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The RNA helicase DDX5/p68 is a key factor promoting c-fos expression at different levels from transcription to mRNA export

Eleonora Zonta[1], Danielle Bittencourt[2], Samaan Samaan[1], Sophie Germann[1], Martin Dutertre[1] and Didier Auboeuf[1,*]

[1]Université de Lyon, Inserm U1052, CNRS UMR5286, Centre de Recherche en Cancérologie de Lyon, Centre Léon Bérard, 28 Rue Laennec, F-69008 Lyon, France and [2]Department of Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA

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ABSTRACT

It is widely accepted that pre-mRNA maturation, including splicing, is tightly coupled to both transcription and mRNA export, but factors linking the three processes are less understood. By analysing the estrogen-regulated expression of the c-fos mRNA that is processed during transcription, we show that the ddx5 RNA helicase, is required throughout the major nuclear steps of the expression of the *c-fos* gene, from transcription to mRNA export. Indeed, ddx5, whose recruitment on the *c-fos* gene was increased upon estrogen treatment, was required for the full transcriptional activation of the *c-fos* gene. In addition, ddx5 was required for c-fos co-transcriptional RNA splicing. When splicing occurred post-transcriptionally in the absence of ddx5, the c-fos mRNA was poorly exported into the cytosol because of inefficient recruitment of the TAP mRNA export receptor. Finally, ddx5 was present in the c-fos messenger ribonucleoprotein together with mRNA export factors, which further supports that ddx5 is a key operator in the c-fos 'mRNA factory'.

INTRODUCTION

Pre-mRNAs are transcribed in the nucleus, where they are processed and packaged into messenger ribonucleoprotein (mRNP) complexes. Proper production of mRNPs requires the addition of a 5′ cap structure, removal of introns, polyadenylation (pA) at the RNA 3′-end and loading of mRNA export factors. It has become clear that these events are integrated and coordinated in space and time as capping, 3′ end processing and to some extent, splicing, are coupled to transcription (1,2). In addition, multiple links have been described between pre-mRNA maturation and mRNA export. For example, the recruitment of the TREX complex, that consists in the THO complex and a set of export factors like the export adaptor ALY, is enhanced by splicing (3–7). The TAP mRNA export receptor is then recruited to the mRNPs, associates with nucleoporins and ensures the efficient translocation of the mRNA across the nuclear pore (5,6). Therefore, correct nuclear processing and recruitment of export factors target mRNA for export from the nucleus and if a transcript is not properly processed, it can be recognized by the nuclear surveillance machinery, retained in the nucleus, and/or degraded by the nuclear exosome, including the Rrp6 exonuclease (8,9). Rrp6 also plays a role in the tethering of unspliced transcripts to RNA Polymerase II (RNAPII), thereby providing a coordination between transcript maturation and either degradation or release (8,9).

While mRNA capping and 3′ end formation are tightly coupled with transcription initiation and termination, respectively, RNA splicing can proceed either during transcription (co-transcriptional splicing) or after transcription and release of the transcript from the DNA template (post-transcriptional splicing) (10–14). This distinction raises several questions. What is the fate of mRNAs that are not spliced during transcription compared with co-transcriptionally spliced mRNAs? What does determine that a splicing event should proceed in a specific mode (co-transcriptional versus post-transcriptional) and which factors are involved in the coupling between transcription and RNA processing?

In this context, the ddx5 (or p68) protein is particularly interesting. This DEAD box RNA helicase acts as a transcriptional co-regulator of several transcription factors,

*To whom correspondence should be addressed. Tel: +33 4 26 55 67 46; Fax: +33 4 78 78 27 20; Email: didier.auboeuf@inserm.fr

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

including the estrogen receptor (ER) (15–18). Ddx5, when recruited to target promoters by transcription factors, can in turn recruit or displace histone modifying enzymes, like CBP/p300 and HDACs, and/or recruit RNAPII, which ddx5 also binds to (19,20). Furthermore, ddx5 is a component of the spliceosome and facilitates the pre-spliceosome to spliceosome transition by unwinding the U1 snRNA/5′ splice site base-pairs thanks to its RNA helicase activity (21). It must be underlined that the splicing of some RNAs (e.g. CD44, Tau, H-ras and NFAT5) seems particularly sensitive to the expression level of ddx5 (22–25). In addition, a role of ddx5 in downstream steps has been suggested. Indeed, ddx5 is recruited early during the splicing process, leaves the spliceosome and comes back on the mRNA after splicing catalysis (26). The Drosophila ddx5 RNA helicase promotes RNA release from chromatin and its sequestration caused by fragile X premutation rCGG repeats could lead to mRNA transport dysfunction (27,28). Finally, ddx5 is a shuttling protein, suggesting that ddx5 might play a role in mRNA export (29,30).

By analysing the estrogen-regulated expression of the c-fos mRNA that is processed during transcription (10,13,14), we showed that ddx5, whose recruitment on the *c-fos* gene was increased upon estrogen treatment, was required for the full transcriptional activation of the *c-fos* gene. In addition, ddx5 was required for c-fos co-transcriptional RNA splicing and, in the absence of ddx5, the c-fos mRNA was poorly exported due to the alteration of TAP recruitment on the c-fos mRNA. Further supporting a role of ddx5 in c-fos mRNA export, ddx5 was present into an mRNP together with ALY and TAP. These data identify ddx5 as a key operator in the c-fos production line and show that a protein factor can impact on multiple steps of the expression process of a given gene, from transcription to mRNA export.

MATERIALS AND METHODS

Cell culture, stable cell lines and treatment

MCF-7 cells were grown in DMEM supplemented with 10% FBS, 1% glutamine and 4.5 g/l glucose (37°C, 5% CO_2). All cell culture reagents were from Gibco. HA-ddx5 was cloned in pTRE2-hyg vector to generate inducible MCF-7/Tet-On stable cell lines (Clontech). Resistant clones were selected with hygromycin (300 µg/ml, Clontech) and protein expression was checked after doxycycline (DOX) treatment (1 µg/ml) for 48 h. Before estradiol (E_2) treatment, 3×10^6 cells were plated per 10-cm dish and were kept for 72 h in red phenol-free DMEM supplemented with 2% charcoal-treated FBS. Cells were treated with a final concentration of 10^{-8} M E_2 for 1 h. Cells were transfected with siRNA (26.6 nM) using Lipofectamine RNAiMax (Invitrogen) following manufacturer's instructions. The siddx5 siRNA is a mixture of siRNA targeting the ddx5 5′ UTR (siUTRddx5) and the ddx5 coding sequence (siCDSddx5) (Supplementary Figure S6). Cells were harvested 48 h after transfection.

RNA preparation and RT-PCR

RNAs from different fractions were prepared as previously described (10,31,32). For nuclear fractionation, cells were lysed in RSB buffer (10 mM Tris–HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$) and the cytosolic fraction was isolated in RSBG40 buffer (10 mM Tris–HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 10% Glycerol, 0.5% NP40, 0.5 mM DTT, 100 U/ml RNase OUT). Nuclei pellet was resupended in 20 mM Tris Hcl [pH 8], 75 mM NaCl, 0.5 mM EDTA [pH 8], 0.85 mM DTT, 0.125 mM PMSF, 0.1 mg/ml yeast tRNA, 50% Glycerol and lysed in 20 mM Hepes, 300 mM NaCl, 0.2 mM EDTA [pH 8], 1 mM DTT, 7.5 mM $MgCl_2$, 1 M Urea, 0.5% NP40, 0.1 mg/ml yeast tRNA: the nucleoplasmic fraction (supernatant) was adjusted with 0.1% SDS before RNA extraction. The chromatin pellet fraction was resuspended in 10 mM Tris–HCl [pH 7.5], 510 mM NaCl, 10 mM $MgCl_2$ and incubated for 30 min at 37°C with 20 U DNase before proceeding with RNA extraction.

RNAs were extracted using TRIpure reagent (Roche) and 1 µl of Glycoblue (Ambion) was added before RNA precipitation with Isopropanol. Each RNA preparation was treated with DNase I (DNAfree, Ambion). Reverse transcription (RT) was performed with 0.1–1 µg of RNA using M-MLV (Invitrogen) and random primers. The RT reactions were diluted and used either in PCR reactions using GoTaq Polymerase (Promega) or in qPCR reactions using Master SYBR Green I (Roche) on a Roche LightCycler 480. Primers are described in Supplementary Figure S6. The relative RNA levels were determined on the basis of the threshold cycle for each qPCR product (Ct). c-fos and cyclin D1 (CCND1) mRNA levels in total cellular extracts were normalized by GADH mRNA levels.

Western blot analysis

Protein extracts were obtained using NP-40 buffer (50 mM Tris–HCl pH 8, 0.4 M NaCl, 5 mM EDTA pH 8, 1% NP40, 0.2% SDS, 1 mM DTT) and Protease Inhibitors (Roche). Between 10 and 30 µg of proteins were separated by SDS-PAGE and western blot was performed by using antibodies against c-fos (sc-7202 Santa Cruz), ddx5 (ab10261 Abcam), ALY (A9979 Sigma), TAP (sc-32319 Santa Cruz), Rrp6 (EXOSC10—ab50558 Abcam), ER (F-10 sc8002 Santa Cruz), actin (I-19 sc1616 Santa Cruz) or H3 (ab1791 Abcam) antibodies.

RNA and DNA chromatin IP

RNA and DNA chromatin immunoprecipitation (IP) was performed as previously described (10,32) using antibodies against RNAPII CTD (05-623 Millipore), ddx5 (ab10261 Abcam), ALY (A9979 Sigma), TAP (sc-32319 Santa Cruz), Rrp6 (EXOSC10—ab50558 Abcam), HA (11 867 423 001 Roche) or control immunoglobulins (Santa Cruz). RNAPII ChIP and RNAPII RNA-ChIP experiments were analysed with E1/2_E3/4, E1_i1, E2_i2, E4, 3′UTR#1, 3′UTR#2, 3′UTR#3, ERBS primers sets. Ddx5, ALY and TAP RNA-ChIPs were analysed with E1/2_E3/4 primer set. All the primers are described in Supplementary Figure S6.

Co-immunoprecipitation

MCF-7 cells were lysed in buffer containing 50 mM Tris–HCl pH 8, 150 mM NaCl, 1% NP40, Protease and Phosphatase inhibitors (Roche) and incubated for 30 min on ice. Cleared cell extracts were incubated overnight with 4 µg antibodies bound to protein G magnetic beads (Invitrogen). The protein-bound magnetic beads were washed three times with PBS 1X, suspended in loading buffer (Invitrogen) before western blot analysis. Inputs correspond to 5% of the cleared cell lysates.

In situ proximity ligation assay

The *in situ* proximity ligation assay (PLA) was conducted using the Duolink II Kit (Olink Bioscience) according to the manufacturer's instructions. Proximity ligation signals were detected with a Zeiss fluorescence microscope (63× objective).

RESULTS

Ddx5 is required for *c-fos* gene transcriptional activation in response to estradiol

As previously shown (33–35), the expression of the *c-fos* gene, which contains an estrogen receptor binding site (ERBS, Figure 1A) downstream of the pA site, was strongly stimulated after 1 h of estradiol (E_2) treatment of the ER-positive MCF-7 breast cancer cells (Figure 1B). While ddx5 depletion (Figure 1C) had no effect on the basal c-fos mRNA level, it almost completely abrogated the E_2-mediated effect (Figure 1B). Similar effects were obtained by comparing different control siRNAs (siCTRL) or different siRNAs targeting ddx5 (siddx5) (Supplementary Figures S1 and S2). To test the specificity of the effect of the ddx5 siRNA used, an siRNA targeting the 5′ UTR of the ddx5 mRNA (siUTRddx5) was transfected into an inducible MCF-7 stable cell line expressing HA-ddx5 cDNA that does not contain the 5′ UTR of the endogenous ddx5 mRNA. The effect of siUTRddx5 was compensated upon treatment of the cells with DOX that induced the expression of HA-ddx5 (Figure 1D and E).

Similar results were obtained after ddx5 depletion when analysing the c-fos pre-mRNA level (Figure 2A and Supplementary Figures S1 and S2). Furthermore, ddx5 depletion inhibited the recruitment of RNAPII induced by E_2 treatment all along the *c-fos* gene (Figure 2B). In addition, ChIP experiments using the MCF-7 inducible stable cell line expressing HA-ddx5 showed that the recruitment of ddx5 on the c-fos ERBS was increased by E_2 treatment (Figure 2C). Finally, ddx5 depletion did not decrease the c-fos mRNA half-life (Supplementary Figure S3). Collectively, these data demonstrated that

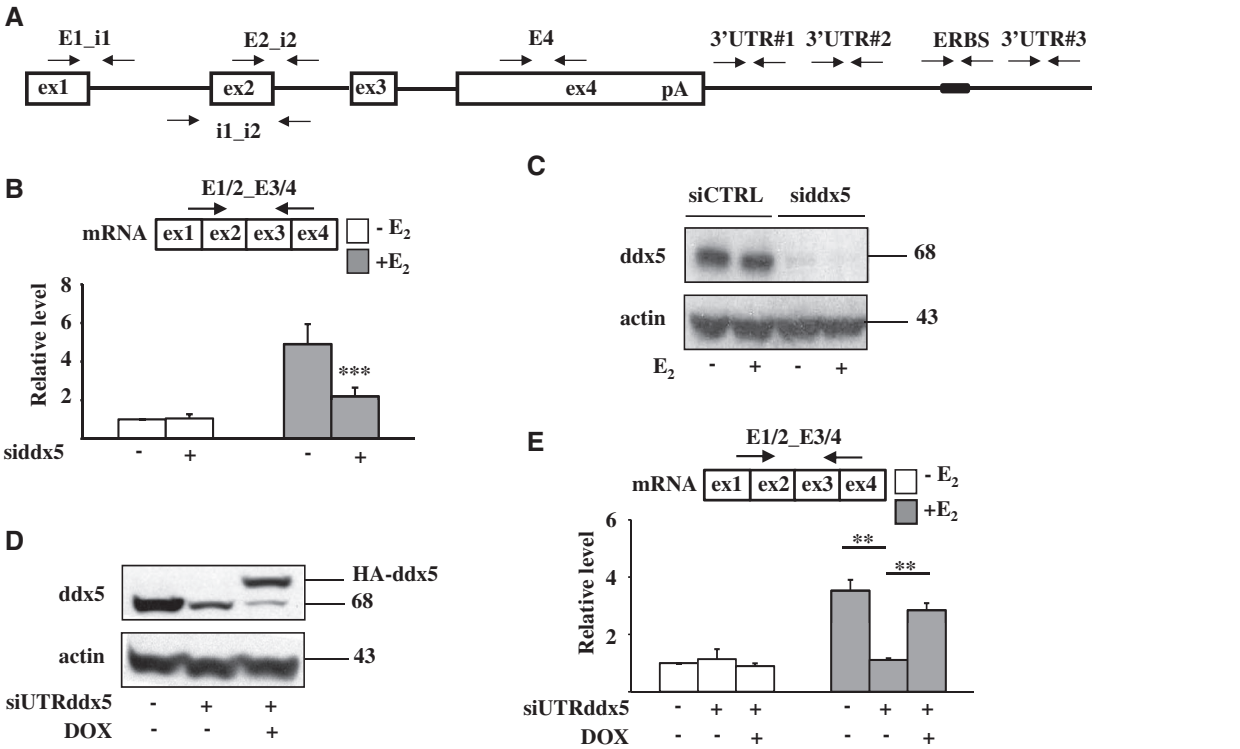

Figure 1. Ddx5 depletion impairs c-fos mRNA production in response to estradiol treatment. (**A**) Schematic structure of the *c-fos* gene and localization of the primers used. (**B**) RT-qPCR measuring the fully spliced c-fos mRNA using primers at the exon 1-exon 2 junction and at the exon 3-exon 4 junction. MCF-7 cells were transfected with a siCTRL or siddx5 before treatment with estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. (**C**) Western blot analysis of ddx5 and actin as a control, using protein extract from MCF-7 cells transfected with either a siCTRL or siddx5 in the presence or absence of estradiol (E_2) for 1 h. (**D**) Western blot analysis of ddx5 and actin as a control, using protein extract from an inducible stable MCF-7 cell line expressing HA-ddx5 after addition of DOX and transfected with an siUTRddx5. (**E**) The fully spliced c-fos mRNA was measured by RT-qPCR using an siUTRddx5 and an inducible MCF-7 stable cell expressing HA-ddx5 after addition of DOX for 48 h, as indicated. Histograms represent the average of at least three independent experiments. Error bars represent S.E.M. (**$P < 0.005$, ***$P < 0.0005$).

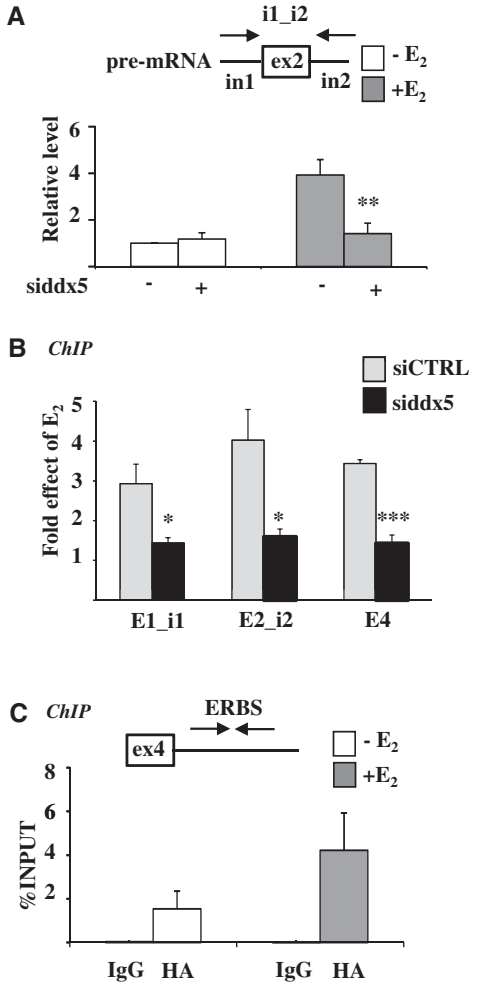

Figure 2. Ddx5 depletion impairs c-fos transcriptional induction by estradiol. (**A**) RT-qPCR measuring the unspliced c-fos pre-mRNA as in Figure 1B. (**B**) qPCR analysis of the RNAPII levels all along the *c-fos* gene as described in Figure 1A. The histograms represent the fold effect of E_2 that is the level of RNAPII (expressed as % of input) measured in the presence of E_2 for 1 h divided by the RNAPII level measured in the absence of E_2 either after cell transfection with siCTRL or siddx5. (**C**) qPCR analysis of ddx5 recruitment to the c-fos ERBS (Figure 1A) using an inducible MCF-7 cell line expressing HA-ddx5. The experiment was conducted after inducing HA-ddx5 expression by DOX treatment and treating cells with estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. ChIP was performed using an HA antibody (HA) or a control antibody (IgG). The data are expressed as % of input. Histograms represent the average of at least three independent experiments. Error bars represent S.E.M. ($*P < 0.05$, $**P < 0.005$, $***P < 0.0005$).

ddx5 is required for *c-fos* gene transcriptional activation in response to E_2.

Ddx5 depletion results in c-fos mRNA nuclear accumulation

Confirming our results, the E_2-induced expression of c-Fos protein was inhibited upon ddx5 depletion (compare lanes 3 and 4, Figure 3A). However, we noticed that the effect of ddx5 silencing was also strong in the absence of E_2 (compare lanes 1 and 2, Figure 3A), that was unexpected since it had no effect on c-fos mRNA and pre-mRNA levels and RNAPII recruitment in the absence of E_2 (Figures 1, 2, 4A and B).

Interestingly, fractionation experiments indicated that ddx5 depletion decreased the c-fos mRNA level in the cytosol while it increased it in the nucleus in the absence of E_2 (Figure 3B, C and Supplementary Figure S4A). After verifying that ddx5 depletion did not affect the sub-cellular localization of small nuclear or cytosolic RNAs (Supplementary Figure S4B), we concluded that in the absence of E_2, ddx5 depletion increased the c-fos mRNA nuclear to cytosolic ratio (N/C ratio) (Figure 3D). A similar effect was observed in the presence of E_2 (Figure 3D). Rescue experiments in the MCF-7 inducible cell line partially restored the initial c-fos mRNA N/C ratio (Figure 3E).

We next tested whether c-fos mRNA was still attached to RNAPII after ddx5 depletion, which would explain the c-fos mRNA nuclear accumulation observed in these conditions. To test this hypothesis, we measured by RNA-ChIP the level of c-fos RNA attached to RNAPII using primers targeting sequences downstream of the pA site (Figure 1A). As shown in Figure 3F, we did not detect any increase in the level of c-fos RNA attached to RNAPII after ddx5 depletion. Another primer set confirmed this result (Supplementary Figure S4C). Furthermore, the level of fully spliced c-fos mRNA associated with RNAPII was also not increased upon ddx5 depletion (Figure 3G). Collectively, these results demonstrated that the c-fos mRNA did not accumulate in the nucleus upon ddx5 depletion due to its retention within the RNAPII complex.

In fact, we observed a decrease in the level of the fully spliced c-fos mRNA attached to RNAPII (Figure 3G), which was confirmed in the MCF-7 inducible cell line (Figure 3H). Based on this observation, we hypothesized that the decrease in c-fos mRNA level attached to RNAPII upon ddx5 depletion result from an inhibition of c-fos splicing as the primers used in this experiment were designed to amplify the fully spliced c-fos mRNA.

Ddx5 is required for efficient co-transcriptional splicing of c-fos pre-mRNA

As shown in Figure 3C, the level of the fully spliced nuclear c-fos mRNA did not decrease upon ddx5 depletion, which suggests that the c-fos mRNA splicing was not globally inhibited. Interestingly, we and others have shown that c-fos mRNA splicing occurs during transcription (10,13,14). Therefore, we tested whether c-fos mRNA splicing was co-transcriptionally impaired upon ddx5 depletion. As ddx5 depletion had effects on c-fos pre-mRNA levels and RNAPII levels on the *c-fos* gene in the presence of E_2 (Figures 1 and 2), we made these experiments in the absence of E_2. In this condition, ddx5 depletion did not change the global nuclear level of c-fos pre-mRNA and did not affect the level of RNAPII on the *c-fos* gene using different primer sets at various locations along the *c-fos* gene (Figures 4A, B and 1A). Strikingly, while ddx5 depletion decreased the amount of fully spliced c-fos mRNA attached to RNAPII (Figure 3G), it increased the level of RNAPII-associated unspliced c-fos pre-mRNA, as measured using primers located in introns 1 and 2 (E1_i1 and E2_i2, Figure 4C). Furthermore, the level of partially

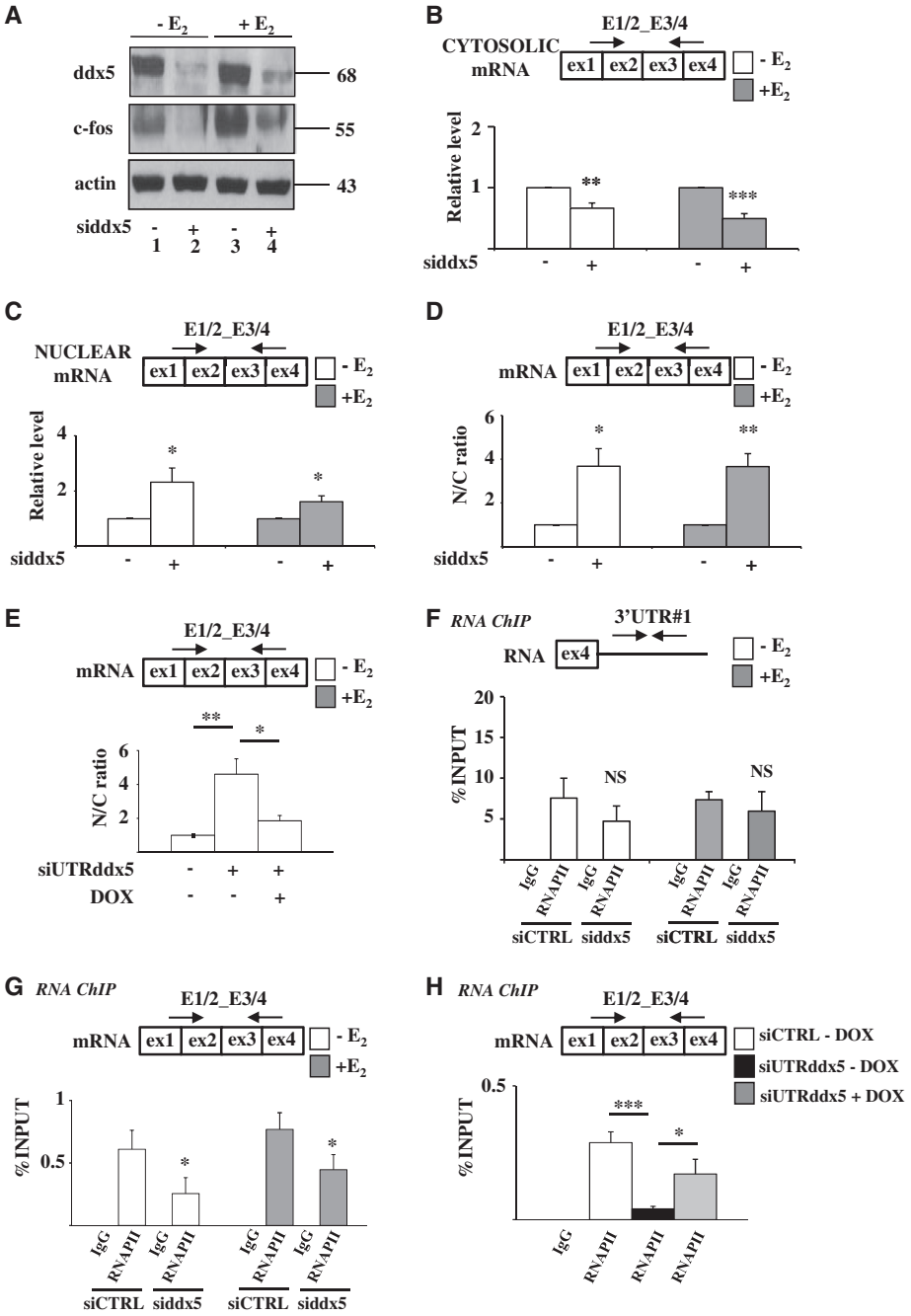

Figure 3. Ddx5 depletion induces c-fos mRNA nuclear accumulation. (**A**) Western blot analysis of c-fos, ddx5 and actin as a control using protein extract from MCF-7 cells transfected with either siCTRL or siddx5 and treated with either estradiol (+E_2) or vehicle (ethanol, −E_2) for 1 h. (**B**) The fully spliced c-fos mRNA was measured by RT-qPCR using cytosolic extracts from MCF-7 transfected with either siCTRL or siddx5 before treatment with either estradiol (+E_2) or vehicle (ethanol, −E_2) for 1 h. The cytosolic level obtained in the control experiments (siCTRL) was used as the baseline. (**C**) Same as above using RNAs from nuclear extracts. The nuclear level obtained in the control experiments (siCTRL) was used as the baseline. (**D**) N/C ratio of c-fos mRNA expression level. The N/C ratio obtained in the control experiments (siCTRL) was used as the baseline. (**E**) The fully spliced c-fos mRNA was measured by RT-qPCR as described above using an siUTRddx5 and an inducible MCF-7 stable cell expressing HA-ddx5 after addition of DOX for 48 h. (**F**) RT-qPCR measuring the c-fos RNA with primers downstream the pA site after cell cross-linking and IP using either a control antibody (IgG) or an antibody against RNAPII (RNA-ChIP). The results are expressed as % of input (nuclear RNA level) after transfection of MCF-7 with either siCTRL or siddx5 before treatment with either estradiol (+E_2) or vehicle (ethanol, −E_2) for 1 h. (**G**) RT-qPCR measuring the fully spliced c-fos mRNA in the same experimental conditions as above. (**H**) RT-qPCR analysis of fully spliced c-fos mRNA associated with RNAPII (RNA-ChIP) after cell cross-linking and IP using either a control antibody (IgG) or an antibody against RNAPII. The results are expressed as % of input (nuclear RNA level) after transfection of inducible stable MCF-7 cell line with either siCTRL or siUTRddx5 and after DOX treatment as indicated. Histograms represent the average of at least three independent experiments. Error bars represent S.E.M. (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

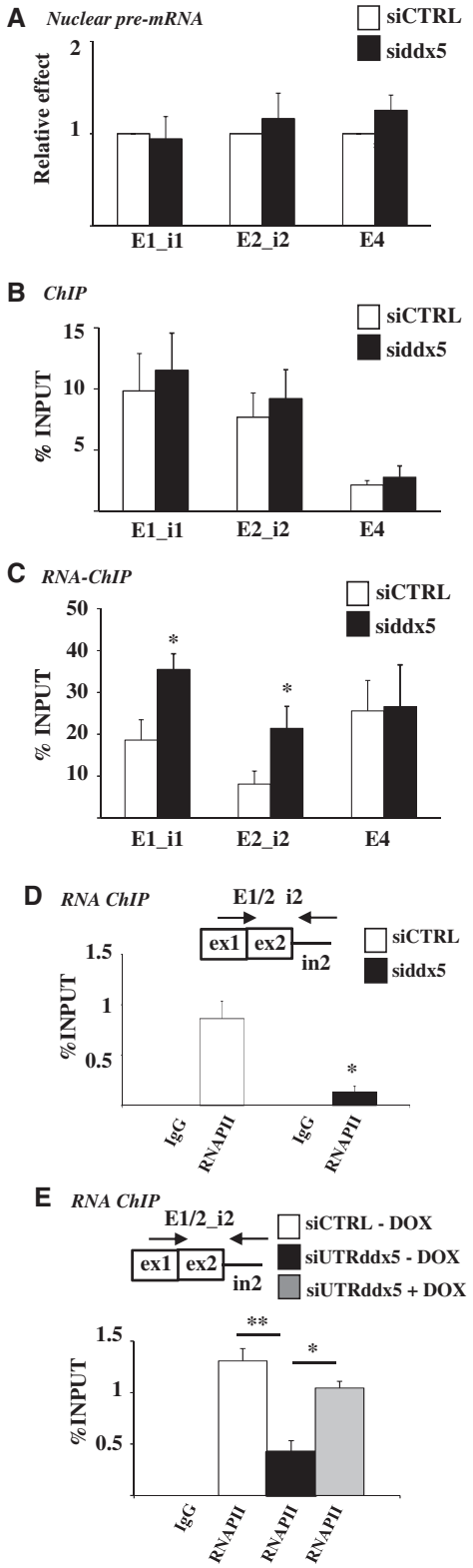

Figure 4. Ddx5 is required for efficient co-transcriptional c-fos splicing. (**A**) RT-qPCR measuring the nuclear c-fos pre-mRNA in the absence of hormone using primers described in Figure 1A. The values obtained in the control experiments (siCTRL) were used as the baseline to quantify the effect of ddx5 depletion (siddx5). (**B**) qPCR analysis of the RNAPII levels measured by ChIP and expressed as % of input using the same primers as above after transfection of MCF-7 with either siCTRL or siddx5. (**C**) RT-qPCR analysis of c-fos pre-mRNA associated with RNAPII (RNA-ChIP) using the same primers as above and expressed as % of input (nuclear level) after transfection of MCF-7 with either siCTRL or siddx5. (**D**) RT-qPCR analysis of partially spliced c-fos pre-mRNA associated with RNAPII (RNA-ChIP) after cell cross-linking and IP using either a control antibody (IgG) or an antibody against RNAPII. The results are expressed as % of input (nuclear RNA level) after transfection of MCF-7 with either siCTRL or siddx5. (**E**) RT-qPCR analysis of partially spliced c-fos pre-mRNA associated with RNAPII (RNA-ChIP) after cell cross-linking and IP using either a control antibody (IgG) or an antibody against RNAPII. The results are expressed as % of input (nuclear RNA level) after transfection of inducible stable MCF-7 cell line with either siCTRL or siUTRddx5 and after DOX treatment as indicated. Histograms represent the average of at least three independent experiments. Error bars represent S.E.M. (*$P < 0.05$, **$P < 0.005$).

spliced c-fos transcripts attached to RNAPII decreased after ddx5 depletion (Figure 4D), and was rescued upon re-expression of inducible ddx5 (Figure 4E). Collectively, these results indicated that ddx5 was required for efficient c-fos co-transcriptional splicing.

Ddx5 is required for making c-fos mRNA competent for export

As shown in Figure 4C, when measuring c-fos RNA association with RNAPII, a primer set (E4) localized in the last exon of c-fos showed no change upon ddx5 depletion, which suggests that c-fos transcript release from RNAPII was not affected by ddx5 depletion. This was unexpected as c-fos co-transcriptional splicing was impaired upon ddx5 depletion, and previous studies indicated that unspliced transcripts can be degraded by the Rrp6 (EXOSC10) exonuclease, a component of the exosome complex (8,9). Intriguingly, purification of ddx5-associated nuclear factors pointed to an interaction with components of the exosome complex, including Rrp6 (www.nursa.org/10.1621/datasets.01001). As shown in Figure 5A, Rrp6 and ddx5 can indeed co-immunoprecipitate. The interaction between endogenous ddx5 and Rrp6 proteins was further addressed using the *in situ* ligation proximity assay (PLA), which generates a signal when two proteins are in close proximity (~40 nm) to each other. As shown in Figure 5B, prominent signals were detected in fixed MCF-7 cells using anti-Rrp6 and anti-ddx5 antibodies but not in control experiments.

Furthermore, ddx5 depletion, which did not affect Rrp6 protein level (Figure 5C), reduced the recruitment of Rrp6 on the *c-fos* gene both in the presence and in the absence of E_2 (Figure 5D). As Rrp6 may travel with RNAPII, we compared RNAPII and Rrp6 patterns along the *c-fos* gene. In the absence of E_2, Rrp6 level was highest at the *c-fos* gene 3′-end, where it was significantly affected by ddx5 depletion (Figure 5E), whereas RNAPII level on the *c-fos* gene was not affected (Figures 5E and 4B). Thus, the decrease in Rrp6 recruitment on the *c-fos* gene after ddx5 depletion was not a consequence of RNAPII decrease at least in the absence of hormone. In the presence of hormone, we cannot exclude that the decrease in Rrp6 recruitment on the *c-fos* gene after ddx5 depletion might be due in part to RNAPII decrease (Figure 2B). Finally, Rrp6 depletion did not increase the c-fos mRNA N/C ratio and did not therefore

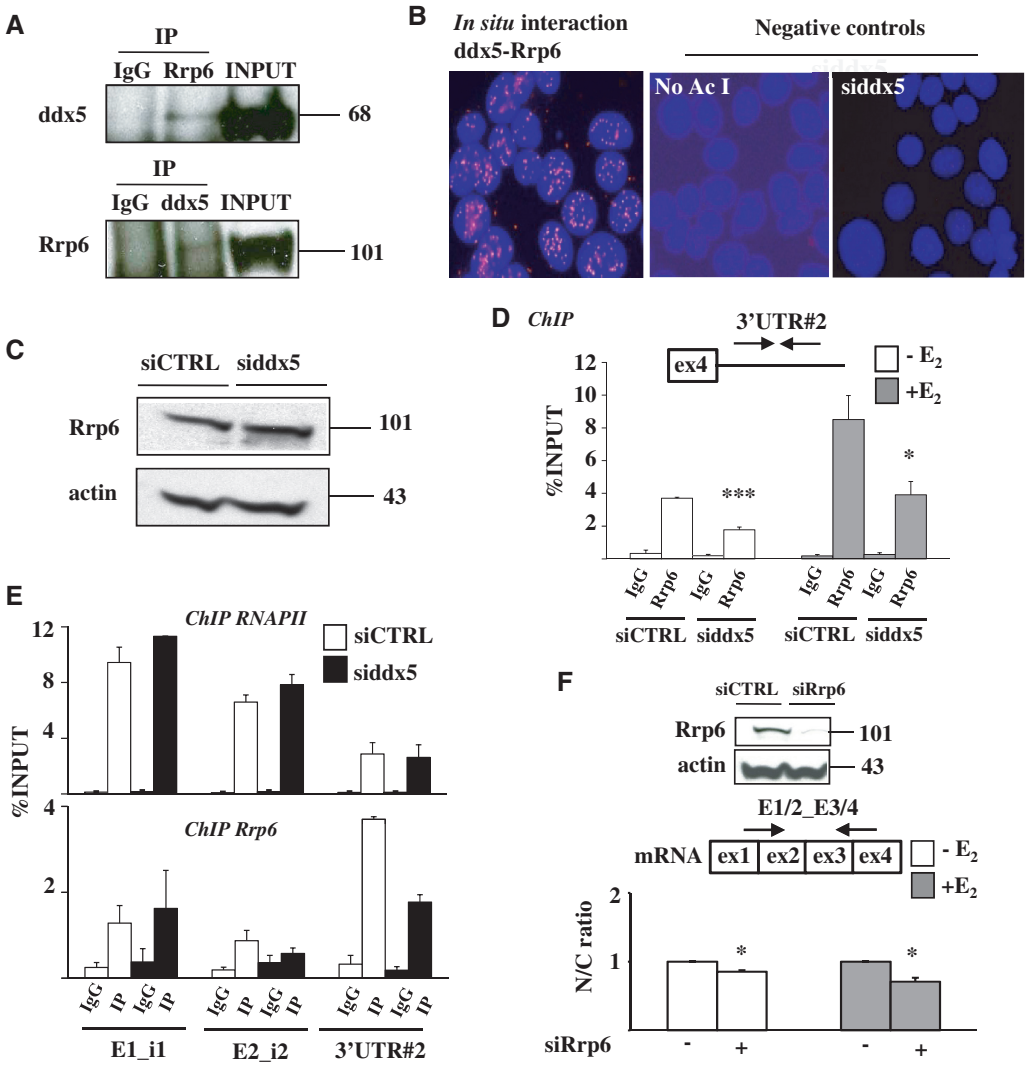

Figure 5. Ddx5 depletion impairs Rrp6 recruitment to the *c-fos* gene. (**A**) Western blot analysis of ddx5 after IP with either a control antibody (IgG) or an Rrp6 antibody and western blot analysis of Rrp6 after IP with either a control antibody (IgG) or an ddx5 antibody. (**B**) Detection of endogenous Rrp6-ddx5 complex by *in situ* PLA. Fixed MCF-7 cells were incubated with antibodies against Rrp6 and ddx5. As negative controls, PLA experiments were performed in the absence of primary antibodies (No Ac I) or after MCF-7 cells transfection with siddx5. (**C**) Western blot analysis of Rrp6 and actin, as a control using protein extract from MCF-7 cells transfected with either a siCTRL or siddx5. (**D**) qPCR analysis of Rrp6 recruitment on the *c-fos* 3′ end gene using either an Rrp6 antibody or a control antibody (IgG) after MCF-7 cell transfection with either siCTRL or siddx5 and cell treatment with either estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. The results are expressed as % of input. (**E**) qPCR analysis of the RNAPII and Rrp6 levels measured by ChIP and expressed as % of input using different set of primers (Figure 1A) after transfection of MCF-7 with either siCTRL or siddx5 in the absence of hormone. (**F**) Western blot analysis of Rrp6 and actin, as a control using protein extract from MCF-7 cells transfected with either an siCTRL or siRNAs targeting Rrp6 (siRrp6). The fully spliced c-fos mRNA was measured by RT-qPCR using cytosolic or nuclear extracts from MCF-7 transfected with either siCTRL or siRrp6 before treatment with either estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. The N/C ratio obtained in the control experiments (siCTRL) were used as the baseline. Histograms represent the average of at least three independent experiments. Error bars represent S.E.M. ($*P < 0.05$, $***P < 0.0005$).

decrease the export of the c-fos mRNA (Figure 5F and Supplementary Figure S5A), suggesting that the nuclear accumulation of c-fos mRNA upon ddx5 depletion is likely not mediated by Rrp6. Altogether, these data suggest a role of ddx5 in Rrp6 recruitment to the *c-fos* gene, and the decrease in Rrp6 recruitment on the *c-fos* gene upon ddx5 depletion could explain why c-fos RNAs were released from RNAPII despite not being spliced in this condition.

We next investigated why the c-fos mRNAs produced in ddx5-depleted cells were poorly exported despite their efficient release from RNAPII. First, we noticed that the nuclear fully spliced c-fos mRNA was enriched in the 'chromatin fraction' compared with the 'nucleoplasm fraction' (Figure 6A and B). It must be underlined that the so-called 'chromatin fraction' may contain nuclear speckles that are enriched in splicing factors and might play a role in post-transcriptional splicing (11,12). Second, a larger proportion of c-fos mRNA was associated with the mRNA export adaptor ALY (Figure 6C), whereas a smaller proportion of c-fos mRNA was found associated with the mRNA export receptor TAP (Figure 6D) after ddx5 depletion. This effect was not due to decrease of TAP expression

level upon ddx5 depletion (Figure 6D). Further supporting a role of TAP in c-fos mRNA export, TAP silencing (Figure 6E and Supplementary Figure S5B) increased the c-fos mRNA N/C ratio (Figure 6F) as ddx5 depletion did (Figure 3D). The effect of TAP depletion was weaker in the presence of E_2, likely because it reduced ER protein expression levels (Figure 6E).

Ddx5 is present in the c-fos mRNP

It has been shown that ddx5 is recruited early during the splicing process, then leaves the spliceosome and comes back after splicing catalysis (26). Supporting a role of ddx5 after splicing, ddx5 was found in mRNP containing mature mRNA and is a shuttling protein (29,30). As shown in Figure 7A, ddx5 was indeed associated with the c-fos mRNA both in the absence and in the presence of E_2. As a control, the inducible stable cell line expressing HA-ddx5 upon DOX treatment was used. The c-fos mRNA was detected after IP with the HA antibody only in the presence of DOX (Figure 7B). In addition, ddx5 co-immunoprecipitated with both ALY and TAP (Figure 7C) in an RNA-dependent manner,

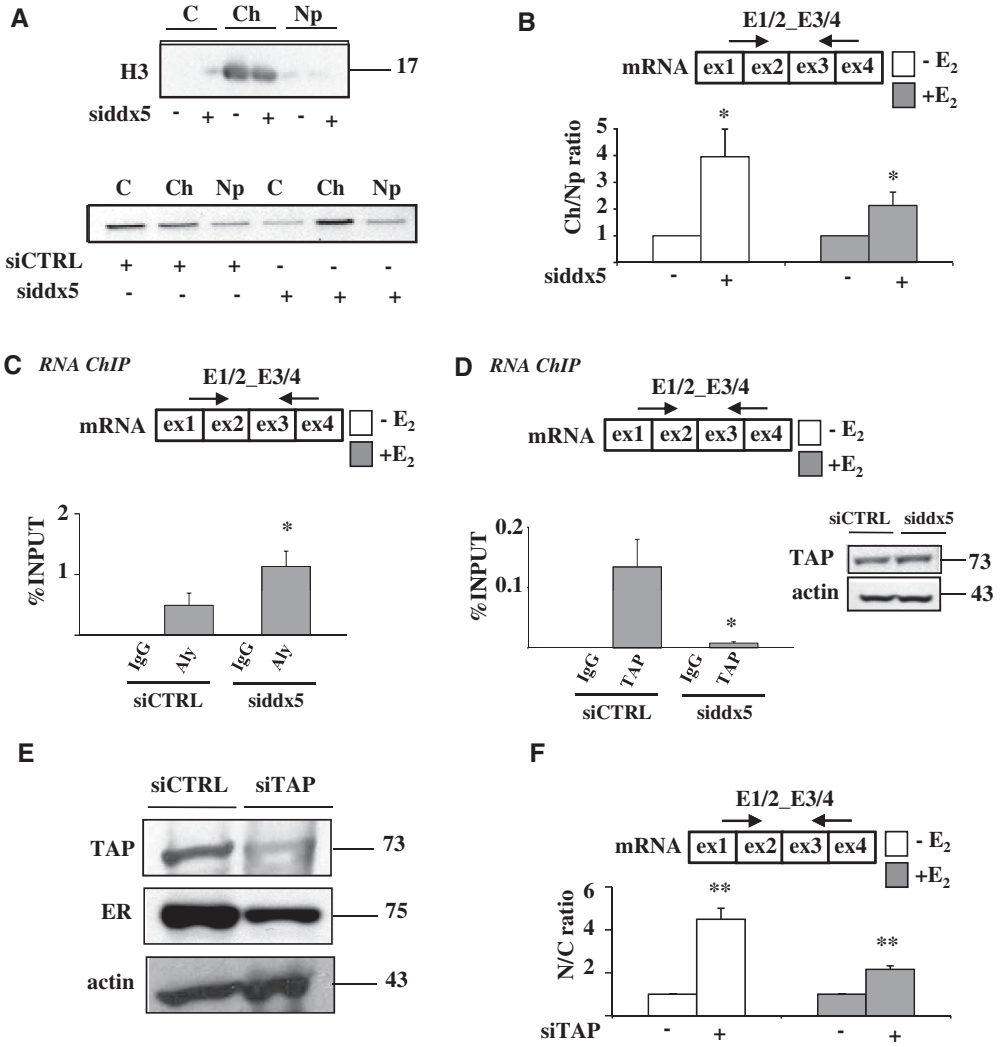

Figure 6. Ddx5 is required for making c-fos mRNA competent for export. (**A**) Western blot analysis of H3 histone (upper panel) using protein extract from cytosolic (C), chromatin (Ch), and nucleoplasmic (Np) fractions of MCF-7 cells transfected with either a siCTRL or siddx5. The c-fos mRNA was amplified by RT-PCR (lower panel) using C, Ch and Np extracts from MCF-7 transfected with either a siCTRL or siddx5. (**B**) The fully spliced c-fos mRNA was measured by RT-qPCR using Ch and Np fractions from MCF-7 transfected with either a siCTRL or siddx5 before treatment with either estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. The chromatin and nucleoplasm ratio (Ch/Np ratio) obtained in the control experiments (siCTRL) was used as the baseline. (**C**) RT-qPCR measuring the fully spliced c-fos mRNA after cell cross-linking and IP using either a control antibody (IgG) or an antibody against ALY (RNA-ChIP). The results are expressed as % of input (nuclear level) after transfection of MCF-7 with either siCTRL or siddx5 before treatment with estradiol for 1 h. (**D**) Same as above using either a control antibody (IgG) or an antibody against TAP. Western blot analysis of TAP and actin, as a control using protein extract from MCF-7 cells transfected with either a siCTRL or siddx5. (**E**) Western blot analysis of TAP, ER and actin as a control, using protein extract from MCF-7 cells transfected with either a siCTRL or siRNAs targeting TAP (siTAP). (**F**) The fully spliced c-fos mRNA was measured by RT-qPCR using cytosolic and nuclear extracts from MCF-7 transfected with either a siCTRL or siTAP before treatment with either estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. The N/C ratio obtained in the control experiments (siCTRL) were used as the baseline. Histograms represent the average of at least three independent experiments. Error bars represent S.E.M. ($*P < 0.05$, $**P < 0.005$).

suggesting that ddx5-containing mRNP complexes also contain ALY or TAP.

Finally, ddx5 depletion also affected the production of CCND1 pre-mRNA in response to estradiol (Figure 7D), which was expected since CCND1 is a well-characterized ER-target gene and was also shown to be regulated by ddx5 (36,37). Like in the case of c-fos, ddx5 depletion increased the nuclear accumulation of CCND1 mRNA both in the presence and absence of hormone (Figure 7E) suggesting that ddx5 is required for the proper synthesis and export of at least a subset of ER-target genes.

DISCUSSION

While it has been shown that some protein factors can physically link the transcriptional and mRNA export machineries and play a role in different steps of the gene expression process, their different functions were often analysed on different mRNA targets (1–4,7,38). In this report, we show that a factor can impact on multiple steps in the expression process of a single gene, from its transcription to the export of the mature mRNA. We propose a model (Figure 7F) in which ddx5 is required for (i) the transcriptional activation of the

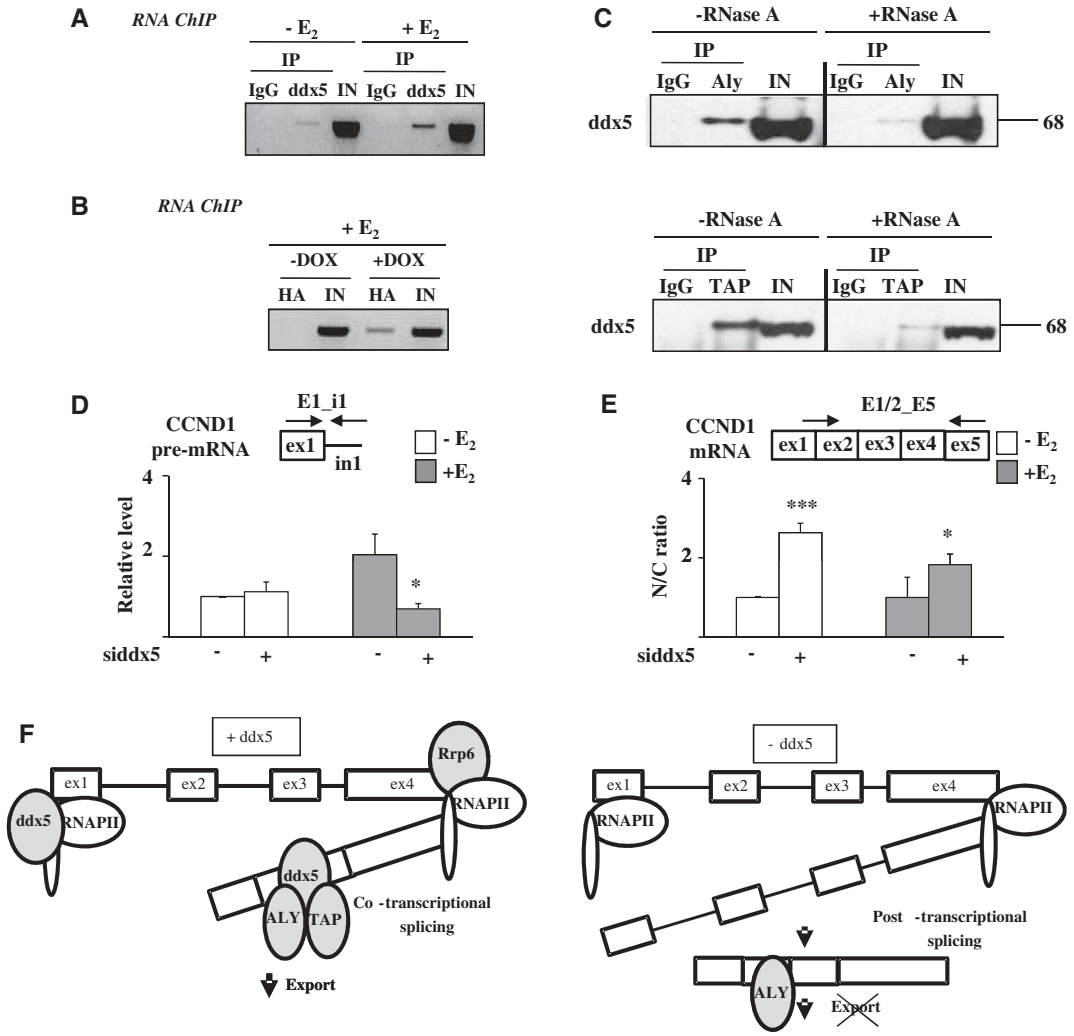

Figure 7. Ddx5 is present in the c-fos mRNP. (**A**) RT-PCR analysis of fully spliced c-fos mRNA associated with ddx5 (RNA-ChIP) after cell cross-linking and IP using either a control antibody (IgG) or ddx5 antibody. Input represents nuclear RNA. MCF-7 were treated with either estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. (**B**) RT-PCR analysis of fully spliced c-fos mRNA associated with HA-ddx5 (RNA-ChIP) using an inducible MCF-7 cell line expressing HA-ddx5 (+DOX) or not (−DOX). After cross-linking, the IP was performed using an HA antibody. Input represents nuclear RNA. (**C**) Western blot analysis of ddx5 after IP with either control antibodies (IgG) or antibodies against ALY, or TAP, as indicated. Cellular extracts were treated or not with RNase A before IP. (**D**) RT-qPCR measuring the unspliced CCND1 pre-mRNA using cellular extracts from MCF-7 transfected with either siCTRL or siddx5 before treatment with either estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. The CCND1 pre-mRNA level value obtained in the control experiment (siCTRL, $-E_2$) was used as the baseline. (**E**) The fully spliced CCND1 mRNA was measured by RT-qPCR using cytosolic or nuclear extracts from MCF-7 transfected with either siCTRL or siddx5 before treatment with either estradiol ($+E_2$) or vehicle (ethanol, $-E_2$) for 1 h. The N/C ratio obtained in the control experiments (siCTRL) was used as the baseline. Histograms represent the average of at least three independent experiments. Error bars represent S.E.M. ($*P < 0.05$, $***P < 0.0005$). (**F**) Ddx5 is required for the transcriptional activation of the *c-fos* gene, the efficient co-transcriptional c-fos RNA splicing and the efficient export of the c-fos mature mRNA. In the absence of ddx5, the c-fos RNA might be spliced after transcription, which disturbs the recruitment of TAP and the c-fos mRNA export.

c-fos gene; (ii) efficient co-transcriptional splicing of c-fos pre-mRNA; and (iii) efficient export of mature c-fos mRNA. In the absence of ddx5, the c-fos transcripts appeared to be spliced after transcription and both the diffusion of the mRNA into the nucleoplasm and the recruitment of TAP were disturbed (Figure 7F).

While the roles of ddx5 as transcriptional co-regulator and splicing factor have been extensively analysed, several reports suggested a role of ddx5 in downstream steps of gene expression. For example, the Drosophila ddx5 promotes RNA release from chromatin (27,28). Remarkably, while this manuscript was under preparation, Dbp2, the yeast ddx5 ortholog, was also proposed to play a role in facilitating RNP assembly and clearance of transcripts from genomic loci (39). Interestingly, it was shown that loss of DBP2 is synthetic lethal with depletion of RRP6 (39). Because ddx5 is a shuttling protein (30) and as we reported here that ddx5 was present on c-fos mRNA, these results collectively demonstrate a role of ddx5 in the control of nuclear mRNA fate.

An important remaining question regards the connection between all ddx5 cellular functions. Addressing this question is likely going to be very challenging as the effect of ddx5 on specific step(s) might be more or less pronounced depending on the target gene and depending on which step(s) will be rate-limiting for each target gene. In addition, because all the steps of the gene expression process can impact on each other, it will be difficult to analyse precisely the molecular action of ddx5 on a selected step. In this context, our results suggest a model where ddx5 depletion inhibited c-fos co-transcriptional splicing (Figure 4), which then impairs c-fos mRNA export. As ddx5 depletion reduced the recruitment of Rrp6 (Figure 5), unspliced c-fos RNAs were not degraded and had a chance to be spliced post-transcriptionally, slowing down the release of Aly and the recruitment of TAP (Figure 6). It is likely that this results in inefficient mRNA export rather than complete nuclear retention of c-fos mRNA. Indeed, despite our efforts, we could not observe any local accumulation of c-fos mRNA by FISH in the nucleus of ddx5-depleted cells (not shown), suggesting that c-fos mRNA are not retained at specific nuclear sites. In addition, c-fos mRNA was still detected in the cytosol after depletion of ddx5 for 48 h (Supplementary Figure S4A) despite its short half-life. Because the c-fos mRNA export becomes inefficient rather than fully inhibited upon ddx5 depletion and because the CCND1 mRNA synthesis and export was also affected by ddx5 depletion, we propose that ddx5 plays a role in the efficient production of a subset of mRNAs.

This assumption is in agreement with the hypothesis that at least some mRNAs might be produced in a very efficient way in what has been referred to as the 'mRNA factory' (1). The efficiency of co-transcriptional RNA processing might play a critical role for gene products whose expression level is rapidly and tightly regulated, like the *c-fos* gene. Indeed, *c-fos* is an immediate early gene that is induced from transcription to protein synthesis within 1 h in response to various stimuli, such as estrogen in breast cancer cells (33–35). As c-fos is a cell cycle activator required during the initial G0–G1 transition stimulated by growth factors, the efficient co-transcriptional processing and export of c-fos mRNA may be a key component of the rapid mitogenic response of MCF-7 cells to estrogen stimulation. Interestingly, ddx5 also seems to play an important role in the synthesis and export of the CCND1 mRNA, which also encodes a key cell cycle regulator whose expression is induced by estrogen (Figure 7D and E).

Collectively, these data demonstrate that, transcriptional co-regulators like ddx5 impact on gene expression not only by controlling the assembly of the transcriptional machinery, but also by coordinating multiple co-transcriptional nuclear gene expression steps.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6.

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