

DDX6 recruits translational silenced human reticulocyte 15-lipoxygenase mRNA to RNP granules

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ABSTRACT

Erythroid precursor cells lose the capacity for mRNA synthesis due to exclusion of the nucleus during maturation. Therefore, the stability and translation of mRNAs that code for specific proteins, which function in late stages of maturation when reticulocytes become erythrocytes, are controlled tightly. Reticulocyte 15-lipoxygenase (r15-LOX) initiates the breakdown of mitochondria in mature reticulocytes. Through the temporal restriction of mRNA translation, the synthesis of r15-LOX is prevented in premature cells. The enzyme is synthesized only in mature reticulocytes, although r15-LOX mRNA is already present in erythroid precursor cells. Translation of r15-LOX mRNA is inhibited by hnRNP K and hnRNP E1, which bind to the differentiation control element (DICE) in its 3' untranslated region (3'UTR). The hnRNP K/E1-DICE complex interferes with the joining of the 60S ribosomal subunit to the 40S subunit at the AUG. We took advantage of the inducible human erythroid K562 cell system that fully recapitulates this process to identify so far unknown factors, which are critical for DICE-dependent translational regulation. Applying RNA chromatography with the DICE as bait combined with hnRNP K immunoprecipitation, we specifically purified the DEAD-box RNA helicase 6 (DDX6) that interacts with hnRNP K and hnRNP E1 in a DICE-dependent manner. Employing RNA interference and fluorescence in situ hybridization, we show that DDX6 colocalizes with endogenous human (h)r15-LOX mRNA to P-body-like RNP granules, from which 60S ribosomal subunits are excluded. Our data suggest that in premature erythroid cells translational silencing of hr15-LOX mRNA is maintained by DDX6 mediated storage in these RNP granules.

Keywords: r15-LOX mRNA 3'UTR; hnRNP K and hnRNP E1; DICE; DDX6; translation regulation; RNP granules

INTRODUCTION

RNA-protein complexes control gene expression at the post-transcriptional level. They regulate the cytoplasmic events, such as mRNA localization, stabilization, and translation, known to be involved in development and differentiation (Thompson et al. 2007). A common feature of these regulatory complexes is the binding of *trans*-acting proteins to

cis-elements located in the 5' untranslated region (UTR) and 3'UTR of mRNAs (Hentze et al. 2007). The regulation of mRNA translation in erythroid precursor cells that undergo nuclear extrusion when they mature to erythrocytes represents a striking example for such processes. While all mRNAs have to be synthesized in bone marrow erythroblasts before the nucleus is lost, those that are not translated until later in erythroid maturation are stored in translational silent messenger ribonucleoprotein particles (mRNPs). Enucleated reticulocytes are released into the blood stream and mature until the mitochondria are degraded and reticulocytes become functional erythrocytes. The breakdown of mitochondria at this final step is initiated by the enzyme r15-LOX, which catalyzes the dioxygenation of phospholipids in mitochondrial membranes (Rapoport and Schewe 1986; van Leyen et al. 1998; Gröllich et al. 2001). The timing of r15-LOX mRNA translation is controlled tightly because synthesis of r15-LOX in premature cells would

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disturb the energy metabolism (Thiele et al. 1981; Höhne et al. 1988). We have identified hnRNP K and hnRNP E1 that bind singly or together to the differentiation control element (DICE) in the r15-LOX mRNA 3'UTR, thereby inhibiting mRNA translation (Ostareck et al. 1997). Interestingly, the hnRNP K/E1-DICE complex interferes with the last step of translation initiation, the joining of the 60S ribosomal subunit to the 40S subunit at the initiation codon (Ostareck et al. 2001). For the first time, we could show how a 3'UTR complex inhibits translation initiation that starts at the 5'UTR. Later, other examples were identified, which act through a similar mechanism. IGF2BP1 binds to the zip-code motif in the β -actin mRNA 3'UTR and inhibits 60S ribosomal subunit joining in neuronal cells (Hüttelmaier et al. 2005).

Previously we have set up an inducible human erythroid cell system based on K562 cells (Naarmann et al. 2008). During induction of erythroid maturation, K562 cells exhibit changes in morphology and protein expression that are characteristic for terminal erythroid cell maturation: nuclear exclusion, expression of hr15-LOX regulated by hnRNP K and hnRNP E1, and loss of mitochondria (Naarmann et al. 2008).

Having established this system, we aimed at the identification of new components of the hnRNP K/E1-DICE silencing complex that maintain translational silencing of hr15-LOX mRNA in erythroid cell maturation. We purified translational silenced mRNPs from translation competent K562 cell extract that recapitulates DICE-dependent translation control, and identified the DEAD box RNA helicase family member DDX6. Employing immunoprecipitation experiments, *in vivo* RNA-binding assays, RNAi, and fluorescence in situ hybridization (FISH), we demonstrate that DDX6 cooperates with hnRNP K and hnRNP E1 in maintaining hr15-LOX mRNA silencing in premature cells. Our data indicate that DDX6 recruits hr15-LOX mRNA into P-body-like RNP granules primed for the storage of translational repressed mRNAs.

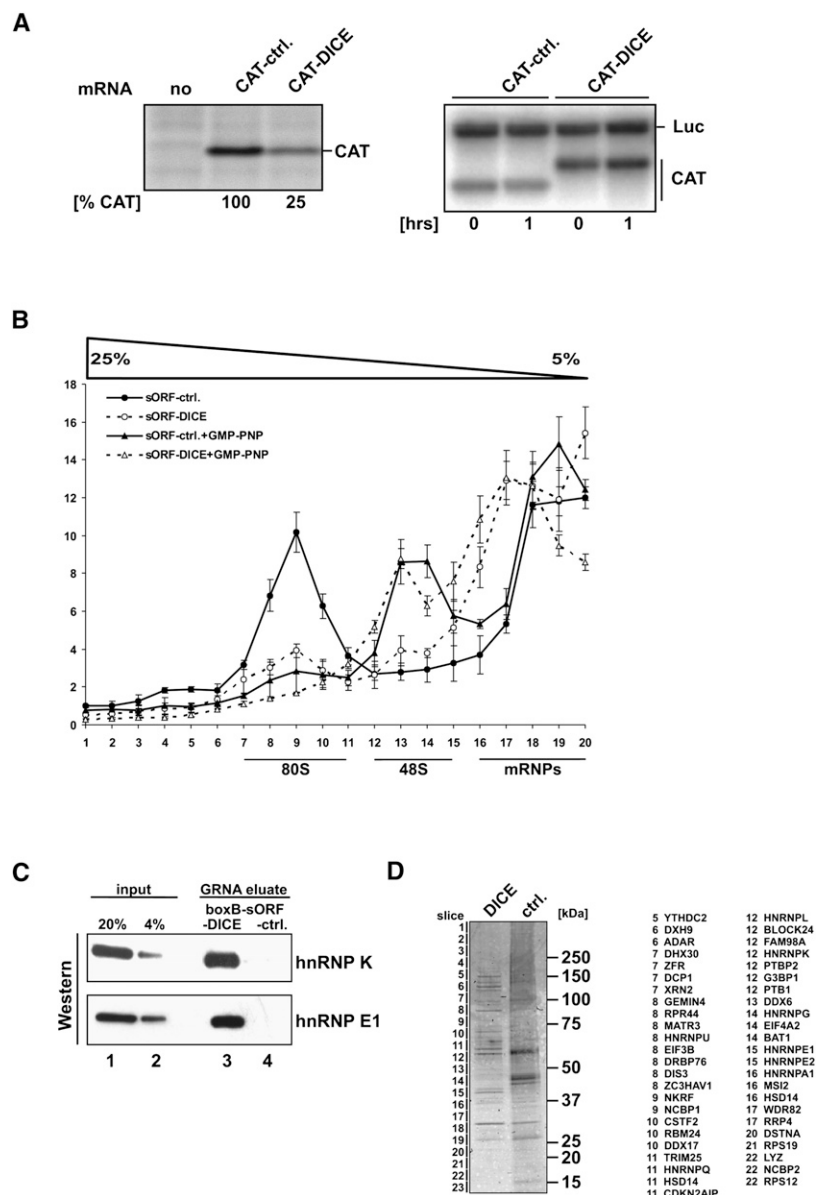


FIGURE 1. Extract of K562 cells, which fully recapitulate translational control of the r15-LOX mRNA, was used for GRNA chromatography. (A, left) [32 P]-Trace-labeled reporter mRNAs CAT-ctrl, or CAT-DICE were translated in cytoplasmic extract of noninduced K562 cells in the presence of [35 S]-methionine. Translation products were resolved by SDS-PAGE and analyzed by autoradiography. The percentage of synthesized CAT protein is indicated below. (Right) [32 P]-Trace-labeled mRNAs were extracted from the translation reactions at time point 0 and after 1 h of translation. [32 P]-Trace-labeled Luciferase (Luc) mRNA was used as an extraction control. The extracted mRNAs were separated on an agarose gel and analyzed by autoradiography. (B) Fractionation of translation initiation reactions containing cytoplasmic extract of noninduced K562 cells and [32 P]-labeled reporter mRNAs sORF-ctrl or sORF-DICE in the presence of cycloheximide or cycloheximide and GMP-PNP on 5%–25% sucrose gradients and analysis by scintillation counting. Positions of 80S-, 48S-, and mRNPs-containing fractions are indicated. (C) Reporter mRNAs containing six boxB stem-loops boxB-sORF-DICE or boxB-sORF-ctrl were incubated with cytoplasmic extracts of noninduced K562 cells to assemble silenced or nonsilenced RNPs and were subsequently purified by GRNA chromatography (lanes 3,4). Enriched proteins were eluted with SDS sample buffer and analyzed in Western blot assays using antibodies against hnRNP K and hnRNP E1, respectively. (D) GRNA chromatography with the indicated mRNAs was combined with an anti-hnRNP K immunoprecipitation, resolved on a 4%–12% NuPAGE Bis-Tris gel, stained with colloidal Coomassie, and analyzed by mass spectrometry. Numbers at the left indicate the position of gel slices used for the identification of proteins shown at the right.

RESULTS

GRNA chromatography employing translation competent K562 cell extract

In order to identify new regulatory components of the inhibitory hnRNP K/E1-DICE complex, we took advantage of glutathione Sepharose RNA affinity (GRNA) chromatography (Czaplinski et al. 2005; Duncan et al. 2006). RNA–protein complex isolation required an appropriate translation competent extract and a bait mRNA, which enabled the specific immobilization of regulatory complexes.

Previously, we could show that expression of endogenous hr15-LOX is translationally regulated by hnRNP K and hnRNP E1 in K562 cells induced for erythroid maturation (Naarmann et al. 2008). To prove that noninduced K562 cell extract is capable to mediate the DICE-dependent translational inhibition, we performed *in vitro* mRNA translation assays (Fig. 1A). [32 P]-Trace-labeled 5′m⁷G-cap CAT reporter mRNAs bearing either the DICE (CAT-DICE) or a control sequence (CAT-ctrl) in their 3′UTR were translated in cytoplasmic extracts of noninduced K562 cells in the presence of [35 S]-methionine (Fig. 1A). Translation of CAT-DICE mRNA was reduced to 25% compared with CAT-ctrl mRNA (Fig. 1A, left panel). The reduced CAT expression from the DICE-bearing mRNA was not due to mRNA degradation, as demonstrated by mRNA isolation with [32 P]-trace-labeled Luc mRNA as extraction control, at time point 0 and after 1 h of translation. (Fig. 1A, right panel). This indicates that the K562 cell extract mediates DICE-dependent translation inhibition.

Next, we performed *in vitro* translation initiation reactions, which were analyzed by sucrose gradient centrifugation. [32 P]-Labeled reporter mRNAs (sORF-DICE or sORF-ctrl) were incubated for 15 min in cytoplasmic extracts of noninduced K562 cells in the presence of cycloheximide, which inhibits the peptidyltransferase reaction and thereby stalls 80S ribosomes at the initiation codon (Obrig et al. 1971). The incorporation of sORF-DICE mRNA in fractions 7–11 that contain 80S ribosomes was strongly reduced compared with sORF-ctrl mRNA (Fig. 1B), consistent with Figure 1A. Interestingly, when DICE-dependent inhibition of 80S complex formation occurs, a shoulder appeared in fractions 12–14 that corresponds to the position at which the 48S initiation complexes sediment. Most of the repressed mRNA were detected in the mRNP fractions, indicating that 48S initiation complexes were unstable. In the presence of the nonhydrolysable GTP analog GMP-PNP, which stalls 43S ribosomal preinitiation complexes at the initiator AUG (Hershey and Monro 1966; Anthony and Merrick 1992), both reporter mRNAs accumulated in 48S fractions (Fig. 1B). A similar pattern had been obtained when recombinant hnRNP K and hnRNP E1 were added to the rabbit reticulocyte lysate, in which 80S ribosome formation was inhibited, whereas 48S initiation complexes were enriched

(Ostareck et al. 2001). In summary, this shows that non-induced K562 cells contain all the factors required for DICE-dependent translation inhibition *in vitro*.

Next we performed GRNA chromatography, which has proven to be highly efficient and specific for the purification of the translational inhibitor UNR, which is recruited to msl-2 mRNA by SXL from *Drosophila* embryo extract (Duncan et al. 2006). For bait, we employed a DICE bearing mRNA and a control mRNA, both carrying box-B hairpin sequences (Fig. 1C). The 80S ribosomal complex formation was not disturbed by the box-B hairpin sequences (data not shown). Each mRNA was incubated with K562 extract under translation conditions, and DICE-dependent inhibitory complexes were allowed to assemble. As shown by Western blot analysis, hnRNP K and hnRNP E1 were specifically bound to the GRNA matrix in a DICE-dependent manner (Fig. 1C, cf. lanes 3 and 4). To isolate specific protein–RNA complexes from the pool obtained through glutathione elution, hnRNP K immunoprecipitation was performed subsequently. Proteins eluted from both matrices were separated on a 4%–12% NuPAGE gel. Following colloidal Coomassie staining (Fig.

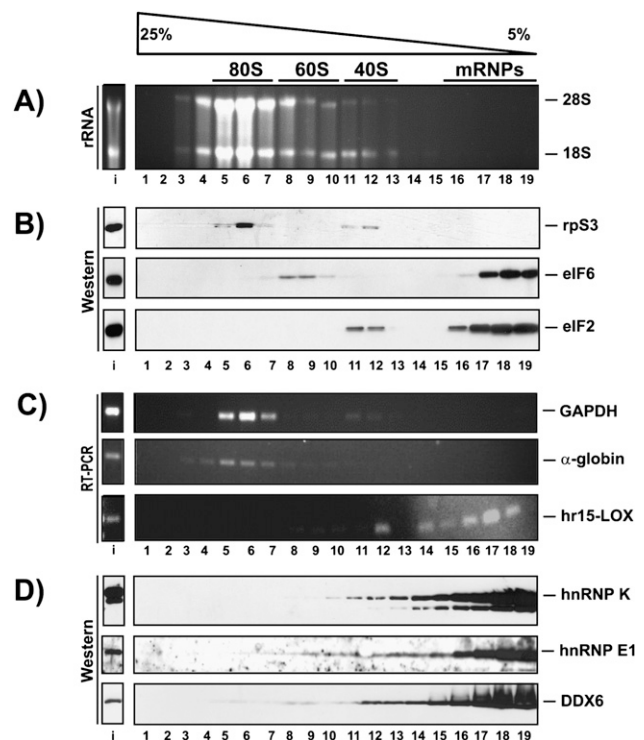


FIGURE 2. DDX6 cosediments with hr15-LOX mRNA, hnRNP K, and hnRNP E1 to 40S ribosomal subunit-containing complexes and mRNP fractions. (A) Sucrose gradient profile of cytoplasmic extract of noninduced K562 cells in the presence of cycloheximide. RNA extracted from the 19 gradient fractions was visualized by ethidium bromide staining of an agarose gel. 18S and 28S rRNA are indicated. (B) The distribution of rpS3, eIF6, and eIF2 in the sucrose gradient fractions was determined by Western blotting. (C) Detection of endogenous mRNAs by RT-PCR, as indicated. (D) The distribution of hnRNP K, hnRNP E1, and DDX6 in the sucrose gradient fractions was determined by Western blotting.

1D), the lanes were cut into 23 slices and subjected to LC/MS/MS. MASCOT-analysis identified 47 proteins specifically eluted from the DICE-matrix (Fig. 1D). Among those were eight hnRNPs, e.g., hnRNP K and hnRNP E1, as expected. Furthermore we identified several DEAD box proteins and RNA-binding/processing proteins.

We focused on the DEAD box RNA helicase DDX6 that was highly and specifically enriched with the DICE as bait RNA and subsequent hnRNP K immunoprecipitation. DEAD-box RNA helicases are involved in RNA-dependent cellular processes, including splicing, ribosome biogenesis, RNA transport, RNA degradation, and mRNA translation, and they influence rearrangements of large RNA structures or protein–RNA interactions (Linder et al. 1989; Jankowsky and Bowers 2006; Linder 2006; Linder and Lasko 2006). Human DDX6 (also known as Rck/p54) (Lu and Yunis 1992) is highly conserved, and homologous proteins have been characterized in *Xenopus* (Xp54) (Ladomery et al. 1997), *Drosophila* (Me31B) (Nakamura et al. 2001), *Caenorhabditis* (CGH-1) (Navarro et al. 2001), and *Saccharomyces* (Dhh1) (Coller et al. 2001; for reviews, see Weston and Sommerville 2006; Rajyaguru and Parker 2009).

DDX6 cosediments with hr15-LOX mRNA, hnRNP K, and hnRNP E1 to 40S ribosomal subunit-containing complexes and to mRNPs

To assess the role of DDX6 as a new component of the complex that associates with the DICE and inhibits hr15-LOX mRNA translation, we first tested whether DDX6 cosediments with hr15-LOX mRNA in translational silenced complexes. For this purpose, we analyzed the cosedimentation of endogenous mRNAs with 80S ribosomes, 60S or 40S ribosomal subunits, and mRNPs. Cytoplasmic extracts prepared from noninduced K562 cells were fractionated on 5%–25% sucrose gradients in the presence of cycloheximide (Fig. 2). The distribution of 18S and 28S rRNA was used to analyze the position of ribosomal complexes and mRNPs (Fig. 2A). 80S ribosomes accumulated in fractions 4–7, while fractions 11–13 contained mainly 18S rRNA of the 40S ribosomal subunits

(Fig. 2A). Ribosomal protein rpS3, a component of the ribosomal 40S subunit, accumulates in 80S and 40S complex-containing fractions (Fig. 2B). We analyzed the distribution of two translation initiation factors that are associated specifically with the individual ribosomal subunits to indicate their position

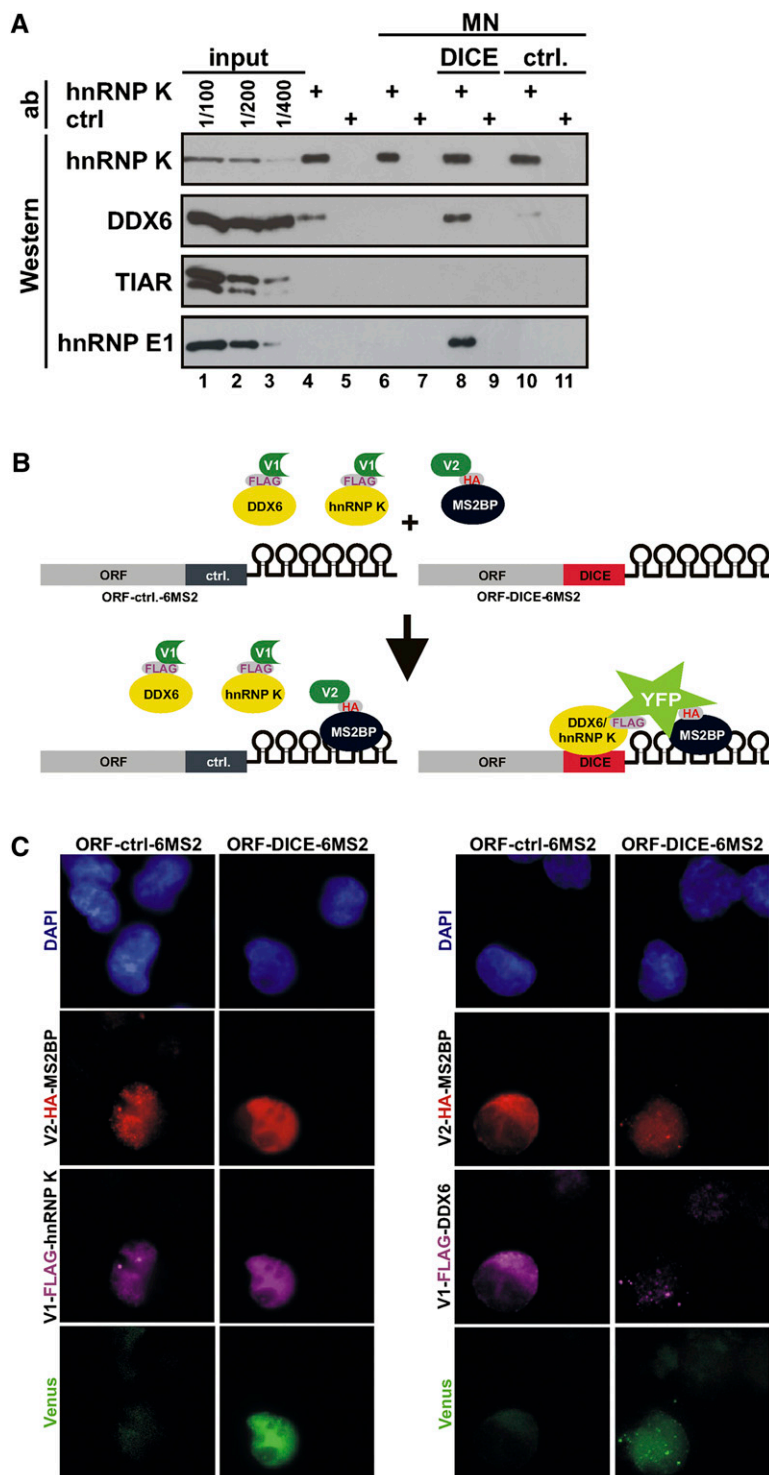


FIGURE 3. (Continued on next page)

in the gradient (Fig. 2B). EIF6 that is bound to the 60S subunit prior to 80S ribosome formation (Ceci et al. 2003) could be detected in the fractions that directly follow those in which 80S ribosomes are enriched and in mRNP-containing fractions. EIF2 that is associated with the 40S ribosomal subunit prior to 80S complex formation (Benne and Hershey 1978; Trachsel and Staehelin 1978) was detected in lighter fractions in which 18S rRNA accumulated and in mRNPs (Fig. 2B).

Furthermore, we monitored the distribution of the endogenous mRNAs. Previously, we could show that GAPDH mRNA and protein is present in noninduced K562 cells (Naarmann et al. 2008). Consistent with this, GAPDH mRNA was detected in 80S-containing fractions (Fig. 2C). In addition, the erythroid-specific α -globin mRNA, which is stabilized by the α -complex formed at the CU-rich sequence in its 3'UTR and is continuously translated during erythroid cell maturation (Weiss and Liebhaber 1994), localized to 80S ribosome fractions (Fig. 2C). In contrast to GAPDH and α -globin mRNAs, hr15-LOX mRNA could not be detected in 80S ribosome-containing fractions (Fig. 2C), consistent with

the presence of hr15-LOX mRNA, but lack of hr15-LOX protein in noninduced K562 cells (Naarmann et al. 2008). Hr15-LOX mRNA was distributed to fractions 12–19 (Fig. 2C), indicating that the endogenous mRNA is associated with 40S ribosomal subunits and mRNPs in its repressed state, as it was found for the DICE-bearing reporter mRNA (cf. Fig. 2C and Fig. 1B). Interestingly, hnRNP K, hnRNP E1, and DDX6 also accumulated in those fractions (Fig. 2D). These results clearly show that hr15-LOX mRNA is excluded from 80S ribosome-containing fractions and cosediments with hnRNP K, hnRNP E1, and DDX6.

DDX6 is a DICE-dependent interaction partner of hnRNP K and hnRNP E1 in vitro and displays DICE-binding activity in K562 cells

To investigate whether the endogenous proteins interact and, if so, whether this interplay is RNA dependent, we performed immunoprecipitation assays (Fig. 3A). hnRNP K was precipitated with the hnRNP K antibody from translation-competent extract of noninduced K562 cells, but not with the control antibody (Fig. 3A, lanes 4–11). DDX6 coprecipitated specifically, whereas the RNA-binding protein TIA1-related protein (TIAR), a component of stress granules in which translational repressed mRNAs are stored (Kedersha et al. 2005), did not (Fig. 3A, lanes 4, 5). Probably due to the low affinity of the antibody, no interaction of hnRNP E1 was detectable (Fig. 3A, lane 4). When the K562 cell extract was treated with micrococcal nuclease, no interaction between hnRNP K and DDX6 could be detected (Fig. 3A, lane 6). The interaction between DDX6 and hnRNP K could be restored and was even enhanced by the addition of in vitro transcribed DICE, but not control RNA, to the extract after inhibition of micrococcal nuclease activity (Fig. 3A, lanes 8–11). A specific interaction of hnRNP K with hnRNP E1 was also detectable in the presence of the DICE (Fig. 3A, lanes 8–11). This indicates that the observed interaction between of hnRNP K, hnRNP E1, and DDX6 is specifically mediated by the DICE.

To analyze DICE binding activity of DDX6 in vivo, we made use of the trimolecular fluorescence complementation (TriFC) assay (Rackham and Brown 2004; Stöhr et al. 2006). In this system, the yellow fluorescent protein, which

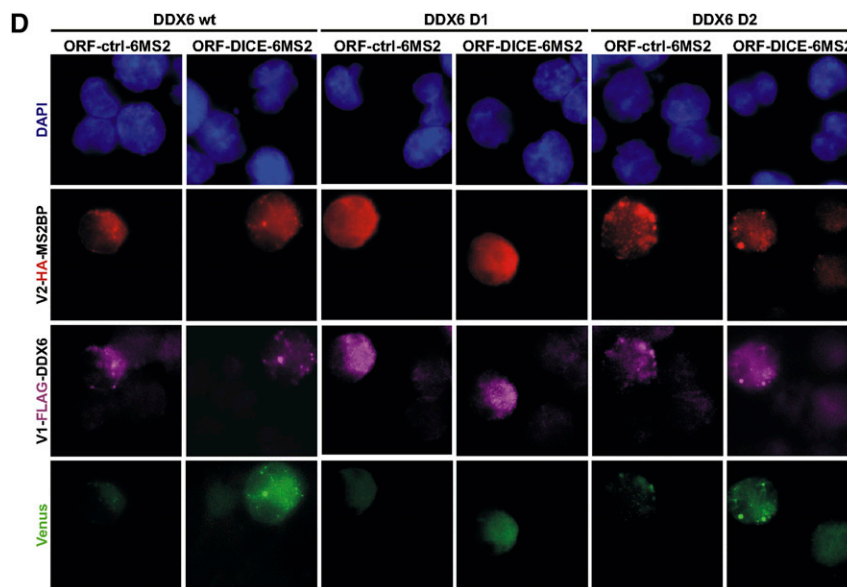


FIGURE 3. DDX6 is a DICE-dependent interaction partner of hnRNP K and hnRNP E1 in vitro and displays DICE-binding activity in K562 cells. (A) Immunoprecipitation with an anti-hnRNP K (lanes 4,6,8,10) or a nonrelated antibody (lanes 5,7,9,11) from cytoplasmic extract of noninduced K562 cells. (Lanes 6–11) Immunoprecipitate was treated with micrococcal nuclease (MN), which was afterward inactivated by EGTA. (Lanes 8,9) Addition of in vitro transcribed DICE RNA. (Lanes 10,11) Addition of in vitro transcribed β -globin RNA as control. After washing, bound proteins were eluted with SDS sample buffer and analyzed by Western blotting, as indicated. (B) Schematic representation of the trimolecular fluorescence complementation (TriFC) assay. (C) TriFC in noninduced K562 cells. (Left) K562 cells were transfected with plasmids encoding Venus1-Flag-hnRNP K, Venus2-HA-MS2BP, and ORF-ctrl-6MS2 or ORF-DICE-6MS2. Cells were analyzed 48 h post-transfection by immunofluorescence staining with specific antibodies against HA and Flag, respectively; nuclei were stained with DAPI. Fluorescence of the reconstituted Venus protein is shown at the bottom. (Right) K562 cells were transfected with plasmids encoding Venus1-Flag-DDX6, Venus2-HA-MS2BP, and ORF-ctrl-6MS2 or ORF-DICE-6MS2. Analysis as described for the left panel. (D) K562 cells were transfected with plasmids encoding Venus1-Flag-DDX6_{wt} or the DDX6 domains D1 or D2, Venus2-HA-MS2BP, and ORF-ctrl-6MS2 or ORF-DICE-6MS2. Analysis as described for panel C.

To analyze DICE binding activity of DDX6 in vivo, we made use of the trimolecular fluorescence complementation (TriFC) assay (Rackham and Brown 2004; Stöhr et al. 2006). In this system, the yellow fluorescent protein, which

shows enhanced fluorescence due to the amino acid exchange F46L (Venus) (Nagai et al. 2002), is separated in two parts (V1 and V2). One part (V2) is tethered by the bacteriophage MS2 coat protein MS2BP (V2-MS2BP) to an mRNA that bears the interaction sequence of interest and six MS2 repeats (Lim and Peabody 1994). The complementary part (V1) is fused to the target RNA-binding protein (V1-X). If the latter is able to associate with the RNA motif of interest, the two parts of Venus will be placed in close proximity to form a fluorescent complex (Fig. 3B). Venus fluorescence was reconstituted from cotransfected V1-hnRNP K, which we used as control, and V2-MS2BP only upon expression of the reporter mRNA comprising the DICE in proximity to the MS2 repeats (ORF-DICE-6MS2), but not when a control sequence was inserted into the 3'UTR (ORF-ctrl-6MS2) (Fig. 3C, left panel). Furthermore, a Venus signal could be detected with V1-DDX6 and the DICE present in the 3'UTR of the reporter mRNA (Fig. 3C, right panel), indicating that DDX6 interacts with the DICE in vivo. Interestingly, DDX6 was localized to granular structures.

DDX6 is composed of two linked RecA-like domains, the N-terminal domain (D1) that contains the Q motif followed by motifs I–III, including the signature sequence DEAD (motif II), and the C-terminal domain (D2) that bears motifs IV–VI (Cordin et al. 2006; Banroques et al. 2008). It has been shown that D2 is sufficient for translational repression employing a tethering assay in *Xenopus* oocytes (Minshall et al. 2009). To investigate the DICE binding activity of domains D1 and D2, we studied DDX6-deletion variants lacking either D1 or D2 in the TriFC assay. A diffuse Venus signal was detectable when domains D1 and D2 were expressed, but characteristic spots appeared only with DDX6_{wt} and D2, indicating that D2 confers DICE-binding and mediates localization to granular structures (Fig. 3D).

DDX6 is required for hr15-LOX mRNA translational repression in vivo

Since DDX6 did interact with the DICE in vitro and in vivo, we next examined

the function of DDX6 in silencing of hr15-LOX mRNA translation (Fig. 4). Previously, we could show that synthesis of hr15-LOX is restricted to late stages of induced K562 cell maturation (days 6 and 8), although the hr15-LOX mRNA is already present in noninduced K562 cells (Naarmann et al. 2008). In noninduced K562 cells, hr15-LOX expression was

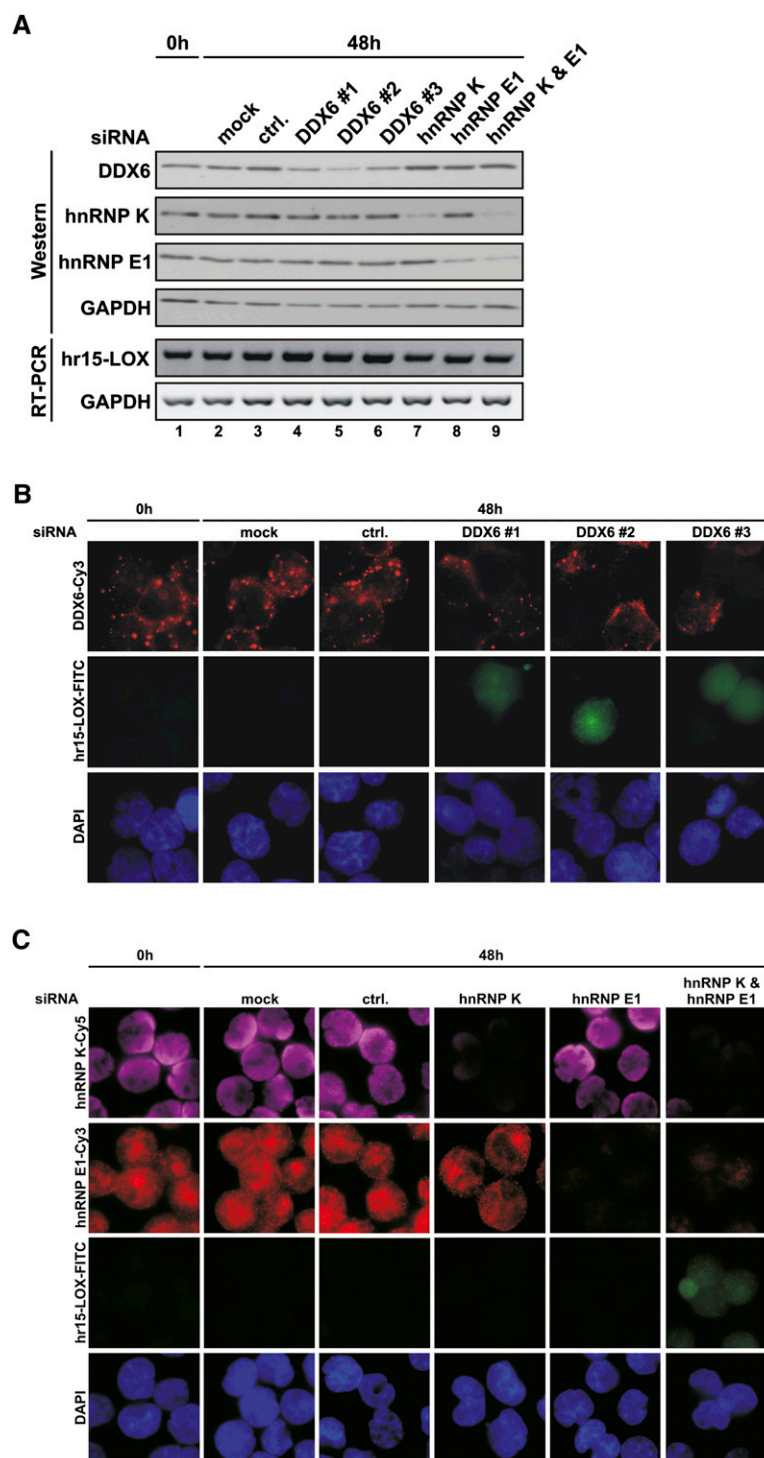


FIGURE 4. (Continued on next page)

only activated when hnRNP K and hnRNP E1 were knocked down simultaneously by RNAi (Naarmann et al. 2008). To address the impact of the DDX6 suppression, we employed three different siRNAs independently (Fig. 4A,B). Transfection of none of the siRNAs resulted in a reduction of DDX6 in all cells. The individual siRNAs suppressed the DDX6 expression in 54% of noninduced K562 cells. In 90% of the DDX6 knockdown cells, hr15-LOX synthesis was de-repressed (Fig. 4B). As shown before, the double knockdown of hnRNP K and hnRNP E1 resulted in hr15-LOX mRNA translation (Fig. 4C; Naarmann et al. 2008). Importantly, the reduction

of DDX6 had no effect on hr15-LOX mRNA levels (Fig. 4A, lanes 1-9). Previously, we have shown that the c-Src mRNA is already present in noninduced K562 cells, whereas c-Src protein could only be detected at days 6 and 8 of induction. hnRNP K binds to the c-Src mRNA 3'UTR and inhibits its translation (Naarmann et al. 2008). In agreement with that, RNAi against hnRNP K, but not hnRNP E1, resulted in de-repression of c-Src synthesis (Fig. 4D). To determine whether DDX6 functions specifically in hr15-LOX mRNA translation control, we tested its impact on c-Src synthesis. When the DDX6 siRNA (#3) (Fig. 4B) was transfected, c-Src mRNA translation remained silenced (Fig. 4E), indicating that DDX6 acts specifically in the hr15-LOX mRNA translation silencing complex.

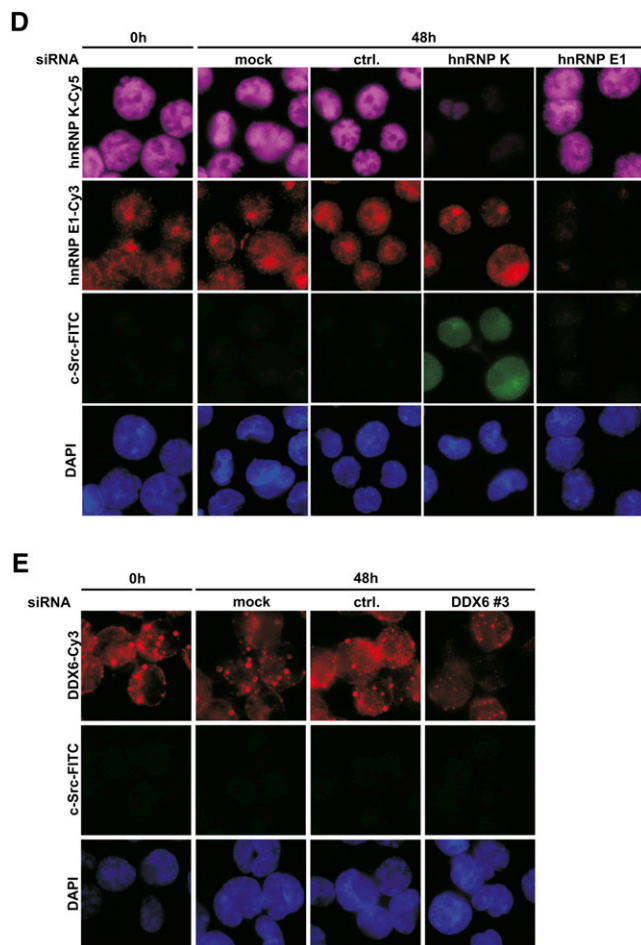


FIGURE 4. DDX6 is required for efficient translation repression of the hr15-LOX mRNA in vivo. K562 cells were transfected with a control siRNA, three different siRNAs against DDX6, or siRNAs against hnRNP K and hnRNP E1 singly or in combination, as indicated. (A, top) Western blot analysis at 0 h and 48 h with antibodies against DDX6, hnRNP K, hnRNP E1, and GAPDH as loading control. (Bottom) Total RNA was isolated from K562 cells. The level of hr15-LOX mRNA and GAPDH mRNA as a control was analyzed by RT-PCR at 0 h and 48 h. (B) Detection of endogenous DDX6 and hr15-LOX by specific antibodies in cells transfected with DDX6-specific siRNAs and control cells. Nuclei were stained with DAPI. (C) Detection of endogenous hnRNP K, hnRNP E1, and hr15-LOX by specific antibodies in cells transfected with hnRNP K and hnRNP E1 siRNAs and control cells. DAPI was used to stain nuclei. (D) Detection of endogenous hnRNP K, hnRNP E1, and c-Src by specific antibodies in cells transfected with hnRNP K and hnRNP E1 siRNAs and control cells. DAPI was used to stain nuclei. (E) Detection of endogenous DDX6 and c-Src by specific antibodies in K562 cells transfected with DDX6 siRNA #3 and control cells. Nuclei were stained with DAPI.

Hr15-LOX mRNA colocalizes with DDX6 to P-body-like RNP granules

Because we detected DDX6 in cytoplasmic granules by immunofluorescence microscopy (Figs. 3, 4), we hypothesized that the function of DDX6 in early stages of erythroid cell maturation could be the sorting of hr15-LOX mRNA into RNP granules, in which translational silenced mRNAs are stored. Therefore we transfected K562 cells and explored the recruitment of ORF-DICE-6MS2 and ORF-ctrl-6MS2 reporter mRNAs to DDX6 granules by confocal laser-scanning microscopy (Fig. 5). In K562 cells, only the DICE bearing reporter mRNA was targeted to the DDX6-containing granules, as observed upon cotransfection of MS2BP fused to the nuclear localization signal (NLS) and GFP (GFP-NLS-MS2BP) and staining of endogenous DDX6 with a specific antibody (Fig. 5, right panel). The intensity peaks for the GFP and Cy3 signals reveal colocalization of GFP-NLS-MS2BP and DDX6. The NLS served to reduce the background of the GFP signal in the cytoplasm, which can only be detected when specific mRNP complexes accumulate in cytoplasmic granules. These cotransfection studies served as a first hint that a DICE-bearing reporter mRNA localizes to DDX6-containing granules. In order to investigate the nature of the DDX6-containing granules and their function in translation control, we analyzed the endogenous hr15-LOX mRNA and DDX6.

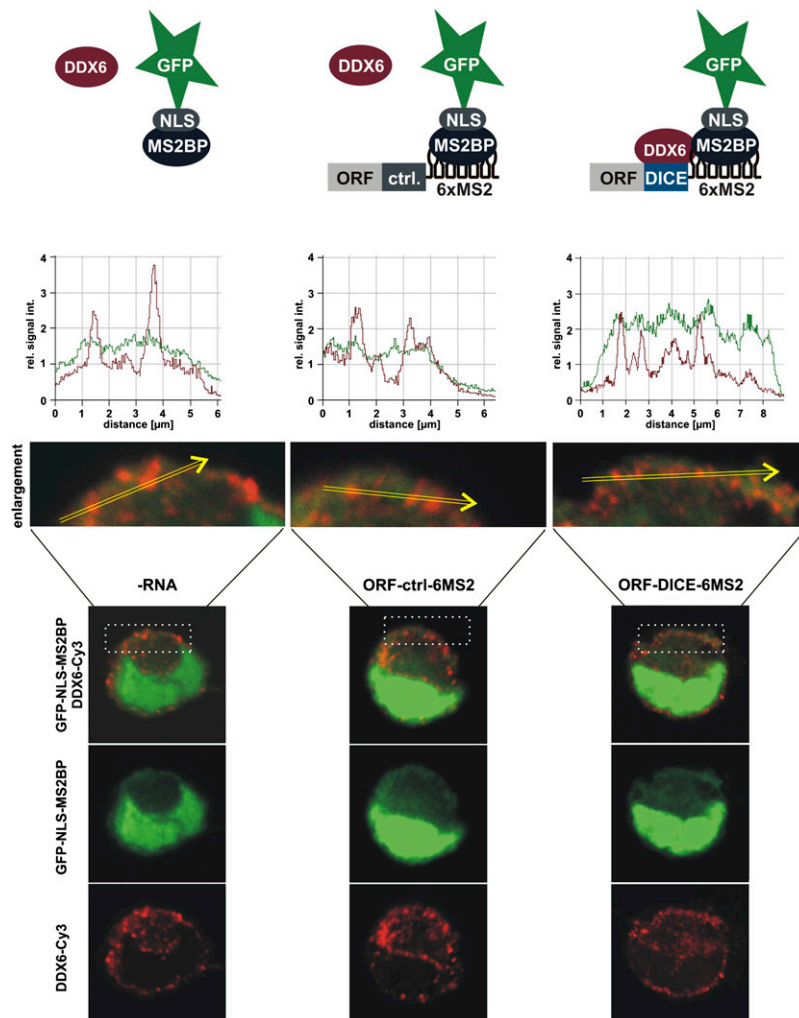


FIGURE 5. DICE-bearing mRNA localizes to DDX6-containing granules. (*Top*) Schematic representation of the assay. (*Bottom*) GFP-NLS-MS2BP was cotransfected with no mRNA, ORF-ctrl-6MS2 (*middle* column), or ORF-DICE-6MS2 (*right* column) into K562 cells. Localization of GFP-NLS-MS2BP traced mRNA and staining of endogenous DDX6 with a specific antibody were monitored by confocal microscopy. Dotted rectangles indicate the magnified cytoplasmic area above. DDX6-Cy3 and GFP fluorescence was monitored along arrows drawn through DDX6-positive granules. The diagrams represent Cy3 (red lines) and GFP (green lines) fluorescence intensities along the length of the arrows.

It has been shown that during development, maternal mRNAs accumulate with specific proteins in cytoplasmic RNA-protein aggregates, referred to as RNP granules or germ granules (Schisa et al. 2001; Anderson and Kedersha 2006; Rajyaguru and Parker 2009). These granules are dynamic and related to stress granules and P-bodies, which are conserved in somatic cells. Stress granules form when translation initiation is compromised, and contain mRNAs that are not translated, some translation initiation factors, and mRNA-binding proteins. P-bodies are distinct cytoplasmic granules that are sites of reversible mRNA translation repression and mRNA decay (Anderson and Kedersha 2006, 2009; Eulalio et al. 2007; Parker and Sheth 2007; Balagopal and Parker 2009; Buchan and Parker 2009). Certain compo-

nents, like DDX6 or its orthologs Rck/p54/CGH-1, localize to stress granules (Wilczynska et al. 2005), P-bodies (Cougot et al. 2004), or germ granules (Navarro and Blackwell 2005).

To investigate whether DDX6 functions in sorting translationally repressed hr15-LOX mRNA to stress granule-like structures, we employed arsenite treatment of K562 cells to induce stress granule formation, for which G3BP1 served as a marker (Kedersha et al. 2005) (Fig. 6A). Interestingly, DDX6 did not localize to stress granules as the lack of colocalization with G3BP1 (Fig. 6A) and another stress granule marker TIAR (data not shown) demonstrated. But transient association of DDX6 with stress granules cannot be excluded because colocalization of transfected RFP-Rck/p54 with anti-eIF3-stained stress granules has been observed in HeLa cells (Wilczynska et al. 2005).

Next we asked whether hr15-LOX mRNA gets localized to arsenite-induced stress granules. By FISH we could detect colocalization of endogenous polyadenylated mRNAs (oligo dT) and GAPDH mRNA with G3BP1, in agreement with studies in arsenite-treated U2OS cells (Stöhr et al. 2006). In contrast, neither hr15-LOX mRNA nor c-Src mRNA, which is translationally repressed in non-induced K562 cells as well (Fig. 4D), did colocalize with G3BP1 (Fig. 6B). Previously, we could show that K562 cells can be induced for erythroid maturation and exhibit changes in morphology (nuclear exclusion, loss of mitochondria) and expression of hr15-LOX regulated by hnRNP K and hnRNP E1, which are

characteristic for terminal erythroid maturation (Naarmann et al. 2008). To discover whether stress granules appear during erythroid maturation, we induced K562 cells for 8 d. During the course of the maturation, no stress granules could be detected, but DDX6-positive granules were well visible (Fig. 6C). DDX6 granules and the protein level decreased during maturation, they were barely detectable at days 6 and 8 of induction (Fig. 6C,D), when hr15-LOX protein is synthesized (Naarmann et al. 2008).

Because stress granule formation could not be detected, we wanted to know if DDX6-containing foci represent P-body-like RNP granules (Fig. 7A). Staining for the P-body and germ granule marker Dcp1A (Ingelfinger et al. 2002; Lall et al. 2005) did show colocalization with DDX6. Interestingly,

neither Dcp1A staining nor the number of foci decreased as dramatically as the DDX6 granules disappeared during maturation (Fig. 7A) and when DDX6 expression is suppressed by RNAi (Fig. 7B). The remaining Dcp1A foci may represent P-bodies. In mouse oocyte injection experiments, two types, small and large, of Dcp1A-bodies were found; Rck/p54 localized only to the former (Swetloff et al. 2009).

Interestingly, by employing FISH analyzed by confocal microscopy, we could show that hr15-LOX mRNA was concentrated in DDX6-containing granules in noninduced K562 cells (Fig. 7C, arrowheads). In contrast c-Src mRNA that is translationally repressed in a DDX6-independent manner (Fig. 4E) did not colocalize (Fig. 7C). The oligo (dT) probe detected polyadenylated mRNA, which appeared to be mainly excluded from DDX6-positive granules in the nucleus and the cytoplasm (Fig. 7C). In addition, translational active GAPDH mRNA did not colocalize with DDX6 (Fig. 7C). Together this indicates that only hr15-LOX mRNA, which is specifically regulated by DDX6 (Fig. 4B,E), colocalizes with DDX6 in P-body-like RNP granules in noninduced K562 cells. On day 8 of maturation, when hr15-LOX mRNA is translated (Naarmann et al. 2008), hr15-LOX mRNA staining was dispersed in the cytoplasm, suggesting that it was released from RNP granules (Fig. 7C).

To further characterize translational silenced hr15-LOX mRNA in DDX6-containing granules, we performed co-staining for hr15-LOX mRNA translation inhibitors and ribosomal proteins, rpS19 and rpL19. The actively translated GAPDH mRNA (Fig. 2C; Naarmann et al. 2008) served as a control. To provide intensity profiles with the images, we changed the color of the molecules analyzed, which now corresponds to that in the diagrams (Fig. 8): Endogenous proteins that differ in panel A to C, hnRNP E1, rpS19, and rpL19, respectively, are represented in green; endogenous hr15-LOX mRNA or GAPDH mRNA, in blue; and endogenous DDX6, in red. The amplitude of the intensity profiles for hnRNP E1, DDX6, and hr15-LOX mRNA revealed colocalization of

the three molecules in noninduced K562 cells, which could not be detected for the respective proteins and GAPDH mRNA (Fig. 8A).

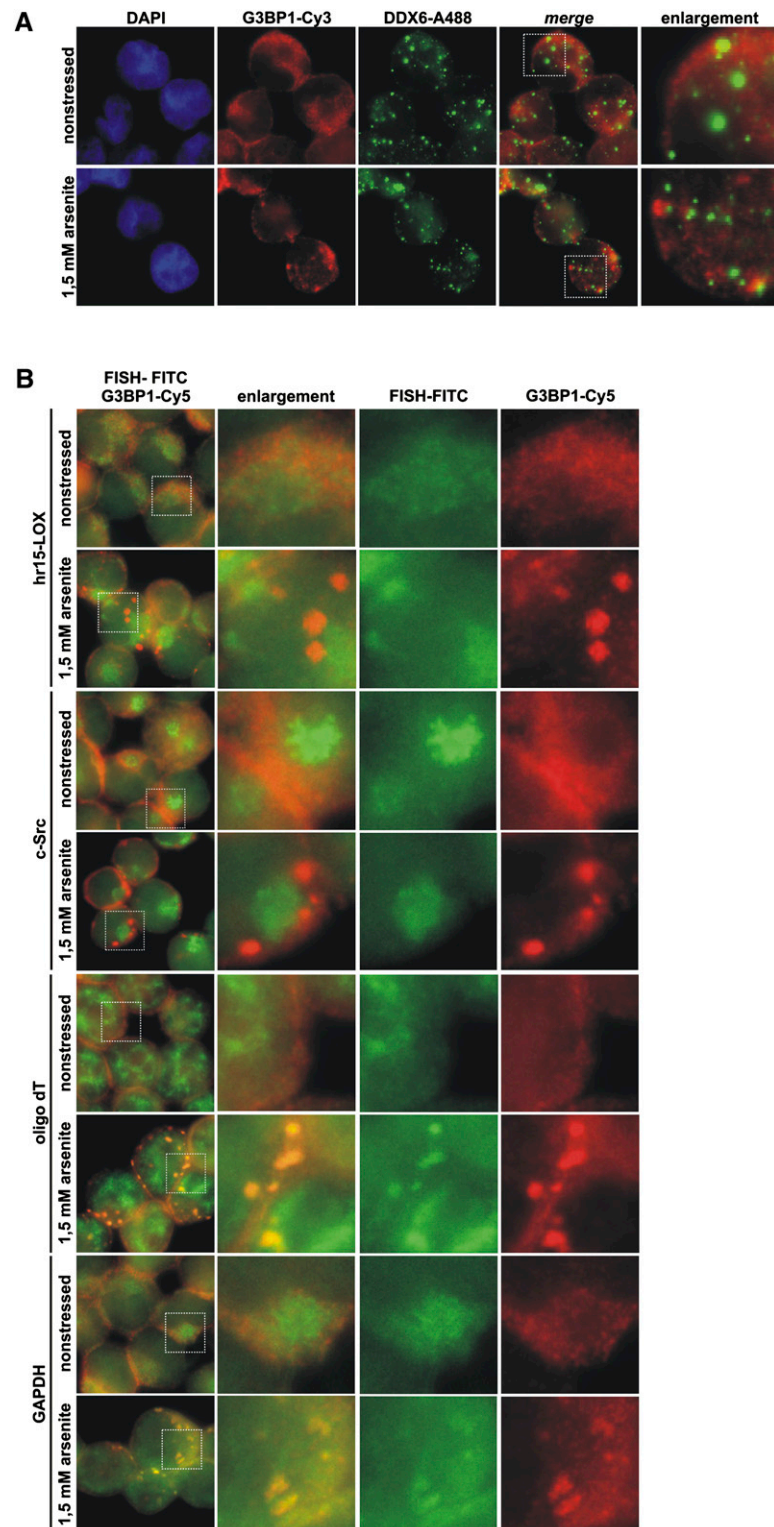


FIGURE 6. (Continued on next page)

Because hnRNP K is mainly localized to the space-filling nucleus in noninduced K562 cells (Naarmann et al. 2008), its colocalization with DDX6 and hr15-LOX mRNA was hard to detect (data not shown).

Previously, we have shown that joining of the 60S ribosomal subunit to the 40S subunit at the AUG of r15-LOX mRNA is inhibited by the hnRNP K/E1-DICE complex (Ostareck et al. 2001). Consistent with this, 43S preinitiation complexes assembled on a DICE-bearing mRNA in its repressed state (Fig. 1B) and endogenous hr15-LOX mRNA sediments to 40S subunit-containing fractions in extracts of noninduced K562 cells (Fig. 2C). Therefore we asked whether 40S subunits colocalize with hr15-LOX mRNA in DDX6-containing granules. Costaining for the 40S subunit protein rpS19 with DDX6 and hr15-LOX mRNA (FISH) could be detected in noninduced cells (Fig. 8B). For GAPDH mRNA, costaining with rpS19, but not DDX6, was detectable, as illustrated by the intensity profiles (Fig. 8B). Interestingly, an

antibody against rpL19, a 60S subunit protein, did not show colocalization with DDX6 and hr15-LOX mRNA in non-induced cells, whereas the GAPDH FISH probe did show colocalization with rpL19, but not with DDX6, as illustrated by the intensity profiles (Fig. 8C). On day 8 of maturation, when hr15-LOX mRNA translation is active (Naarmann et al. 2008), hr15-LOX mRNA staining was dispersed in the cytoplasm, suggesting that it was released from RNP granules (Supplemental Fig. S1).

Taken together, our data suggest that DDX6 mediates the storage of translational silenced hr15-LOX mRNA in P-body-like RNP granules, from which 60S ribosomal subunits are excluded.

DISCUSSION

Employing a native mRNA–protein complex purification approach, we identified a novel component of the hr15-LOX mRNA translation silencing complex in premature erythroid cells. The mammalian RNA helicase DDX6 (Rck/p54) was specifically purified together with hnRNP K and hnRNP E1 on a DICE–RNA matrix (Fig. 1). Consistently, in sucrose gradient analysis of cytoplasmic K562 cell extract, DDX6, hnRNP K, and hnRNP E1 cosediment with the translational silenced hr15-LOX mRNA to fractions that contain 40S ribosomal subunits and mRNPs, but no 80S ribosomes (Fig. 2). Earlier we have shown that hr15-LOX mRNA is present in K562 cells prior to induction of erythroid maturation, whereas neither hr15-LOX protein, nor its enzymatic activity could be detected (Naarmann et al. 2008). In conjunction with this, the DICE-specific purification of DDX6 and its sedimentation profile indicate that this RNA helicase might be functional relevant for hr15-LOX mRNA translational silencing during erythroid cell maturation.

Employing immunoprecipitation, we show specific interaction of hnRNP K and DDX6 from K562 cell extract (Fig. 3A). Microcococcus nuclease treatment of K562 cell extract demonstrated that the interaction of DDX6 with hnRNP K is RNA dependent. Probably due to the low affinity of the antibody, hnRNP E1 was not detectable. Importantly, re-addition of in vitro transcribed DICE, but not of control RNA, to the nuclease-treated extract restored and enhanced the binding of both proteins DDX6 and hnRNP E1 to hnRNP K (Fig. 3A).

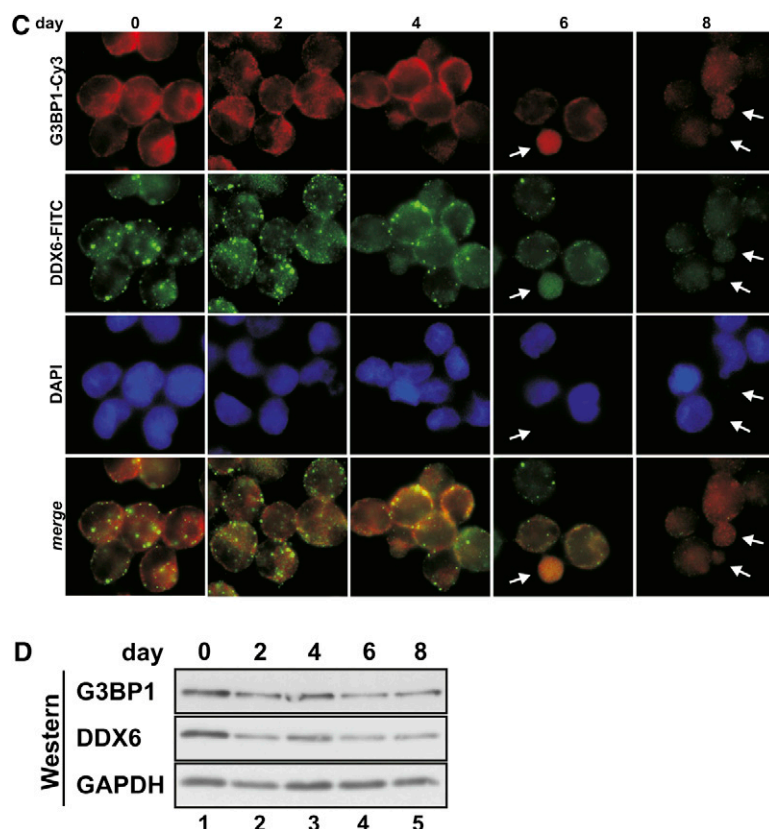


FIGURE 6. DDX6 and hr15-LOX mRNA are not localized to stress granules. (A) Induction of stress granules by arsenite treatment of K562 cells. Detection of G3BP1 and DDX6 with specific antibodies. DAPI was used to stain nuclei. Enlarged areas are indicated by dotted squares. (B) Combined immunofluorescence and fluorescence in situ hybridization (IF-FISH) analysis of nonstressed and arsenite-treated K562 cells. Cells were stained with an anti-G3BP1 antibody and hybridized with hr15-LOX, c-Src, oligo (dT), and GAPDH probes, as indicated. Enlarged areas are indicated by dotted squares. (C) Immunofluorescence microscopy of K562 cells from day 0 to day 8 of erythroid maturation. Cells were stained as in A. Cells lacking the nucleus are indicated by arrows. (D) Western blot analysis from day 0 to day 8 with antibodies against G3BP, DDX6, and GAPDH as loading control.

Consistent with this finding, RNase-sensitive protein–protein interactions in translational regulatory complexes have been described for the DDX6 orthologs p47 in *Spisula* (with cytoplasmic polyadenylation element-binding protein [CPEB]) (Minshall et al. 2001), Me31B in *Drosophila* (with Exuperantia [Exu] and Ypsilon Schachtel [Yps]) (Wilhelm et al. 2000; Nakamura et al. 2001), and CGH-1 in *Caenorhabditis* (with cytokinesis/apoptosis/RNA binding [CAR-1]) (Boag et al. 2005). Furthermore, specific interactions of Nos-2 mRNA and other developmentally regulated mRNAs have been shown with CGH-1 (Boag et al. 2008; Noble et al. 2008) and the *Trypanosoma* ortholog DHH1 (Kramer et al. 2010).

In TriFC assays, we could show a specific interaction of DDX6 with the DICE in vivo (Fig. 3B). DDX6 binding to the DICE and its accumulation in granular structures is mediated by the C-terminal Rec A–like domain D2 of DDX6 (Fig. 3C). Domain D2 of the *Xenopus* ortholog Xp54 is sufficient for translational repression in tethering assays and for complete accumulation in P-bodies (Minshall et al. 2009).

Interestingly, when the amount of DDX6 is strongly reduced by the application of three individual siRNAs in noninduced K562 cells (Fig. 4A), hr15-LOX mRNA translation is specifically de-repressed (Fig. 4B,E). We show for the first time that human DDX6 is important for the translational regulation of a specific mRNA involved in cellular differentiation. Previously, it has been shown that loss of Me31B in *Drosophila* germline cells causes de-repression of oskar and BicD mRNA translation during their transport to the oocyte (Nakamura et al. 2001). Furthermore, during oogenesis in *Caenorhabditis*, Nos-2 mRNA is translated prematurely in the absence of CGH-1 (Gallo et al. 2008).

When we further investigated the function of DDX6 for hr15-LOX mRNA translation inhibition by FISH, we found that DDX6 colocalized with hr15-LOX mRNA in P-body-like RNP granules (Fig. 7), in which hnRNP E1 and rpS19, a 40S ribosomal subunit protein, but not rpL19, a protein of the 60S subunit, could be detected (Fig. 8). This indicates that DDX6-containing granules, in which translationally repressed hr15-LOX mRNA is concentrated, are related to P-body-like granules. Recently, P-body-related RNP granules, in which specific maternal mRNAs are stored during development, were described in *Caenorhabditis* (Boag et al. 2008; Noble et al. 2008).

During erythroid maturation of K562 cells, we could show that DDX6-containing granules disappear, accompanied by a reduced staining for the P-body marker Dcp1A (Fig. 7A). This can be recapitulated through RNAi against DDX6 in noninduced K562 cells (Fig. 7B). Therefore it is feasible that the loss of DDX6 in premature erythroid cells by RNAi (Fig. 4) and during erythroid cell maturation (Fig. 7; Supplemental Fig. S1) leads to the loss of RNP storage granules and concomitantly causes rearrangements of interactions in the hnRNP/E1–DICE complex, which result in the de-repression of hr15-LOX mRNA translation.

In yeast extract, the DDX6 homolog Dhh1 has been shown to repress 43S preinitiation complex accumulation on a capped reporter mRNA when added as recombinant protein (Coller and Parker 2005). Different effects of DDX6 homologous on translational initiation might be modulated by their N and C termini, for which interactions with other factors have been proposed (Weston and Sommerville 2006).

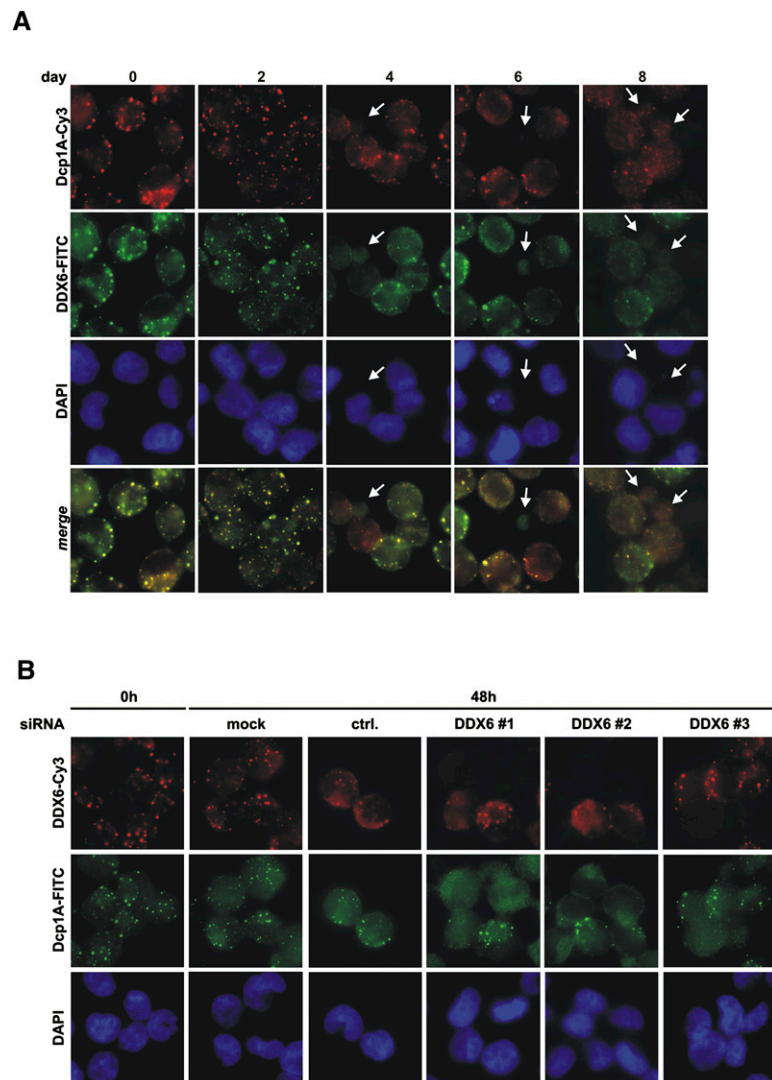


FIGURE 7. (Continued on next page)

The characterization of DDX6 as a regulator of hr15-LOX expression, which interacts with hnRNP K and hnRNP E1 in a DICE-dependent fashion sheds light on how the translational inhibition of hr15-LOX mRNA is maintained in early

stages of erythroid cell maturation. The mechanism that underlies the inhibition of 60S ribosomal subunit joining and thereby prevents translation initiation is still under investigation.

C

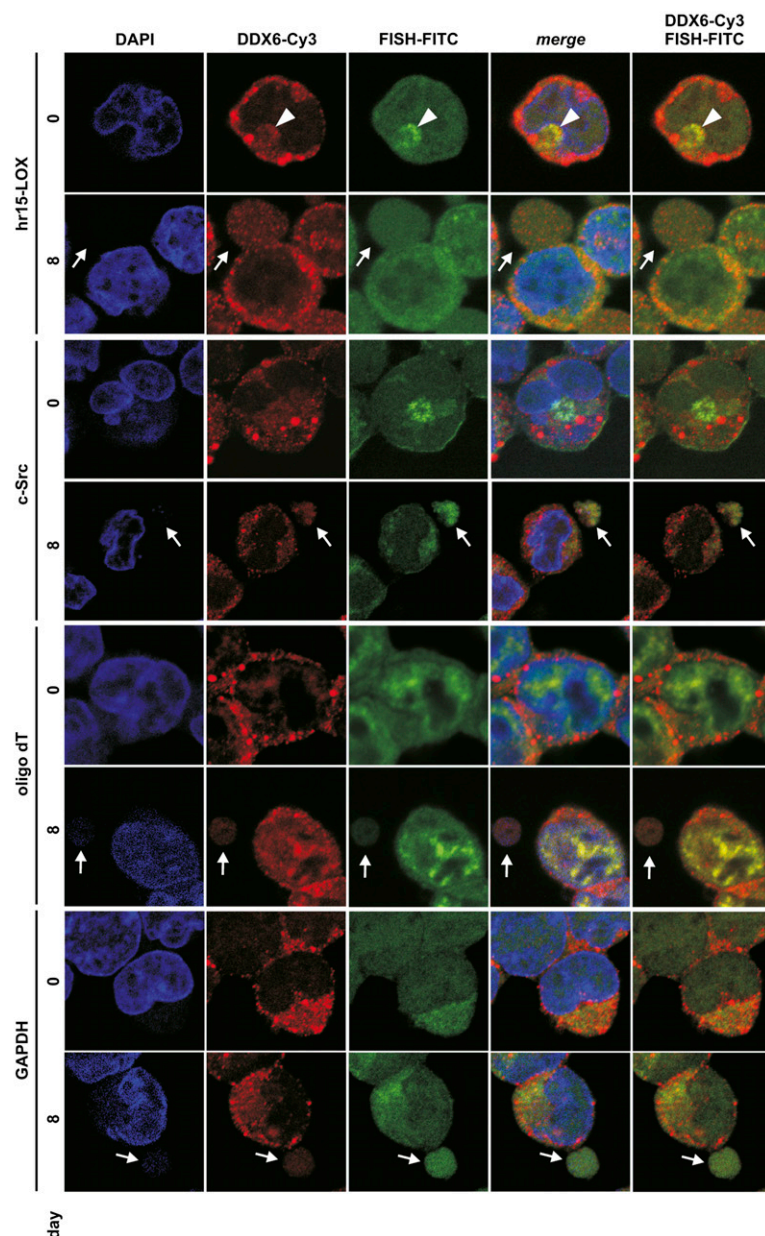


FIGURE 7. Hr15-LOX mRNA colocalizes with DDX6 to P-body-like RNP granules. (A) Immunofluorescence microscopy of K562 cells during erythroid maturation. Dcp1A and DDX6 were detected with specific antibodies. Nuclei were stained with DAPI. Arrows indicate enucleated cells. (B) K562 cells were transfected with a control siRNA or three different siRNAs against DDX6. Staining of cells as described in A. (C) Confocal microscopy analysis of non-induced K562 cells and cells induced for 8 d of erythroid maturation by IF-FISH. Cells were hybridized with hr15-LOX, c-Src, oligo (dT), and GAPDH probes, as indicated. DAPI was used to stain nuclei. Enucleated cells are marked by arrows. Cells were costained with an anti-DDX6 antibody. Arrowheads indicate DDX6-positive and hr15-LOX mRNA-positive RNP granules.

MATERIALS AND METHODS

Plasmids

pGEM-CAT-ctrl and pGEM-CAT-DICE (Ostareck et al. 2001) and pBSII SK-LUC (Iizuka et al. 1994) were described previously. pBSII SK-DICE (Ostareck-Lederer et al. 1994), pBSII KS- β -globin (Weinlich et al. 2009), and sORF-ctrl (Naarmann et al. 2008) were described previously. sORF-DICE originates from sORF-ctrl, to which the Klenow-treated BamHI/PstI DICE fragment of pBSII SK-DICE was added into EcoRV. To generate boxB-sORF-ctrl and boxB-sORF-DICE, six boxB oligonucleotides were inserted into the BamHI and PstI site of sORF-ctrl or sORF-DICE, respectively. DDX6 cDNA was amplified by RT-PCR from K562 cell RNA and inserted into HincII of pBSII KS. pET16b-DDX6 was generated by DDX6 amplification from pBSII KS-DDX6 and cloned into XhoI of pET16b. Plasmids Venus1C, Venus2-HA-MS2BP, Luc- Δ zip-6MS2, and GFP-NLS-MS2BP were kindly provided by S. Hüttelmaier (Department of Medicine, Martin-Luther-University Halle-Wittenberg, Halle, Germany) (Stöhr et al. 2006). To create Venus1-Flag-hnRNP K, a flag-tag was cloned between XhoI/EcoRI of Venus1-hnRNP K (Adolph et al. 2007). To generate Venus1-Flag-DDX6 as well as domains -D1 and -D2, first the Flag-tag was cloned into Venus1C, and DDX6 was amplified from pET16b-DDX6 with primers DDX6-KpnI-fw and DDX6-KpnI-rv. DDX6 domain D1 was amplified using DDX6-KpnI-fw and D1-KpnI-rv; domain D2, with D2-KpnI-fw and DDX6-KpnI-rv. To complete Venus1-Flag-DDX6, -D1 and -D2, the PCR products were inserted into KpnI of Venus1-Flag. To generate ORF-ctrl-6MS2, the XhoI β -globin fragment of pBSII KS- β -globin was blunt end ligated into BamHI of Luc- Δ zip-6MS2 (Stöhr et al. 2006). ORF-DICE-6MS2 originates from Luc- Δ zip-6MS2, to which the BamHI/PstI DICE fragment of pBSII SK-DICE was blunt end ligated into BamHI.

Cell culture and cytoplasmic extract preparation

K562 cells were maintained in RPMI 1640 (10% FBS). Cytoplasmic K562 cell extract

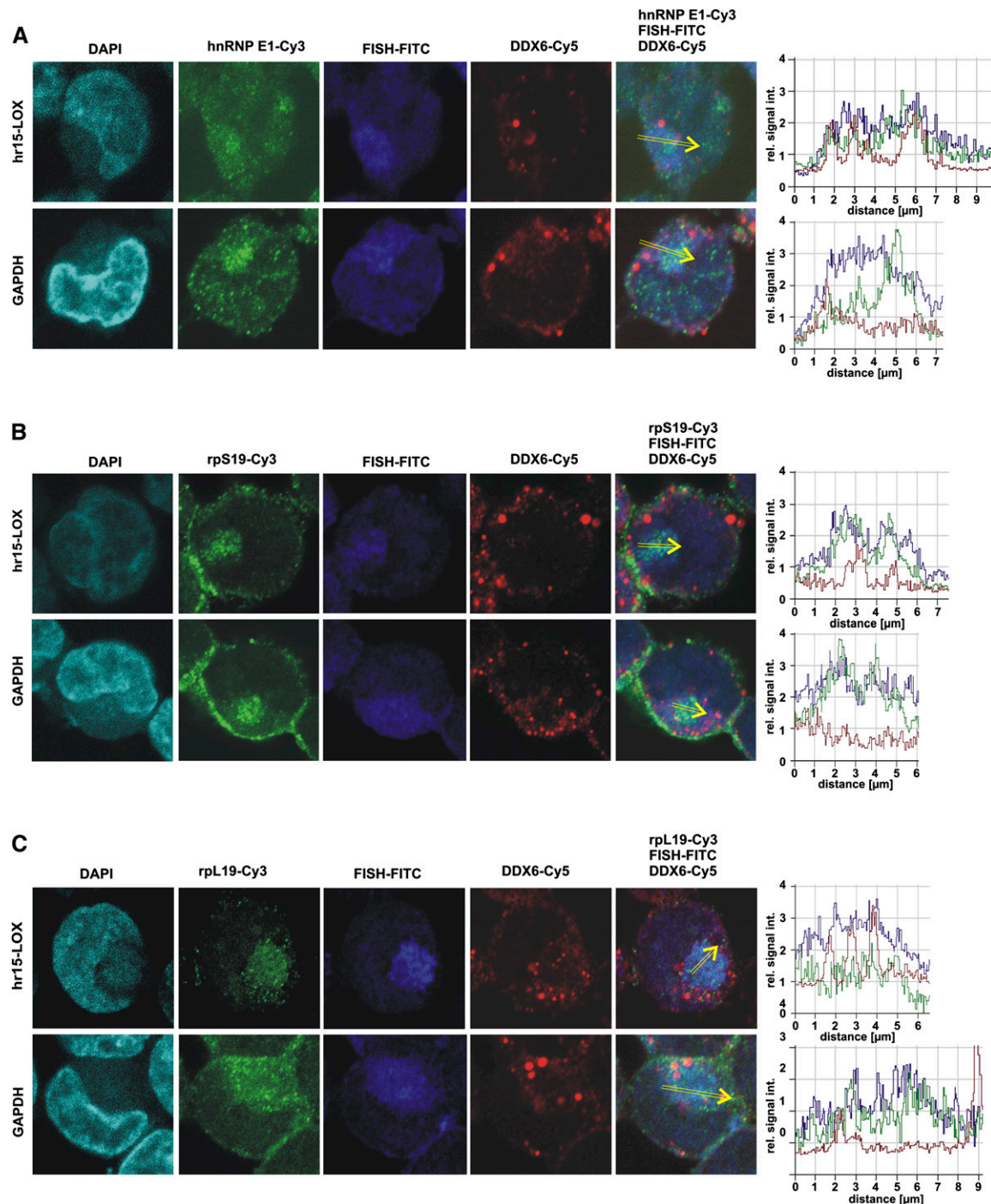


FIGURE 8. HnRNP E1 and rpS19, but not rpL19, colocalize with hr15-LOX mRNA to DDX6-containing RNP granules. (A–C) Confocal microscopy analysis of noninduced K562 cells by IF-FISH and with specific antibodies. Cells were hybridized with hr15-LOX or GAPDH probes (FITC, blue). Endogenous proteins were detected with antibodies against hnRNP E1, rpS19, rpL19 stained with Cy3 (green), and DDX6 stained with Cy5 (red), as indicated. Nuclei were stained with DAPI (light blue). In merged images (right column), Cy3, Cy5, and FITC fluorescence was monitored in cytoplasmic areas along the length of the arrows. The diagrams represent FISH-FITC (blue lines), Cy3 (green lines), and DDX6-Cy5 (red lines) fluorescence intensities along the arrows. (A) Cells were stained with antibodies against hnRNP E1 and DDX6. (B) Staining with antibodies against rpS19 and DDX6. (C) Staining with antibodies against rpL19 and DDX6.

was generated according to the method of Naarmann et al. (2008). Stress granules were induced by incubation with 1.5 mM sodium arsenite for 30 min. Induction of erythroid cell maturation was described previously (Naarmann et al. 2008).

In vitro transcription

[³²P]-Trace-labeled 5' capped mRNAs were generated according to the method of Ostareck et al. (2001). For translation initiation

assays, capped [32 P]-labeled mRNAs were synthesized and purified as detailed by Ostareck et al. (2001). Competitor RNA for immunoprecipitation experiments was generated as described (Ostareck-Lederer et al. 1994).

In vitro translation

In vitro translation reactions were performed as described previously (Naarmann et al. 2008).

Sucrose gradient analysis of translation initiation complexes

Cytoplasmic K562 extract (300 μ g) was incubated with 500 fmol of [32 P]-labeled mRNA in the presence of 1 mM cycloheximide for 15 min. Where indicated, 3.5 mM GMP-PNP was added alongside to cycloheximide. Translation initiation complexes were resolved on linear 5%–25% sucrose gradients (Ostareck et al. 2001; Naarmann et al. 2008).

To investigate the distribution of endogenous RNAs and translation initiation factors, cytoplasmic K562 extract (2 mg) was incubated with 1 mM cycloheximide and resolved on a linear 5%–25% sucrose gradients. Nineteen fractions were collected from the bottom of the gradient.

GRNA chromatography and mass spectrometry

To purify mRNPs from translational active K562 cell extracts, which contain 3'UTR DICE-bearing mRNA in its translational silenced state, we adapted GRNA chromatography (Czaplinski et al. 2005; Duncan et al. 2006); 100 pmol of each mRNA was incubated on ice with 100 μ L of K562 cell extract containing the translation reaction components in a total volume of 250 μ L. Proteins eluted from the RNA matrices were applied to hnRNP K immunoprecipitation. Coprecipitated proteins were separated on a NuPAGE 4%–12% Bis-Tris gel (Invitrogen) and stained with colloidal Coomassie blue. The entire lanes were cut in 23 slices; proteins were in gel-digested with trypsin and extracted according to the method of Shevchenko et al. (1996). The extracted peptides were analyzed in a capillary HPLC coupled electrospray ionization quadrupole time of flight (ESI-Q-TOF, Ultima, Waters) mass spectrometer. Peptide assignments and data analysis were performed by Mascot.

RNA isolation and RT-PCR

Total RNA was isolated using Trizol (Invitrogen). Two micrograms of RNA (Fig. 6) or equal volumes of RNA (Fig. 2) and random primers or oligo dT₍₁₀₎ (Fig. 2) and 150 U MMLV-RT (Promega) were used for reverse transcription. Aliquots of the reverse transcription were used for PCR with GoTaq-Flexi DNA polymerase (Promega) and respective primer pairs described by Naarmann et al. (2008).

Antibodies

Antibodies were purchased from Santa Cruz (hnRNP K, Fyn, G3BP1, hnRNP E1, rpL19), Oncogene (v-Src), Abcam (eIF3B, GAPDH, rpS19), Abnova (eIF2, Dcp1A), BD Transduction Laboratories (TIAR), Cell Signaling (rpS3), Novus Biologicals (DDX6), and Sigma (α -tubulin, Flag, HA) and used according to the manufacturer's protocol. The eIF6 antibody was a kind gift of

Stefano Biffo (Molecular Histology and Cell Growth, San Raffaele Scientific Institute, Milano, Italy) (Ceci et al. 2003) and the r15-LOX antibody raised in guinea pig was kindly provided by Hartmut Kühn (Institute of Biochemistry, University Medicine Berlin-Charité, Berlin, Germany) (Rapoport et al. 1979). For the detection of hnRNP E1, a peptide antibody was used (Naarmann et al. 2008).

Western blot assays

Western blot assays were performed as described previously (Ostareck-Lederer et al. 2002).

Immunoprecipitation

Forty microliters of protein A–Sepharose coupled with the hnRNP K or Fyn antibody was incubated with 1 mg cytoplasmic K562 cell extract for 1.5 h at 4°C in IPP buffer (20 mM HEPES at pH 7.4, 100 mM KCl, 5 mM magnesium acetate, 1 mM DTT, 0.025% Triton X-100) and protease inhibitors. To digest endogenous RNAs, beads were resuspended in IPP buffer containing 1 mM CaCl₂ and 600 U micrococcal nuclease and incubated for 10 min at room temperature. We added 2.5 mM EGTA to inactivate the enzyme. One microgram micrococcal nuclease-treated cytoplasmic K562 cell extract was added back together with 10 μ g DICE or ctrl RNA and incubated for 30 min at 4°C in IPP buffer. Beads were washed twice in IPP buffer and boiled in SDS sample buffer, and the supernatant was analyzed in Western Blot assays.

Immunofluorescence microscopy

K562 cells were spun on poly-L-lysine-coated coverslips. Staining was performed according to the method of Naarmann et al. (2008). Cells were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). For microscopy, an Axiovert 200M or AxioStar (Zeiss) microscope equipped with cameras was used. Images were acquired by AxioVision (Zeiss) software and compiled with Corel Technical Suite 14. Confocal microscopy was performed at a LSM 710 laser-scanning microscope using a Plan Apochromat 63x/1.4 oil objective (Zeiss). Multicolor fluorescence was recorded in the multitrack modus using a single track for each dye. The following laser lines and filter settings were used: DAPI, excitation 405 nm and detection 410–492 nm; FITC, excitation 488 nm and detection 493–542 nm; Cy3, excitation 514 nm and detection 552–650 nm; and Cy5, excitation 633 nm and detection 638–759 nm. The pinhole was adjusted separately for each track resulting in optical slices of 1- μ m thickness.

Fluorescence in situ hybridization

To visualize RNAs by FISH, K562 cells were fixed in 4% PFA in PBS after immunostaining (10 min, room temperature). Cells were washed twice with PBS and 2 \times SSC. Incubation with 200 ng biotinylated dT₃₀ or RNA specific biotinylated probes (MWG) Supplemental Table 1) was performed on at 42°C in hybridization buffer (2 \times SSC, 1% BSA, 1 mg/mL yeast tRNA, 35% formamide, 2.5% dextran sulfate). Cells were washed three times with 2 \times SSC, once with 4 \times SSC, 0.1% triton X-100 and incubated with ExtrAvidin-FITC (Sigma) in 4 \times SSC, 1% BSA (1 h, room temperature). After washing, cells were mounted with ProLong Gold anti-fade reagent with DAPI (Invitrogen).

Transfections

For RNAi in K562 cells (1×10^6 cells in RPMI without FBS and antibiotics) were transfected by electroporation at 0.36 kV, 100 μ F (GenePulser II, BioRad) with 500 pmol of siRNAs against hnRNP K, hnRNP E1, or a nonspecific control siRNA (Naarmann et al. 2008) or DDX6: #1, #2, #3 (all MWG) (Supplemental Table 1). Cells were harvested 48h post-transfection for immunofluorescence microscopy, Western blot assays, and RT-PCR. Plasmids were transfected into K562 cells (5×10^6 in RPMI without FBS and antibiotics) by electroporation at 0.36 kV, 950 μ F (GenePulser II, BioRad). Cells were harvested 48 h post-transfection for fluorescence microscopy.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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