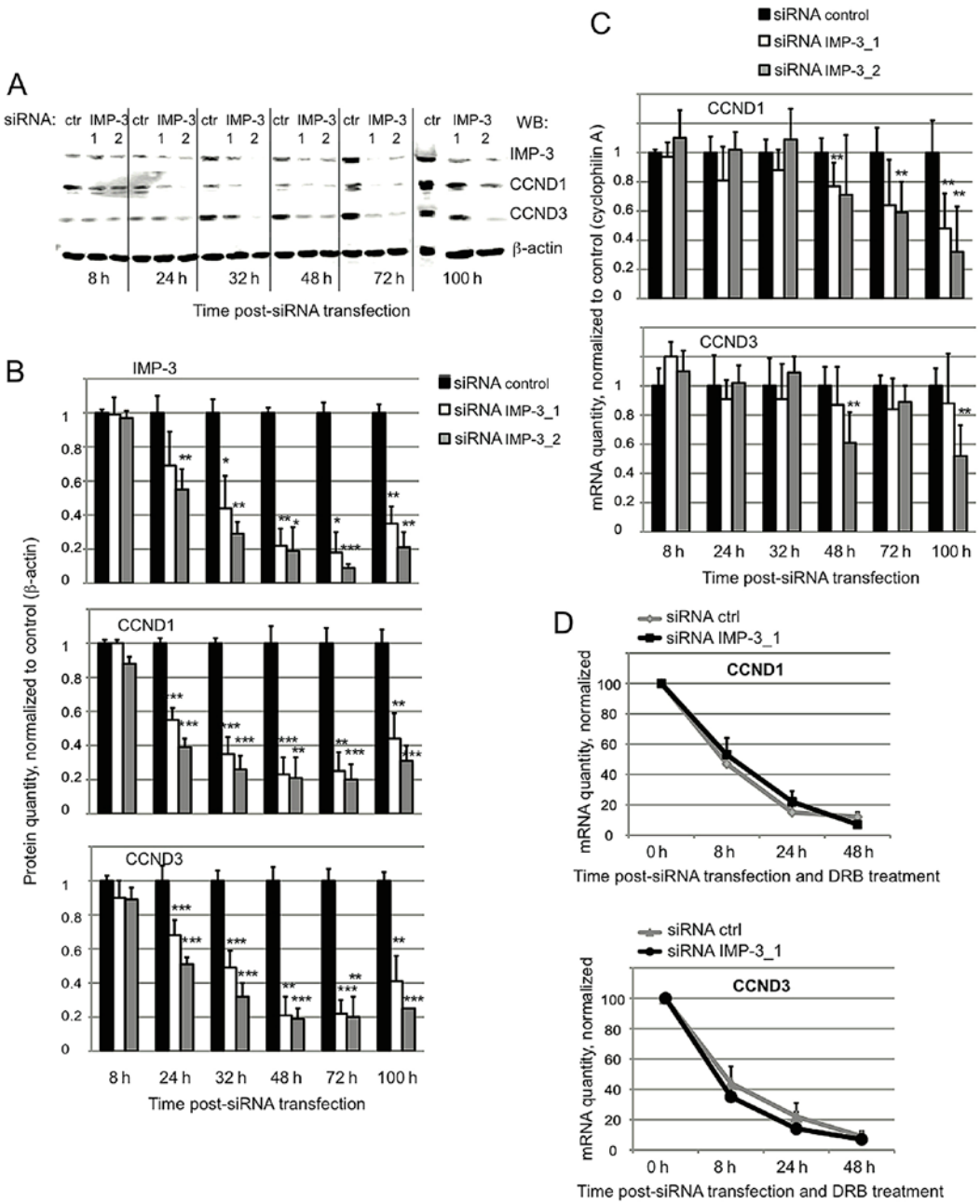

Figure 1. The expression of CCND1 and D3 is regulated on post-transcriptional level in IMP-3 KD cells. (A-C) Kinetics of inhibition of CCND1 and D3 in RD cells transfected with the indicated siRNAs and collected at 8, 24, 32, 48, 72 and 100 h post-transfection. All values have been normalized to actin (B) or cyclophilin A expression, and the control (ctrl) siRNA values for all the time-points have been set to 1. (A) Protein levels (western blot analysis); (B) quantification of three independent western blots; (C) mRNA levels (RT-qPCR). Error bars represent SEM from 3 (A and B) or 6 (C) independent experiments. P-values (above each bar) were calculated using the Mann-Whitney test and R software: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (D) Relative rate of mRNA degradation in control vs. IMP-3 KD cells under the conditions of general transcription inhibition by DRB.

and D3 with polyribosomes did not decrease in the absence of IMP-3 (Fig. 2A). On the contrary, a slight accumulation of these mRNAs in heavy polyribosomal fractions was observed for the mRNAs of CCND1 and D3, but not for the control mRNA of cyclophilin A. Taken together with the decrease of protein levels of the cyclins in IMP-3 KD cells, these observations suggested that the translation of the mRNAs of CCND1 and D3 can be slowed down in the absence of IMP-3, but this event does not involve the dissociation of the mRNAs from the ribosomes. In agreement with this hypothesis, when we inhibited the protein translation in IMP-3 KD or control cells using the mRNA-ribosome 'freezing' drug cycloheximide, we observed a strong decrease of the cyclins in control cells, whereas in IMP-3 KD cells, these was no further decrease of the cyclin levels when the translation was arrested by the drug. We conclude that in IMP-3 KD cells, the translation of the mRNAs of CCND1 and D3 is already strongly repressed by a mechanism that does not involve mRNA degradation, erroneous mRNA localization or disassembly of polyribosomes as an initial event.

The expression of CCND1 and D3 in IMP-3 KD cells can be fully restored by inactivating the RISC complex. The mechanisms of the rapid translational arrest occurring on the mRNAs

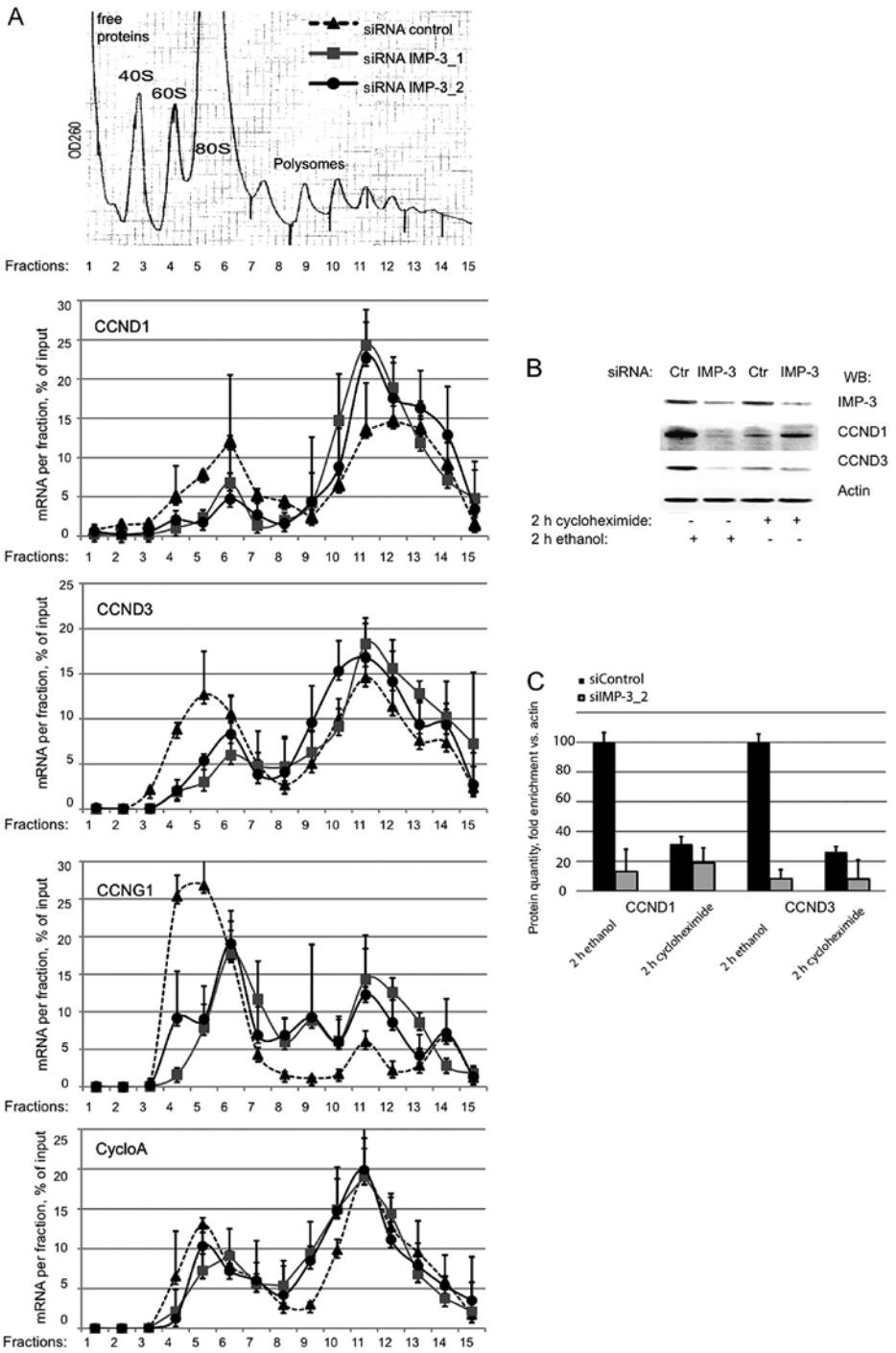

Figure 2. The mRNAs of CCND1 and D3 are associated with polyribosomes in IMP-3 KD cells, but their translation is repressed. (A) Sucrose gradient fractionation of cytoplasmic lysates of RD cells transfected for 48 h with control siRNA or with two distinct anti-IMP-3 siRNAs. The mRNAs of CCND1 and D3 and cyclophilin A (cycloA) were quantified in each fraction using RT-qPCR. (B and C) RD cells were transfected with control or IMP-3 siRNA, and treated at 48 h post-transfection with 20 mg/ml cycloheximide or ethanol for 2 h. Expression of CCND1 and D3 was evaluated by western blotting and quantified using Chemi-Capt (Vilber Lourmat). Shown are typical results (B) or a mean (A and C) of 3 independent experiments.

of CCND1 and D3 in IMP-3 KD cells were unclear. However, the observed phenomenon strongly resembled the translational repression caused by miRNA-dependent recruitment of argonaute (AGO) proteins and/or of GW182/TNRC6A on target mRNAs (reviewed in ref. 14). Multiple publications have earlier identified the mRNAs of CCND1 and D3 as miRNA targets (15,16). In addition, the perinuclear localization of the mRNAs of CCND1 and D3 in IMP-3 KD cells observed in our experiments (Fig. 1D) strongly resembled the typical subcellular localization of activated RISC complexes, as previously reported (17). Therefore, we attempted to simultaneously knock down key RISC components GW182, AGO1 or AGO2 in our IMP-3 KD cells, and to study the expression levels of CCND1 and D3 proteins. The results presented in Fig. 3A and B clearly show that a KD of GW182 and of AGO2, but not of AGO1, fully restored the levels of CCND1 and D3, even when IMP-3 remained downregulated. The KD of other GW182 family members, TNRC6B and C, which are usually

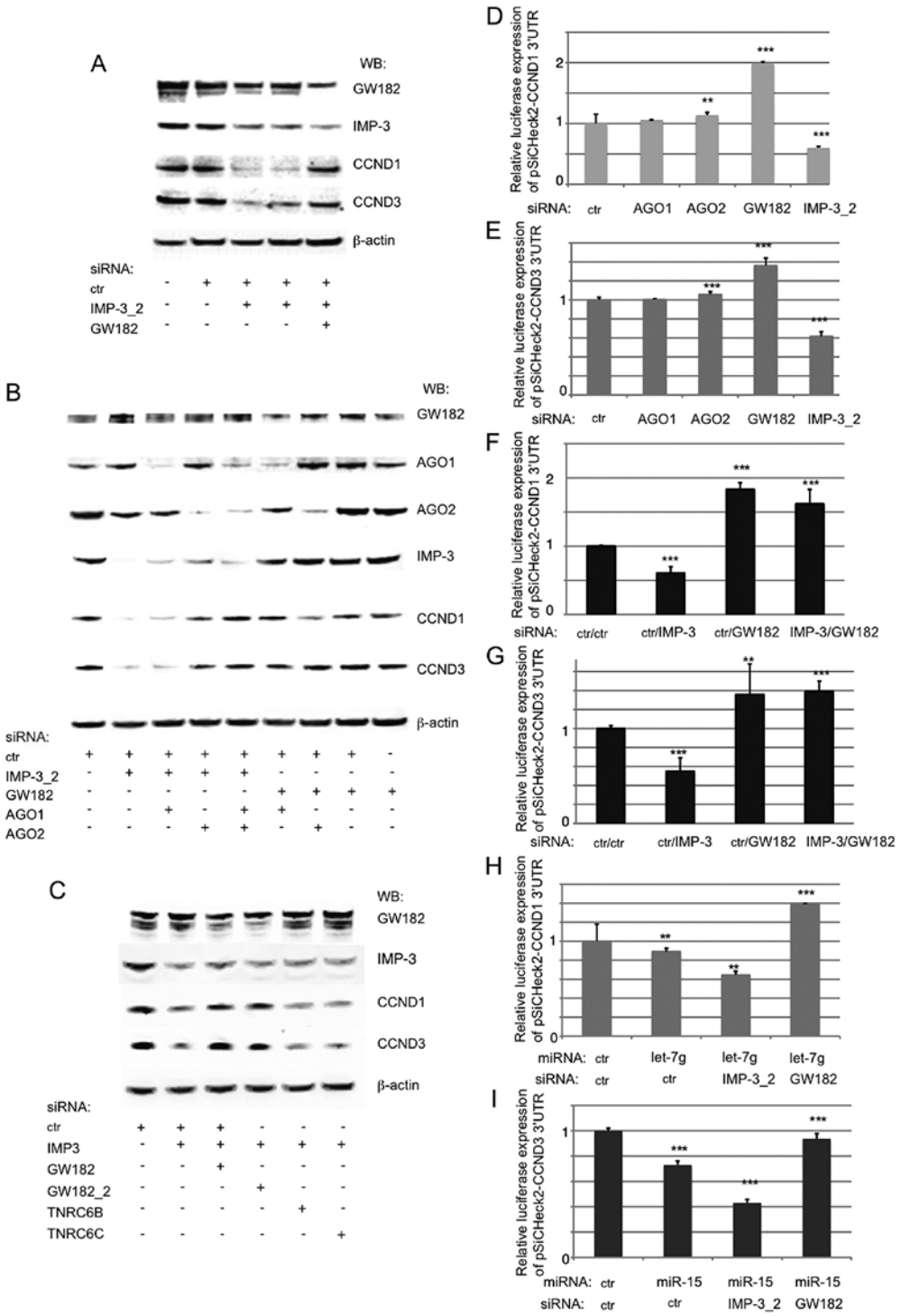

Figure 3. The expression of CCND1 and D3 in IMP-3 KD cells can be fully restored by inactivating the RISC complex. (A) A western blot analysis shows a rescue of CCND1 and D3 expression in IMP-3 KD cells when GW182 is knocked down. (B) The expression of CCND1 and D3 in IMP-3 cells can be rescued by a KD of AGO2, but not of AGO1 (lanes 1-5). In control cells, the KD of AGO1, AGO2 or GW182 has no effect on the levels of CCND1 and D3 (lanes 5-9). (C) Only a KD of GW182/TNRC6A, but not of other family members, TNRC6B and C, can rescue the expression of CCND1 and D3 in IMP-3 KD cells. (D-I) The regulation of luciferase expression (psiCHECK-2 reporter vector) under the control of 3'UTRs of CCND1 (D, F and H) and CCND3 (E, G and I) depends on IMP-3, GW182, AGO2 and miRNAs let-7g (CCND1) and miR-15 (CCND3). The luciferase expression of each vector co-transfected with control siRNA used was set to 1 and used for normalization of all the respective assays. Error bars represent SEM from 3 independent experiments. P-values (above each bar) were calculated using the Mann-Whitney test and R software: $^{**}P<0.01$; $^{***}P<0.001$.

expressed at low levels, did not rescue the expression of the cyclins in IMP-3 KD cells (Fig. 3C). We concluded that IMP-3 could protect the mRNAs of CCND1 and D3 from RNAi in human cancer cells. To find out whether this regulation was direct, we cloned the 3'UTRs of CCND1 and D3 into the psiCHECK-2 luciferase reporter vector, and first studied the roles of AGO1, AGO2, GW182 and IMP-3 in the regulation of the luciferase expression. Consistent with the results observed on endogenous CCND1 and D3, the expression of the luciferase under the control of the 3'UTRs of the cyclins was

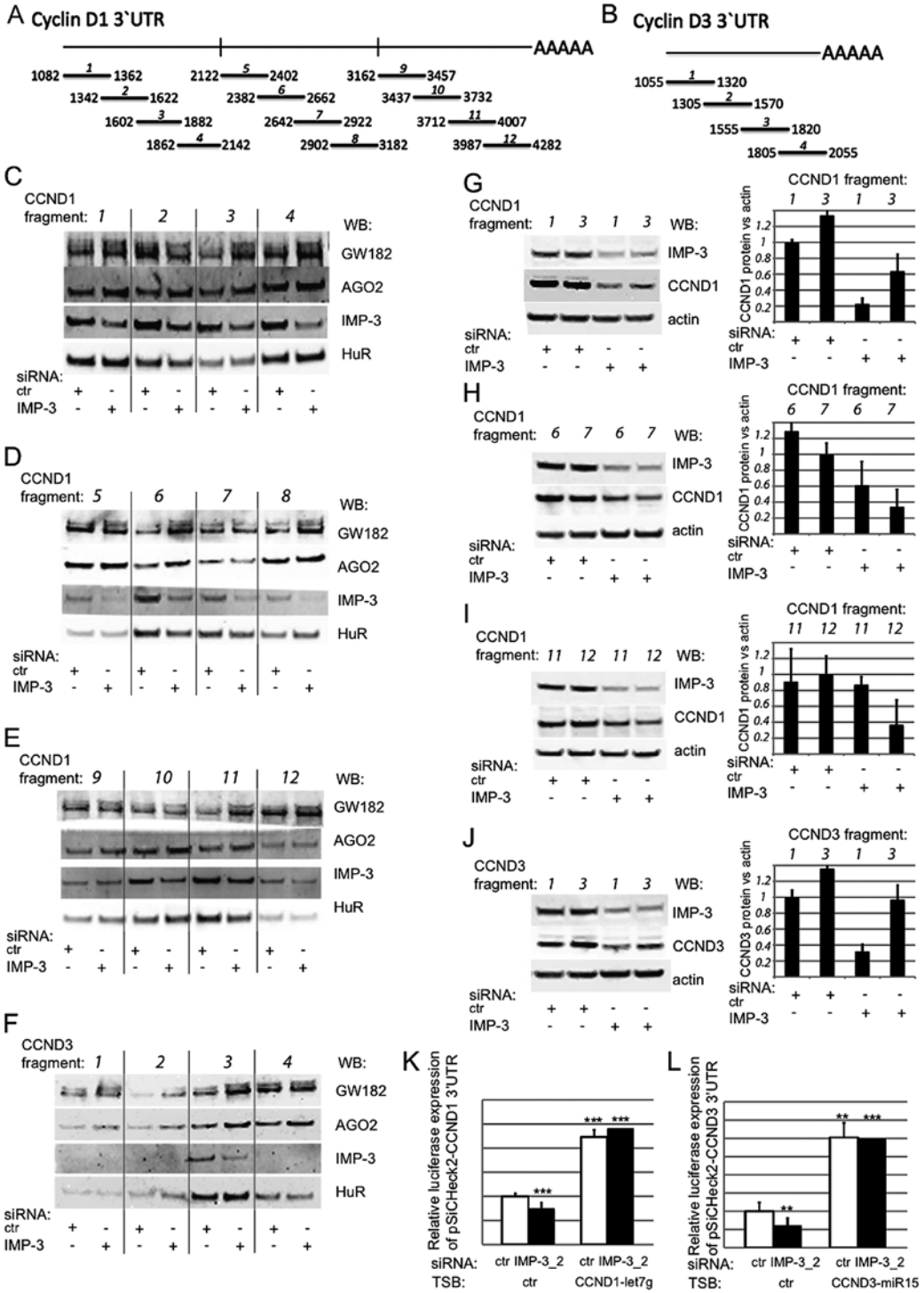

Figure 4. IMP-3 and GW182/AGO2 bind to and compete within the regions of the 3'UTRs of the cyclins that are critical for their expression. (A and B) A schematic representation of the 3'UTRs of CCND1 (A) and D3 (B) with the coordinates of the fragments used for pull-down experiments. (C-F) Indicated fragments of the 3'UTRs of CCND1 and D3 transcribed *in vitro*, biotinylated, and incubated with total cell extracts from conrol of IMP-3 KD cells. The proteins bound to the RNA fragments were revealed using western blot analysis. (G-J) RNA fragments characterized by IMP-3-RISC competitive binding, or control fragments from the CCN 3'UTRs (identified in C-F) were transfected into control or IMP-3 KD RD cells, and the expression of CCND1 and D3 proteins was quantified by western blot analysis in three independent experiments. Left, a representative experiment out of three, right, quantification of three experiments. (K and L) The regulation of luciferase expression (psiCHECK-2 reporter vector) under the control of 3'UTRs of CCND1 (K) and CCND3 (L), transfected with let-7g or miR-15, does not depend on IMP-3 in the presence of specific target site blockers for these miRNAs.

dramatically downregulated by a KD of IMP-3, upregulated by a KD of GW182, and slightly increased by a KD of AGO2, but not of AGO1 (Fig. 3D and E). A KD of GW182 in IMP-3 depleted cells released the inhibition of the luciferase in these conditions (Fig. 3F and G). These results clearly indicated that IMP-3 and the RNAi machinery were competing on the 3'UTRs of CCND1 and D3, thus, regulating the expression of the cyclins. We have subsequently co-transfected miRNAs let-7g and miR-15, known to regulate CCND1 and D3, respectively, and have further confirmed that while a KD of IMP-3

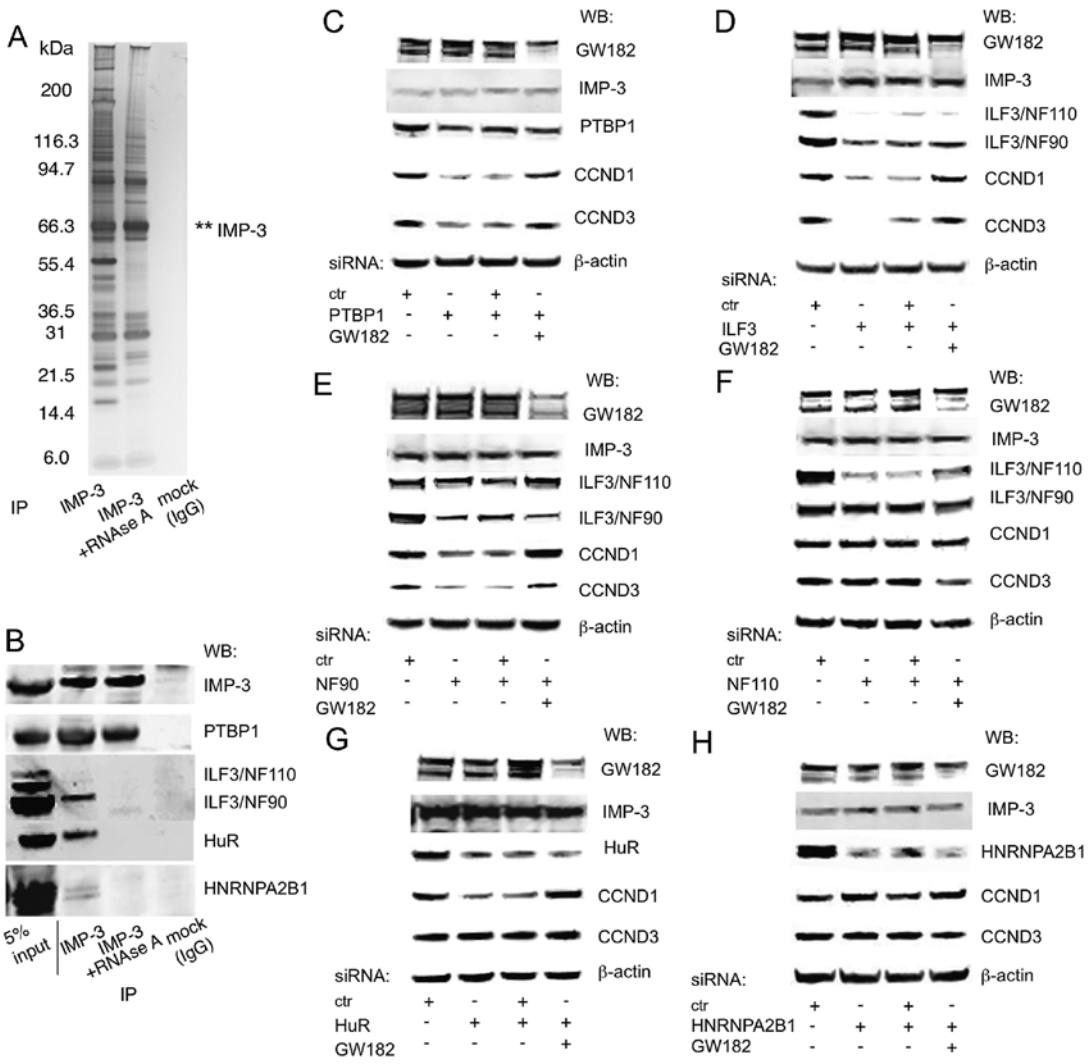

Figure 5. The expression of CCND1 and D3 is co-regulated by IMP-3 protein partners. (A) Silver staining of IMP-3 protein complexes immunopurified from RD cells, untreated or treated with RNase A. Mock, IgG. (B) Co-immunoprecipitation analysis of endogenous IMP-3 interaction with the indicated partners. (C-G). A western blot analysis shows the rescue levels of CCND1 and D3 expression in cells depleted of IMP-3 protein partners PTBP1 (C), ILF3 (D), ILF3/NF90 (E), ILF3/NF110 (F), and HuR (G) when GW182 (lower band on the western blot analysis) is knocked down. (H) An IMP-3 protein partner HNRNPA2B1 does not influence the expression of CCND1 and D3.

increased the repressive effect of endogenous or ectopic miRNAs on the cyclins, a KD of GW182 fully reversed this effect (Fig. 3H and I).

IMP-3 and GW182/AGO2 bind to and compete within the regions of the 3'UTRs of the cyclins that are critical for their expression. The IMP proteins do not have a universal binding motif, and were reported to bind to various RNA sequences depending on the cell type and experimental model used in different studies. Various miRNA-binding sites, both experimentally proven or predicted, are numerous throughout the 3'UTR sequences of CCND1 and D3 (see Introduction for details). Therefore, no prediction was possible as to the functionally important binding sites of IMP-3 protein within these mRNAs. We decided to identify these regions by a two-step experimental approach (Fig. 4). First, we synthesized a number of short, partially overlapping mRNA fragments covering the 3'UTRs of the cyclins (Fig. 4A and B), biotinylated them, and used them in an *in vitro* pull-down assay together with total cell extracts from control or IMP-3 KD cells (Fig. 4C-F). In order to test our hypothesis that IMP-3 could hinder the binding of RISC complex to the mRNAs of CCND1 and D3, we were looking for RNA fragments where IMP-3 binding would compete with GW182 and/or AGO2. We used the HuR protein, a very well-characterized post-transcriptional regulator of the expression of CCND1 and D3, as an internal control for the assay, because the binding of HuR to target RNAs depends on the presence of AU-rich sequences, but not on IMP-3 (1,18). We were able to identify three fragments within the 3'UTR of CCND1 where the binding of RISC complex components was increased in the absence of IMP-3: fragments 3, 6 and 11. In the case of CCND3, IMP-3 competed with GW182 and AGO2 within fragment 3 of 3'UTR.

In the second step of the assay, we attempted to evaluate the functional importance of the identified binding regions of IMP-3 within the 3'UTRs of CCND1 and D3, where it competed with RISC *in vitro*. To this end, we have transfected the relevant RNA fragments, or the flanking regions of 3'UTRs, where no

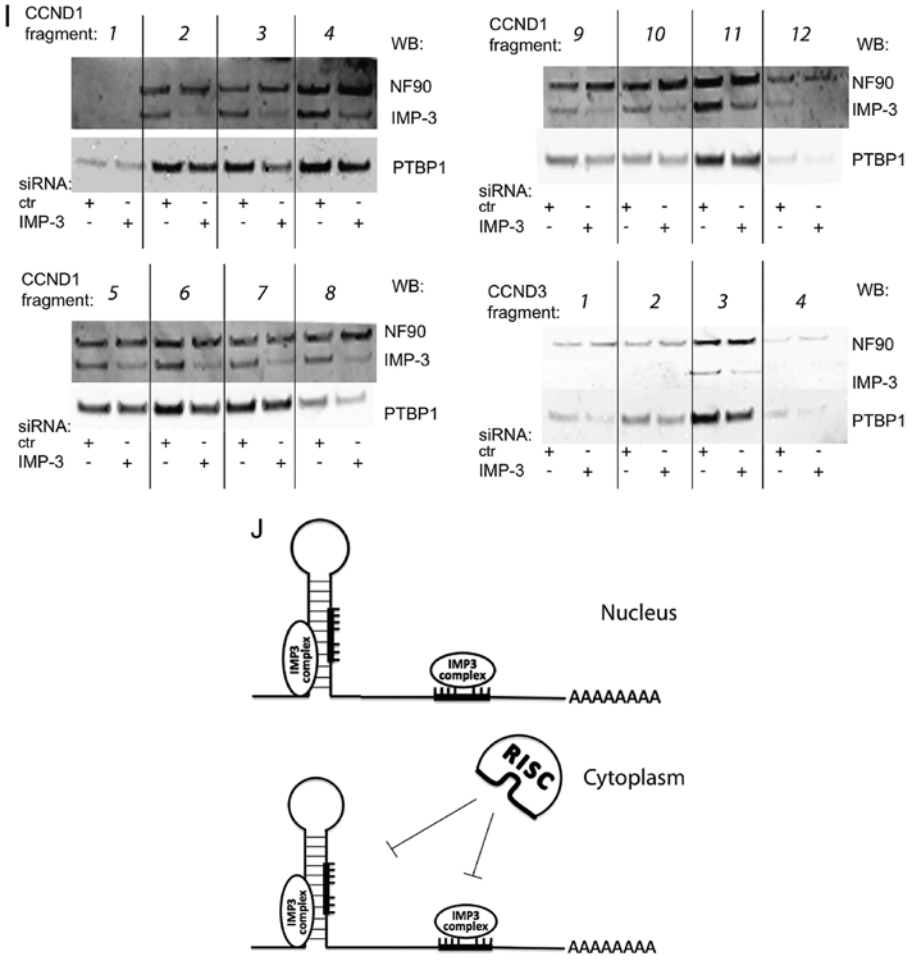

Figure 5. Continued. (I) Indicated fragments of the 3'UTRs of CCND1 and D3 were transcribed *in vitro*, biotinylated, and incubated with total cell extracts from control of IMP-3 KD cells. The IMP-3 protein partners bound to the RNA fragments were revealed using western blot analysis. (J) A model of action for IMP-3/NF90/PTBP1 complex in the inhibition of RISC-dependent translational repression. Antagonistic interactions between IMP-3/NF90/PTBP1 and RISC complexes: IMP-3 complex can interfere with miRNA function via competition for the same binding site (on the right). In the context of non-overlapping binding sites IMP-3 complex can change local mRNA structure and prevent RISC binding (on the left).

competition between IMP-3 and RISC had been observed, and quantified the expression of CCND1 and D3 proteins under the control of IMP-3 KD conditions (Fig. 4G-J). If our hypothesis is correct and the depletion of IMP-3 leads to excessive presence of RISC on the identified regions of the 3'UTRs of the cyclins, and to translational repression, then the transfection of the corresponding RNA fragments in molar excess should lead to the binding of RISC to the transfected fragments, and to a complete of partial release of the repression. These were the results we have observed in at least three independent experiments (an example of each experiments is shown on the left, and a quantification of three experiments is shown on the right). The transfection of the relevant RNA fragments, identified in Fig. 4C-F, led to a partial (Fig. 4G and H) or complete (Fig. 4I and J) release of the expression of the cyclins, even in IMP-3 KD cells. Moreover, even in control cells, the transfection of these fragments led to a slight increase of the level of CCND1 and D3, respectively. Contrary to our previous observations concerning the binding sites for all three members of IMP family within the 3'UTRs of the cyclins, obtained with GST-tagged recombinant proteins *in vitro* (11), the region 1064-1252 of the CCND3 3'UTR was very weakly bound by endogenous IMP-3 in cell lysate, and was not functionally important for the competition between IMP-3 and RISC for the regulation of CCND3 translation (Fig. 4F and J).

Notably, there is a binding site for the miRNA let-7 within the fragment 11 of CCND1 3'UTR, (positions 3955-3961), and miR-15 binds within fragment 3 of CCND3 3'UTR (positions 1813-1819). Blocking these miRNA target sites by specific LNA antisense inhibitors led to a release of luciferase expression under the control of the 3'UTRs of CCND1 and D3, and in this case, a KD of IMP-3 did not change the luciferase expression (Fig. 4K and L).

Therefore, we have experimentally proven that IMP-3 can protect the mRNAs of CCND1 and D3 from RISC-induced translational repression, and have identified a number of regions of 3'UTRs targeted by these regulatory mechanisms. The other regions, such as fragments 1, 7 or 12 of CCND1 3'UTR, might titrate the RISC complex to some extent when transfected into live cells, but they will equally titrate it in the presence or in the absence of IMP-3. Therefore, no rescue of the cyclin expression is observed following the transfection of these fragments in IMP-3 KD cells.

The expression of CCND1 and D3 is co-regulated by IMP-3 protein partners. A competition between IMP-3 and miRNA-

guided GW182 or AGO2 was a novel finding. However, IMP proteins interact with a large amount of other proteins in the cell, both RNA-binding and non-RNA binding (Fig. 5A) (11,12). Thus, it was interesting to determine the exact composition of the IMP-3 subcomplex that was functionally relevant to this new regulatory mechanism. In our previous study, we identified IMP-3 partners that were important or not for the expression of CCND1, D3 and G1 (11). Among them, PTBP1 was a direct IMP-3 partner, and ILF3, HuR and HNRNPA2B1 depend on the presence of RNA to co-precipitate with IMP-3 (Fig. 5B). Now, we have systematically tested for the involvement of these proteins in competition with GW182. IMP-3 partners PTBP1/HNRNPI and ILF3 were shown to be necessary for the expression of CCND1 and D3, and their KD was compensated by a simultaneous KD of GW182 (Fig. 5C and D). ILF3 gene gives rise to two distinct RNA-binding proteins, NF110 and NF90, which are results of alternative splicing and do not have similar functions in translational regulation (19). Therefore, we have used isoform-specific siRNAs and have identified the known regulator of cell growth NF90, but not NF110, as the partner of IMP-3 that regulates the expression of CCND1 and D3 in GW182-dependent manner (Fig. 5E and F). HuR/ELAVL1 interacts with IMP-3 in an RNA-dependent manner, and regulates the expression of CCND1, but not CCND3 (Fig. 5G). However, the majority of IMP-3 interacting RNA-binding proteins, such as HNRNPA2B1, do not regulate the expression of the cyclins (Fig. 5H; data not shown). Neither of these IMP-3 protein partners regulated the protein levels of IMP-3 (Fig. 5C-H). The protein partners of IMP-3 that have been shown to be important for the expression of the cyclins, NF90 and PTBP1, demonstrate strong binding along the 3'UTRs of CCND1 and D3, and this binding is dependent on PTBP1 or independent of NF90 in the presence of IMP-3, especially within the functionally important fragments we have described above (Fig. 5I). These results have highlighted the specificity of our experiments and confirmed the existence of a distinct specific subcomplex of IMP-3 that contains PTBP1 and ILF3/NF90, and is critical for the protection of the mRNAs of CCND1 and D3 from RNAi.

IMP proteins are known to interact with multiple other proteins and to regulate the fate of numerous mRNAs, most frequently by regulation of mRNA stability, but also by impacting on such diverse processes as mRNA translation, mRNA subcellular localization and others (3,13,20-24). These observations suggest the existence of multiple, functionally diverse IMP-dependent RNP complexes, and highlight the necessity to study these complexes individually and target-wise. We have recently demonstrated that the mRNAs of CCNs D1, D3 and G1 are direct and functionally important targets of IMP-3, as these mRNAs were found within IMP-3 RNP complexes, and in IMP-3 KD cells the expression of these cyclins on protein level was sharply downregulated. The proliferation of IMP-3 KD cells was arrested due to accumulation of the cells in G1 phase of the cell cycle. This function is specific to IMP-3, depends on the nuclear-cytoplasmic shuttling of IMP-3 and its partner HNRNP M, and is not shared by IMP-1 and IMP-2 (11). Now, we have identified the molecular mechanisms of this regulation, which does not depend on regulation of mRNA stability, but involves miRNA and RISC-dependent, reversible translational arrest in the absence of IMP-3 and/or its partners PTBP1 and ILF3/NF90. The capacity of IMP-3 protein complex to bind the cyclin mRNAs in the nucleus (11) might give a specific advantage to these mRNAs that are subsequently translocated to the cytoplasm, already protected from miRNA-RISC complexes, and are thus translationally active (Fig. 5J). This ability to bind the cyclin mRNAs and to protect them from RISC is a novel function for a member of the IMP family, showing an important function for IMP-3 in rapid, sensitive and reversible cell cycle regulation of human cancer cells.

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