Unr defines a novel class of nucleoplasmic reticulum, involved in mRNA translation

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Abstract

Unr is a cytoplasmic RNA-binding protein with roles in the regulation of mRNA stability and translation. In this study, we have identified a novel function for Unr, which acts as a positive regulator of placental development. Unr expression studies in the developing placenta revealed the presence of Unr-rich foci apparently localized in the nuclei of trophoblast giant cells (TGCs). We determined that these foci are actually cross sections of a network of double-wall nuclear membranes invaginations containing a cytoplasmic core, related to the nucleoplasmic reticulum (NR), and accordingly named Unr-NRs. Unr-NRs constitute a novel type of NR, because they contain high levels of poly (A) RNA and translation factors and are sites of active translation. In murine tissues, Unr-NRs are only found in two polyploid cell types, TGCs and hepatocytes. In vitro, their formation is linked to stress and polyploidy, since in three cancer cell lines, cytotoxic drugs known to promote polyploidization induce their formation. Finally, we show that Unr is required, in vivo, for Unr-NRs formation, because these structures are lacking in Unr-null TGCs.

Keys words
Unr / polyploid cells / Nucleoplasmic Reticulum /mRNA translation / unr knock out
Background

Unr (upstream of N-ras), also known as Csde1 (cold shock domain containing E1), was identified as a transcription unit located immediately upstream of N-ras in the genome of several mammalian species (Jeffers et al., 1990; Nicolaiew et al., 1991). The Unr protein is a member of the family of proteins that contain an evolutionarily conserved nucleic acid-binding domain, termed cold shock domain (CSD), which binds single-stranded DNA and RNA (Graumann and Marahiel, 1998), and are involved in transcriptional and/or post-transcriptional control of gene expression (Mihailovich et al., 2010; Wolffe, 1994). The mammalian Unr proteins, composed of five CSDs, are highly similar, sharing > 90% amino acid identity. Unr is a cytoplasmic RNA-binding protein that, in vitro, interacts preferentially with purine-rich motifs located in RNA loops (Jacquemin-Sablon et al., 1994) (Triqueneaux et al., 1999). Unr has been characterized as a regulator of mRNA turnover (Grosset et al., 2000) and translation. In translation, Unr acts as a positive or negative regulator of specific transcripts. Unr either stimulates or represses the translation driven by internal ribosome entry sites (IRESs) (Boussadia et al., 2003; Dormoy-Raclet et al., 2005; Hunt et al., 1999; Mitchell et al., 2003) or represses cap-dependent translation (Abaza et al., 2006; Duncan et al., 2006; Patel et al., 2005). Recent studies have identified numerous direct Unr mRNA targets in Drosophila (4) and in human melanoma (Wurth et al., 2016). In melanoma Unr regulates its specific target genes mainly at the level of elongation or termination (Wurth et al., 2016).

Genetic and biochemical studies have linked Unr to several cellular processes and to human diseases. Unr has been implicated in the control of cell death (Dormoy-Raclet et al., 2007), cell differentiation (Elatmani et al., 2011) and cell migration (Kobayashi et al., 2013). In human, recent studies have identified important roles for Unr, in promoting melanoma cell invasion and metastasis (Wurth et al., 2016) and in pathological diseases such as autism or Diamond-Blackfan anemia (Sanders et al., 2012; Xia et al., 2014) (Horos and von Lindern, 2012). Unr has also been characterized as a critical regulator of embryonic development. In Drosophila, Unr inhibits dosage compensation in female flies, and Unr overexpression results in predominant male lethality at the larvae stage (Patalano et al., 2009).

Here, we report that Unr is critical for mouse embryonic and placental development. We also report the identification of an Unr-rich structure, forming a network of cytoplasmic invaginations into the nucleus, reminiscent of the nucleoplasmic reticulum (NR, reviewed in (Malhas et al., 2011). These so-called Unr-NRs constitute a novel type of NR, found in polyploid cells, in which active translation takes place.
Results

Disruption of the unr gene in mice causes placental defects that coincide with embryonic lethality occurring at mid-gestation.

We previously generated mice carrying an inactivated unr allele as a result of the unr promoter deletion and we reported that the homozygous mutation of the unr gene resulted in a null mutation (Boussadia et al., 2003). The lack of Unr led to an embryonic lethality occurring at mid-gestation, since unr-/− mutant embryos could be detected close to the expected Mendelian ratios at 9.5 days of gestation (24%), but were fewer by day 10.5 (14%) and absent at 12.5 days of gestation or later (Fig. 1A). unr-/− embryos were indistinguishable from normal littermates at E 7.5. Between E 8.5 and E 10.5, mutant embryos could be identified morphologically based on their smaller size, delayed growth and, at E 9.5-10.5, by the absence of neural tube closure (Fig. 1B). The heart maturation was delayed in unr-/− embryos, which presented a defect of ventricular trabeculation and smaller atrioventricular cushions at the atrioventricular canal (Fig. S 1A). This organ however was not critically abnormal.

Malfunctions of extra-embryonic tissues are primary causes of embryonic lethality at mid-gestation (Copp, 1995; Ihle, 2000; Rossant and Cross, 2001). Histological analyses of unr−/− yolk sac did not reveal obvious structural changes at E 8.5 or later (Fig. S 1B). In contrast, at E 9.5-11.5 obvious placental defects were detected in unr KO placentas, when compared to wild type littermates. The murine placenta consists of maternal and embryonic parts, with the latter being composed of three distinct trophoblastic cell layers, namely, an outermost layer of trophoblast giant cells (TGCs), an intermediate spongiotrophoblast layer, and the innermost labyrinthin layer. In the unr KO placentas, there was a marked atrophy of the spongiotrophoblast and labyrinthin layers and a ~60-75% decrease in the number of TGCs (Fig. 1C). These results show that Unr is essential for the placental development between E 8.5 and E 11.5.
Identification of an Unr-rich Nucleoplasmic Reticulum (Unr-NR) in placental trophoblast giant cells

We examined the expression of the Unr protein within wild-type placentas at day 10.5. Immunofluorescence (IF) analyses of placental sections revealed that Unr is distributed throughout the cytoplasm of the three trophoblastic sub-populations (Fig. 2A, left panel). The specificity of the Unr antibody used in this study was shown by the absence of Unr staining in IF analyses of unr K.O. placentas (fig. 2B). In addition, we found that Unr also localized to distinguishable foci within the nuclei of a subset of TGCs, but not of Spongiotrophoblasts (Fig. 2A, zooms). This finding was unexpected because no nuclear expression of Unr has been reported so far, prompting us to further characterize these Unr foci through physiological, molecular and functional analyses.

First, we performed by IHC a temporal analysis of their presence in TGCs. The results (Fig. 2 C, D) show that Unr foci were undetectable in TGCs at E 8.5. The proportion of Unr foci-containing TGCs increased up to 45% at E 10.5 and declined later. This temporal analysis revealed that Unr foci are transient structures, regulated during placental development.

We next wanted to know if Unr foci represent a novel type of intra-nuclear structure or if they correlate with known intra-nuclear domains. In view of the RNA-binding properties of Unr, we focused on nuclear structures known to be involved in RNA metabolism. We first observed by IHC staining that Unr foci were large (average diameter 4,03 µM ± 1,07, n= 100, Fig. 2A) and clearly distinct from nucleoli (shown in Fig. 2C).

We then thought to determine if the Unr foci are membraneless sub-nuclear bodies, or if they are nuclear bodies wrapped by the nuclear envelope (Mao et al., 2011). Co-labelling of TGCs with antibodies against Unr and lamin A/C, and with Dapi (Fig. 3A) revealed that the Unr foci were limited by the nuclear lamina and devoid of DNA (absence of Dapi staining, Fig. 3A, right panel). Of note, Unr was highly concentrated within these foci as revealed by the comparative quantification of the Unr protein in nucleoplasm, cytoplasm and Unr foci (Fig. S 2A). Because Unr foci were surrounded by the nuclear lamina and devoid of DNA, they might correspond to either the nucleoplasmic reticulum (NR) or to the inner nuclear membrane (INM) infoldings. The NR consists in nuclear invaginations of the double nuclear membranes with embedded nuclear pores, continuous with the endoplasmic reticulum (ER) and enclosing a cytoplasmic core (Fricker et al., 1997; Malhas et al., 2011). INM infoldings consist in invaginations of the INM alone that do not contain nuclear pores and do not represent a continuum with the cytoplasm (Jokhi et al., 2013; Speese et al., 2012).
Using a combination of confocal immunofluorescence (IF) and transmission electronic microscopy (TEM) imaging of TGC sections, we determined that Unr foci were: (i) lined by a double wall nuclear membrane containing nuclear pores, marked by antibodies against the nuclear pore complex (NP) and visible in TEM images (Fig. 3B), (ii) contained ER, marked by antibodies against the ER resident protein Calnexin, and visible in TEM images (Fig. 3C) and (iii) were continuous with the cytoplasm (Fig. 3D). These results indicated that Unr foci were structurally similar to the NR. In agreement, we found that Unr foci contained cytoskeletal cytoplasmic elements (Vimentin and α-Tubulin, Fig. S 2B) frequently found in NRs (Gehrig et al., 2008). We next investigated whether Unr foci, as the NR, form a network of invaginations connected to the nuclear envelope. We performed 3D reconstruction using confocal serial sections through the nucleus of a TGC co-stained with antibodies against Unr and Lamin A/C. Visualizing Unr foci from xy and z vantage points revealed that the observed dots (xy plane) are cross section cuts of tubular extensions of the nuclear membrane, clearly observed in the xz and z images (Fig. 3D and movie 1). All the Unr foci that we visualized in 3D appeared to form a network of branched tubular and vesicular structures in continuity with the nuclear membrane.

These results show that Unr is not expressed in nuclear bodies. They identified a pronounced structural similarity between Unr foci and the NR, a network of cytoplasmic invaginations in the nucleus. Accordingly, we named Unr-NRs, for Unr-Nucleoplasmic Reticulum, the nuclear foci containing Unr.

**Unr-NRs concentrate poly (A) RNA, translation factors and Ribosomes**

The high concentration of Unr throughout the tubular network suggested a function of Unr-NRs in mRNA metabolism, which has never been described for the NR. To determine whether Unr-NRs contained poly (A) RNA, we combined RNA in situ hybridization, using a fluorescent FITC-oligo (dT) probe, and IF, using antibodies against lamin A/C to outline the Unr-NR. An intense oligo (dT) signal was detected in 100% of the Unr-NRs examined, and the signal was sensitive to RNAse treatment (Fig. 4A). To better characterize the spatial organization of the RNA within Unr foci, we performed triple-staining analyses combining RNA FISH and IF, using antibodies marking the NPC and the ER (Fig. S 2C). The fluorescent signals corresponding to the poly(A) RNA (green) and the ER (red) did not coincide within the Unr-NR, but were closely apposed, revealing an intermingle organization of these components within the Unr-NR.

This spatial organization i.e. poly(A) RNA concentrated in the vicinity of nuclear
pores and the ER suggests that Unr-NRs might be sites of active translation of newly exported mRNAs. As mRNAs exit the nucleus, they undergo a pioneer round of translation, initiated by the cap-binding CBP80-CBP20/NCPB2 complex (CBC, (Maquat et al., 2010)). (CBC) is then replaced by the major translation initiation factor 4E (eIF4E), which directs steady-state rounds of mRNA translation. Poly(A) binding protein nuclear 1 (PABPN1) is then replaced by Poly (A) binding protein cytoplasmic 1 (PABPC1) at the poly(A) tail (Hosoda et al., 2006; Lemay et al., 2010). We examined the sub-cellular localization in TGCs of NCBP2/CBP20, eIF4E, PABPN1, PABPC1 and of the elongation factor eEF2, through co-immunostaining experiments using Lamin A/C antibodies to delineate Unr-NRs. The specificity of these co-localization experiments was demonstrated by the absence in Unr-NRs of the survival of motor neurons (SMN) protein, an RNA-binding protein not linked to mRNA translation (Fig. 4G). We found (Fig.4B-F) that all these factors, except NCBP2/CBP20, were localized and concentrated within Unr-NRs. Because of the substitution of NCBP2 by eIF4E, it is likely that the pioneer round of translation has been completed for most of the transcripts. The presence of both PABPN1 and PABPC1 is surprising, and might be related to the reported cytosolic function of PABPN1 (Lemay et al., 2010).

Next, we used TEM to determine if the ER present in Unr-NRs was studded with ribosomes. High magnification TEM micrographs showed that free ribosomes as well as ER-bound ribosomes were clearly visible within these nuclear bodies (Fig. 4H).

Altogether, these results showing that poly (A) RNA, translation factors as well as ribosomes are concentrated within Unr-NRs support a role of these structures in mRNA translation. Since a role of NRs in mRNA metabolism has never been reported, our next efforts were aimed at determining (i) whether Unr-NRs are present in other tissues and cell lines and (ii) whether Unr-NRs are sites of active mRNA translation.

In vivo, Unr-NRs are specifically found in polyploid cells

We next used Unr IHC to analyse Unr expression in a murine tissue microarray with 27 cores and in paraffin-embedded embryos (examples are shown in Fig. S1 C-F). This screening revealed that none of the examined tissues contained Unr-NRs except the liver that we further studied using IF. The Unr-NRs present in hepatocytes were highly similar to those of TGCs, i.e. consisting in nuclear membrane invaginations concentrating Unr, poly(A) RNA and translation factors (Fig. 5A, B). A tubular structure reaching deep in the nucleus was also visualized, (Fig. 5C and movie 2), with however a less branched organization as compared to TGCs. As a result, the number of Unr-NRs per nucleus appears lower in
hepatocytes than in TGCs. That the presence of Unr-NRs was restricted to the liver in addition to the placenta sustained the idea that Unr-NR formation was linked to polyploidy. In agreement, a quantitative analysis of nuclear areas and Dapi intensities (Fig. 5D) showed that hepatocyte nuclei containing Unr-NRs were \approx two fold larger and had a \approx two fold higher DNA content than hepatocytes devoid of Unr-NRs.

**In vitro, Unr-NRs are related to both stress and polyploidy**

NRs have been described *in vitro* in a variety of cultured cells (Malhas et al., 2011), but their Unr content has not been investigated. It was therefore conceivable that Unr-NRs and NRs were identical structures, and NRs might have a role in mRNA metabolism not yet explored. To address this question, we examined by confocal microscopy MDA-MB-231 cells, reported to present the highest ratio of NRs per cell (Johnson et al., 2003). Although we detected double-wall nuclear membrane invaginations devoid of DNA (Fig. S 3A), and containing microtubules (Fig. S 3B), these structures neither concentrate Unr nor eIF4E (Fig. S 3A and C). Based on their expression profile, Unr-NRs therefore do not correspond to the classical NRs.

Since *in vivo* the presence of Unr-NRs was restricted to polyploid cell types, we reasoned that, *in vitro* also, hyperploidy might be essential for Unr-NRs formation. To this end, we selected two cancer cell lines, BeWo and Hep3B, derived from trophoblasts and hepatocytes, respectively, and tested the capacity of cytotoxic treatments known to induce polyploidy to trigger Unr-NRs formation. The drugs we used included etoposide, a DNA damaging drug and taxol (paclitaxel), a spindle toxin (Litwiniec et al., 2013; Marth et al., 1995). Whereas Unr-NRs were detectable in untreated cultures, their incidence markedly increased following drug treatment in both cell cultures (Fig. 5E and 6A). The proportion of Unr-NR containing cells reached \approx 23% in etoposide-treated Hep3B cells and \approx 70% in taxol-treated BeWo cells (Fig. 5F and 6B, left panels). A quantitative analysis of nuclear areas revealed that, simultaneously, etoposide and taxol induced a \geq two-fold enlargement of nuclei (Fig. 5F and 6B, middle panels). Moreover, among this cell population, the Unr-NR positive cells exhibited \approx two fold larger nuclei (Fig. 5F and 6 B, right panels).

We then extended our investigations to two cancerous cells of other origin, MDA-MB231 and HCT116, derived from breast and colon cancer, respectively. We found that Unr-NRs, barely detectable in untreated cell cultures, were efficiently induced by etoposide in MDA-MB231 cells (Fig. S 3 D,E). As in BeWo and Hep3B cells, Unr-NRs appeared in MDA-
MB231 cells with enlarged nuclei (Fig. S3E). Figure S3F presents a clear Unr-rich nuclear invagination, observed in MDA-MB231 cells. We did not detect Unr-NRs in HCT116 cells, whatever the dose of etoposide or taxol used.

In summary, from these results, we inferred that, in vitro, Unr-NR formation is related to both stress and polyploidy. Moreover, cancer cells treated with anticancer drugs seem prone to form Unr-NRs (3 positive cell lines of 4 tested).

**Unr-NRs are sites of active translation**

The development of in vitro cell culture models made possible to decipher the translational status of mRNAs localized in Unr-NRs. Since taxol treatment of BeWo cells turned out to be the most effective in UNR-NRs induction, we selected this condition for further analyses. First, we confirmed that the in vitro formed Unr-NRs were highly similar to their in vivo counterpart. Indeed, in taxol-treated BeWo cells, Unr, eIF4E, and poly (A) RNA were concentrated in nuclear membrane invaginations (Fig. 6A,C).

To directly visualize localized translation in live cells, we used the ribopuromycylation method (RPM). RPM is based on incorporation of puromycin (PMY) into nascent chains, whose association with ribosomes is maintained by the presence of the chain elongation inhibitor emetine (David et al., 2012). In control BeWo cells, RPM coupled with PMY staining produced an intense signal, distributed throughout the cytoplasm, and co-localized with Unr (Fig. 6D, E, left panels). Blocking translation by anisomycin before the puromycin pulse abrogated the PMY signal (Fig. 6D, right panels), demonstrating the specific labeling of ribosome-associated nascent chains by the PMY antibodies. In taxol-treated BeWo cells, the distribution of the Unr and PMY signals clearly changed, Unr being mostly localized in Unr-NRs, whereas the PMY signal was observed both in the cytoplasm and in Unr-NRs (Fig. 6E and S4 B,C). The PMY and the ER marker calnexin showed a similar distribution, present in the cytoplasm of control cells, and in both the cytoplasm and Unr-NRs of taxol treated cells (Fig. S5C).

These results demonstrated that an active mRNA translation process is taking place within Unr-NRs (more than 80% of the Unr-NRs were PMY positive). However, we could not exclude that Unr-NRs also represent a storehouse for non-translating mRNAs. Indeed, a number of studies has provided evidence that cytoplasmic RNA granules are structures in which mRNAs are « masked », i.e. in a translational repressed state (Anderson and Kedersha, 2009) (Eulalio et al., 2007). To test whether Unr-NRs might harbor non-translating mRNAs, we used antibodies against TIA-1 and rck/p54, markers shared by SGs, PBs and PGs.
(Buchan and Parker, 2009). We observed that, in TGCs, most of the Unr-NRs appeared positive for TIA-1 and rck/p54 (Fig. S 2D), suggesting that Unr-NRs store a pool of non-translating mRNAs. Does then active and repressed mRNA translation take place within Unr-NRs? To investigate this hypothesis, we performed RPM in live BeWo cells, coupled with PMY, Unr and TIA-1 triple staining. As expected, TIA-1 was undetectable in control unstressed cells (Fig. S 4A). In taxol-treated cells, TIA-1 was expressed in most of the Unr-NRs and the PMY and TIA-1 signals colocalized within Unr-NRs (Fig. S 4B).

Altogether, the aforementioned data suggest a dual role of Unr-NRs. On one side, they are sites of active mRNA translation, and on the other side, they likely maintain a pool of mRNAs in a translational repressed state.

Unr is required for Unr-NRs formation

To address the role of Unr in the formation of Unr-NRs, we examined whether Unr-NRs were formed in TGCs null for Unr. To be able to visualize a nuclear tubular network in the absence of Unr, we used antibodies against the nuclear pore. Five wild type and unr KO placentas at E 10.5, and three at E 11.5, were analyzed. Whereas the perinuclear membrane was normally stained in unr KO TGCs, intranuclear invaginations visible as granules in cross sections were completely absent, using either 2D or 3D confocal imaging (Fig. 7B). Moreover, the nuclei of unr/− TGCs neither accumulate ER resident proteins nor translation factors in NR-like structures in (Fig. 7C). unr +/− TGCs did not differ from unr+/+ TGCs, regarding the proportion of Unr-NR positive cells or their molecular composition. These results demonstrate that Unr is required for the formation of NR-like structures that concentrate polyadenylated RNA and translation factors in the proximity nuclei of TGCs.
Discussion

In this study, we have used an unr knock out model to determine the role of this RNA binding protein during mouse development. Two main findings are that (a) Unr is required for mouse embryonic and placental develop, and (b) Unr defines a novel type of NR, having a role in mRNA translation.

Unr is required for placental and embryonic development in mice.

unr KO embryos die between E 10.5 and E 12.5, but the phenotypic defects they exhibit before dying, i.e. an absence of neural tube closure, a delay in heart maturation and a small size are unlikely to be the cause of their death (Copp, 1995). In contrast, major placental defects were evident and are presumably the primary cause for growth retardation and death of unr KO embryos, as a result of insufficient materno-foetal nutrient exchanges. Thus, Unr as several other RNA-binding proteins (Katsanou et al., 2009; Lu et al., 2005; Shibayama et al., 2009; Stumpo et al., 2004) has important roles during mouse development.

Unr defines a novel class of Nucleoplasmic Reticulum involved in mRNA translation

Unr expression studies revealed that, surprisingly, Unr was localized to bright foci punctuating the nuclei of a subset of TGCs. We determined that Unr foci were cross sections of a tubular network of nuclear membrane invaginations enclosing a cytoplasmic core. These structures are related to the nucleoplasmic reticulum (NR) (Fricker et al., 1997; Malhas et al., 2011) and accordingly named Unr-NRs.

What is the functional role of Unr-NR? Several studies have provided evidence that NRs are involved in \( \text{Ca}^{2+} \) signaling in localized sub-nuclear regions (Echevarria et al., 2003{Lui, 1998 #45). Because of the RNA-binding properties of Unr, we thought that Unr-NRs might have a role in mRNA metabolism that had not yet been explored in NRs (Malhas et al., 2011). And indeed, we found that Unr-NRs present in TGCs are structures that contain an impressive amount of poly (A) RNA and translation factors. We then considered two possibilities: (i) either a function in mRNA metabolism had not yet been explored in classical NRs, and Unr-NRs are not functionally different from classical NRs (ii) or they are two types of NRs: the first one, described in many cell types, do not concentrate the translational machinery and has been shown to be involved in calcium signaling; the second one, Unr-NR, is restricted to specific cell types and has a function in mRNA metabolism. To answer this question, we went in
search of Unr-NRs in a variety of cell types, and we generated in vitro cell culture models in
order to analyse Unr-NR function.

Unr-NRs are related to both stress and polyploidy

In an effort to link Unr-NRs to cell physiology, we went in search of these structures in
murine tissues. A tissue microarray screen combined with IHC analyses of some murine
tissues revealed their scarcity, since none of the examined tissues contained Unr-NRs, except
the liver, a tissue rich in polyploid cells (Chen et al., 2012; Pandit et al., 2012). The low
incidence (3%) of Unr-NRs suggests that, in liver, the increased ploidy, modest as compared to
that reached in TGCs (up to 1,024 C), is necessary but not sufficient to produce Unr-NRs. An
adverse context, such as oxidative stress, might also contribute to their formation. It will be
important to scrutinize other polyploid cell types not examined here, such as megakaryocytes
and cardiomyocytes, to confirm the restriction of Unr-NRs to enlarged polyploid cells.

These findings helped us to generate cell culture models exhibiting Unr-NRs. The
hypothesis linking Unr-NRs to polyploidy was right, since Unr-NRs were inducible in the
hepatoblastic Hep3B cell line treated by etoposide, and in the trophoblastic BeWo cell line
treated by taxol/paclitaxel (Litwiniec et al., 2013; Marth et al., 1995). Unr-NRs were
preferentially formed in cells with enlarged nuclei, their frequency in the surviving cell
population reaching up to 23 % in etoposide-treated Hep3B cells and 70 % in taxol-treated
BeWo cells. Unr-NRs are not restricted to cell lines derived from placenta and liver, since they
are also efficiently induced by etoposide in MDA-MB231 cells, derived from breast cancer.
Neither etoposide nor taxol led to Unr-NRs formation in the colorectal HCT116 cells. An
hypothesis is that HCT116 cannot endoreplicate their genome because they express wild-type
p53 (Liu, 2006 #73), assumed to prevent cells from undergoing endoreduplication and
polyploidy (Di Leonardo et al., 1997). Bewo, Hep3B and MDA-MB231 cells either express an
inactive p53 protein, or carry p53 mutations or are p53-null (Hau et al., 2006; Lin et al., 2000;
Negrini et al., 1994).

Unr-NRs are involved in mRNA translation

Using ribopuromycylation (RPM), we demonstrated that Unr-NRs are sites of active mRNA
translation. Nevertheless, a number of studies has provided evidence that mRNAs, aggregated
as microscopically visible RNA granules, are translationally repressed (Anderson and
Kedersha, 2009; Eulalio et al., 2007). Specific examples include germ cells perinuclear P
granules (PGs), P-bodies (PBs) and Stress Granules (SGs) that share protein components including TIA-1 (Kedersha and Anderson, 2007). TIA-1 was found in most (but not all) Unr-NRs, suggesting that a pool of untranslated mrnas is also herein localized. As proposed for P granules, (Sheth et al., 2010), newly exported mrnas could be transiently stored in Unr-NRs because their diffusion rate into the cytoplasm is low.

That Unr-NRs are sites of active mRNA translation and storage is a quite unusual situation, since most of RNA granules are dedicated to the storage of non-translating mrnas. Two recent reports however have documented RNA granules either translationally active, or having a dual translational status (Buchan, 2014; Weil et al., 2012; Yasuda et al., 2013).

Benefits of Unr-NR formation

A diagram illustrating the structure and function of Unr-NRs is presented in Fig. 8. We propose that the formation of Unr-NRs is an adaptative response of the cells to metabolic or cytotoxic stress situations. TGCs encounter a metabolic stress condition because of their vastly amplified genome, producing an amount of mRNA exceeding with their capacity of translation. We propose that, to cope with this stress, polyploid TGCs expand the nucleo-cytoplasmic interface and the ER compartment by forming the Unr-NR network, which facilitates mRNA export and translation at the rough ER. They also maintain a pool of untranslated mrnas localized within the core of the Unr-NR network. This model is consistent with recent reports having established that, under stress conditions, mRNA translation on free ribosomes is repressed, whereas mRNA translation on ER-bound ribosomes is sustained (Lerner and Nicchitta, 2006; Unsworth et al., 2010).

Many questions arise from the results reported here. Unr-NRs are regulated structures, appearing at a precise developmental stage of the placental development, or in response to cytotoxic stresses. A current theme is that polyploidy confers resistance to environmental stresses not tolerated by diploid cells (review in (Schoenfelder and Fox, 2015). It will be very interesting in the future to see whether Unr-NRs facilitate survival of giant cancer cells subjected to chemotherapy.

Unr is required for the formation of Unr-NRs

Finally and importantly, we determined that Unr-NRs do not form in Unr-null TGCs in the absence of Unr. What is the role of Unr in Unr-NR formation? Although the role of Unr is a matter of speculation, we propose that Unr could (a) promote the nuclear and membrane
expansion required for the acquisition of a NR, by stimulating lipid and protein synthesis (Gehrig et al., 2008), (b) recruit mRNAs fated to be translated on ER-bound ribosomes. This later possibility relies on the localization of Unr at the ER, conserved between Drosophila and mammals (Abaza et al., 2006; Jacquemin-Sablon et al., 1994).

Conclusion

In conclusion, the main finding of this study is the identification of an Unr-rich structure, named Unr-NR, because of its structural similarity with the nucleoplasmic reticulum (NR). The novelty of this structure, identified in vitro and in vivo, relies on the high amount of poly (A) RNA and translation factors herein localized and on its function in mRNA translation that has never been reported. Unr-NRs are not found in normal cells, as reported for NRs, but are restricted to polyploid cells (in vivo), and to stressed cells (in vitro). We propose that this novel type of NR provides an extended surface for mRNA translation at the rough ER, helping cells to ensure proper translational control. Our results linking Unr-NRs to cancerous cells subjected to anticancer drugs might be of importance for drug resistance.
Methods

Unr-Deficient Mice and Genotyping

Unr+/− mice were obtained as previously described (Boussadia et al., 1997). Unr+/− mice were maintained on a C57BL/6 genetic background. Mice were routinely genotyped by PCR of tail DNA. Embryos were genotyped by PCR of yolk sac DNA at E9.5, and by PCR at earlier stages. This study was performed in accordance with the European Community Standards on the Care and Use of the Animals and was approved by the Animal Care and Use Committee of the University of Bordeaux. Adult livers were obtained from C57-BL/6, Nog or NMRI mice.

Primers. Primers used for genotyping. The targeted allele was detected by using neo primers: (neoFo 5’-CGTGTTCCGGCTGTCAGCGCAGG-3’; neoRo 5’-CAACGCTATGTCTCTGATAGCGGTCC-3’), which give a product of 565 bp. The normal allele was detected by using unr promoter primers: (unrFw 5’ -AACGCAGAGATTGCTTCTGG-3’; unrBw 5’- CCACCTTAAACAGTGAGGTCG-3’), which give a product of 290 bp.

Cell culture and drug treatment

BeWo (human trophoblast-derived choriocarcinoma) cells were maintained in Ham’s F-12 K medium (Gibco) supplemented with 2mM l-glutamine, 20%fetal bovine serum and 100 U/ml penicillin-streptomycin (Invitrogen). Hep3B (human hepatocellular cell carcinoma), MDA-MB231 (human breast cancer) and HCT116 (human colorectal carcinoma) cells were maintained in DMEM containing 4.5 g/l glucose, supplemented with 10%fetal bovine serum and 100 U/ml penicillin-streptomycin. For Unr-NR induction, BeWo cell, Hep3B and MDA-MB231 cells were plated in 24-well dishes (4x10^4 cells/well). 24 h after, Bewo cells were treated with 0.5 μM taxol (Sigma) or 0.2% DMSO (as vehicle) for 48 h. The medium with taxol or 0.2% DMSO was changed every 24 h. Hep3B and MDA-MB231 cells were treated with 1.5 μM etoposide for 24 h (Hep3B) or 48h (MDA-MB231), and cultured for an additional 48 h in drug-free medium (Hep3B).

Histology and Immunohistochemistry

Embryos, yolk sacs, placentas and adult livers were fixed in 4% paraformaldehyde in PBS for
2 hours (except livers fixed overnight) at 4°C, rinsed in PBS, dehydrated in graded ethanol, cleared in toluene and paraffin embedded. Sections (4 µm) were stained with Hematoxylin and Eosin following standard protocols, or used for immunohistochemistry. Antigens were retrieved by a 30 minutes incubation at 98°C in target retrieving solution (Tris 10mM EDTA 1mM pH9) followed by a 30 minutes cooling at room temperature. Sections were incubated for 2 hours at room temperature with primary antibody (anti-Unr (1:100, HPA018846, Sigma), and bound antibodies were visualized using anti-rabbit or anti-mouse RTU Vectastain Kit (Universal Elite ABC Kit, PK-7200, Vector) and NovaRED Substrate Kit (SK-4800, Vector). Nuclei were detected by light counterstaining with Mayer’s hemalun.

**Immunofluorescence**

Tissue sections. After deparaffinization and antigen retrieval, sections were incubated with a blocking solution (1% BSA and 10% SVF in PBS) for 30 minutes and with primary antibodies for 1 hour, at room temperature. Sections were then incubated with Alexa Fluor-conjugated secondary antibodies for 1 hour before mounting in DAPI-Fluoromount-G (SouthernBiotech). Cell cultures. Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 for 10 min before incubation with antibodies as described above.

**Antibodies.** The following primary antibodies were used in this study: anti-Unr (1:100, HPA018846, Sigma), anti Lamin A/C (1:500, provided by H. Wodrich), anti-nuclear pore complex (1:100, clone Mab414, ab24609, Abcam), anti-Calnexin (1:100, ab93355, Abcam), anti-KDEL (1:100, clone 10C3, Enzo Life Sciences), anti-vimentin (1:40, clone V9, Dako), anti-alpha tubulin (1: 100, clone DM1A, Sigma-Aldrich), anti-eIF4E (1:100, clone 87, BD Transduction Laboratories), anti-NCP2 (1:100, ab124632, Abcam), anti-PABPN1 (1:100, AJ1580b, Abgent), anti-PABPC1 (1:100, clone 10E10, Sigma-Aldrich), anti-eEF2 (1:100, ab40812, Abcam), anti-SMN (1: 100, 610647, BD Transduction Laboratories), anti-PMY (1:100, Millipore, MabE343), Anti-TIA1 and anti-p54/rck were gifts from H.Moine and C.Tomasetto (1:500 each). Secondary antibodies used were: Alexa Fluor 488-, 594- and 647-coupled anti-mouse and anti-rabbit, anti-goat and anti-guinea-pig (1:400, Life Technology). DNA was visualized using DAPI (0.5 µg/ml).
**RNA-FISH**

RNA-FISH on paraffin-embedded tissue sections was performed according to de Planell-Saguer, 2010 (de Planell-Saguer et al., 2010). (a) RNA FISH on tissue sections. After deparaffinization and antigen retrieval, sections were incubated with a prehybridization solution (4x SSC, 10% formamide) for 20 minutes at room temperature. Where prehybridization RNase treatment was applied, sections were treated with 0.1 g/ml RNase A in PBS for 2 h at room temperature. Hybridization was performed for 1 h at room temperature, in hybridization solution (4xSSC, 10% formamide, 0.5 mg/ml ssDNA, 0.5 mg/ml tRNA, 10% dextran) containing FITC-conjugated oligo (dT)\textsubscript{40} probe (Eurogentec) at a final concentration of 0.5 ng/\mu l. After washes, sections were either mounted and examined, or subjected to immunofluorescence before mounting. (b) For RNA FISH on cell cultures, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed in PBS and permeabilized for ≥ 24h in 70% ethanol at 4°C. Prior to hybridization, the coverslips were incubated in 2x SSC, 15% formamide, at 65°C for 10min. The following steps (hybridization and washes) were proceeded as described above, except that hybridization was performed for 16 h at 37°C.

**Ribopuromycylation (RPM) method**

To visualize newly synthetized proteins within cells, we used the RPM method as described by David et al. (David et al., 2012). BeWo cells grown on coverslips were incubated for 15 min at 37°C in complete H12 medium supplemented with 208 \mu M emetin (EMD, Sigma). In protein synthesis inhibitor control experiments, cells were pretreated with 40 \mu M anisomycin (Sigma) for 30 min at 37°C before incubation with EMD. Cells were then treated with 355 \mu M cycloheximide (Sigma) for 2 min on ice in permeabilization buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl\textsubscript{2}, 25 mM KCl, 0.015% digitonin, EDTA-free protease inhibitor, 10 U/ml RNaseOut (Invitrogen). Cells were then washed and incubated in polysome buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl\textsubscript{2}, 25 mM KCl, 0.2 M sucrose, EDTA-free protease inhibitor, 10 U/ml RNaseOut (Invitrogen) supplemented with 91\mu M puromycin (PMY, Sigma) for 10 min on ice. After rapid washing in in polysome buffer, cells were fixed in 4% formaldehyde for 15 min at room temperature. After fixation, cells were washed twice with PBS and immunostaining with anti-PMY antibody was performed as described above.
Image acquisition.

(a) Histological and IHC data. Slides were scanned using a digital slide scanner (Pannoramic Scan; 3D HISTECH Ltd, Budapest, Hungary) with a Zeiss objective (Plan Apochromat 40x; numerical aperture 0.95) and a high-resolution color camera (CIS VCC-FC60FR19CL, 4MP). The images were read using the Pannoramic Viewer software (3D HISTECH Ltd).

(b) Confocal images. Cells were imaged as previously described (Juin et al., 2014). Cells were imaged with a SP5 confocal microscope (Leica, Leica microsystems GmbH, Wetzlar, German) using a 63x/numerical aperture (NA) 1.4 Plan Neofluor objective lens. To prevent contamination between fluorochromes, each channel was imaged sequentially using the multitrack recording module before merging. Z-stack pictures were obtained using LAS AF, Leica software. Three-dimensional reconstructions were obtained from Z-cut pictures, by using Imaris software (Bitplane, Zurich, Switzerland).

Quantifications

Unr-NRs. We used a macro with ImageJ software that allowed measurement of Unr-NRs number and diameter. Nuclear DNA content was quantified using total DAPI fluorescence intensity per nucleus using ImageJ. A minimum of 100 cells was counted for each field.

Statistics

Data were reported as mean ± SD. Significance was measured using a two-tailed Student’s test (p<0.05).

Electron Microscopy

For transmission electron microscopy (TEM), placentas were washed with ice cold PBS, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) for 2h at 4°C, and post-fixed in 1% OsO₄ in PB for 2 hours at room temperature. Tissue blocks (1-2 mm³) were then processed following standard procedures. After fixation, tissue blocks corresponding to the embryonic part of the placenta were dehydrated, embedded in Epoxy resin and sectionned through a series of graded ethanol and propylene oxide, and. Blocks were sectionned at a thickness of 60nm using an ultramicrotome Leica UCT. Sections were collected onto copper grids, contrasted with 3% uranyl acetate and lead citrate, and examined using a transmission electronic microscope (HITACHI H7650).
List of Abbreviations

TGC: Trophoblast Giant Cell; NR: Nucleoplasmic Reticulum; ER: Endoplasmic Reticulum.

Declarations

Ethics approval and consent to participate
This study was performed in accordance with the European Community Standards on the Care and Use of the Animals and was approved by the Animal Care and Use Committee of the University of Bordeaux.

Consent for publication
Not applicable

Availability of data material
All data generated or analyzed during this study are included in this published article (and its supplementary information files). Request for material should be made to the corresponding author.

Competing interests
The authors declare that they have no conflict of interest

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Author Contributions
Conceived and designed the experiments: FS, AG, LA, OB, PD and HJS.
Performed the experiments: FS, AG, LA, ZE, HE, PC, NDS, and HJS.
Wrote the manuscript: FS, HW and HJS.
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### Figures

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Figure 1. *unr* disruption causes embryonic and placental abnormal development. (A) Genotype analysis of the progeny from *unr*<sup>+/−</sup> intercrosses. The embryos genotypes were determined by PCR using DNA extracted from the whole embryo or from the yolk sac, using primers a, b, c and d as shown in Figure S1B. In parentheses appear the percentages corresponding to the different genotype classes. (N.D.) Genotype not determined. (B) Morphological defects in *unr<sup>−/−</sup>* embryos at E 9.5. Micrographs of *unr<sup>−/−</sup>* embryos and control littermates at E 9.5 are shown. Note the marked reduction in size of the mutant relative to the control littermate (left) and the absence of neural tube closure (right, the unclosed head is indicated by the arrow). (C) Placental defects in *unr<sup>−/−</sup>* embryos. Histological analysis of wild-type and *unr* KO placentas at E 8.5, E 9.5 and E10.5. Sections through the central regions of placentas were (H&E) stained and imaged at 40x. Maternal decidua (Ma), Spongiotrophoblast (Sp), trophoblast giant cell (TGC), and Labyrinth (Lab) layers are indicated.
Figure 2. Unr localizes to discrete foci in nuclei of trophoblast giant cells. (A) Unr Immunofluorescent staining of representative sections from wild-type placentas at E 10.5. DAPI was used to visualize nuclei. (A) Unr staining (green) is detected in the cytoplasm of spongiotrophoblasts (Sp) and trophoblast giant cells (TGCs) of wild-type placenta. Middle and panel show enlarged views (x5 and x25 that allowed measurements of the Unr foci size of TGCs (boxed region). Right panel shows enlarged view (x5) of SpS.. Scale bar: 150 μm. (B) A confocal image of unr K.O. TGCs co-stained with antibodies against Unr (green) and the nuclear pore (NP, red) is shown. (C) Immunohistochemical staining for Unr (brown staining) of a wild-type placental section, showing the localization of Unr to nuclear foci in TGCs. The inset is a zoomed image of the boxed region. x40 (left) and x100 (inset) magnifications. Scale bar: 50 μm. The counterstain was haematoxylin. (C) Histogram representing the quantification of TGCs positive for nuclear Unr foci between E 8.5 and E 11.5. Data are the average of the percentage of Unr-foci positive cells per x 20 fields ± SD, four placentas analyzed at each stage.
Figure 3. Immunostaining and Transmission electron microscopy analyses showing that the Unr foci are related to the Nucleoplasmic Reticulum (NR) structure. (A) Unr foci are surrounded by nuclear lamina (LN) and are devoid of DNA. TGCs were double-stained with antibodies against Unr (green) and Lamin A/C (red). Scale bar: 5 μm. Middle panel: zoom
(x5) of the boxed region. Right panel: Dapi staining corresponding to the confocal image shown in (A). (B) Unr foci are wrapped by nuclear envelope studded by nuclear pores. Confocal images of TGCs double-stained with antibodies against Unr (green) and the nuclear pore complex (NP, red), scale bar: 10μm, and TEM micrograph showing a nuclear pore (black arrow). (C) Unr foci contain ER. Left: Confocal images of TGCs double-stained with antibodies against lamin A/C (LN, red) and the ER protein Calnexin (CNX, green), and TEM micrograph, showing ER sheets in nuclear invagination. Right panel: zoom (x2) of the boxed region. Scale bar: μm (D) Unr foci consist in a tubular structure, continuous with the cytoplasm. Left panel: TEM micrograph showing an invagination of the nuclear envelope enclosing a cytoplasmic (Cyto) core (black arrow). The inset is a zoom image (x1.75) of the boxed region. Scale bar: 0.5 μm (left). Right panel: 3D analysis of a representative TGC with Unr granules co-stained with anti-Unr and anti-lamin A/C antibodies. A unique 0.3 μm confocal section shows the XY plane; serial confocal sections (1 μm each) spanning the nucleus were used to reconstruct the Y-Z plane (right side) and X-Z planes (bottom). The white dashed lines represent the cutting position for the analysis. Note the continuity between the Unr granule and the nuclear membrane (white arrow). Scale bars: 5μm.
**Figure 4.** Unr-NRs concentrate poly (A) RNA, translation factors and ribosomes. (A) Immunostaining for lamin A/C (red) was combined with FISH using an FITC-oligo(dT) probe (green) to localize poly (A) RNA in TGC nuclei (N). Left panels: Absence of RNase treatment. Right panels: the section was treated with RNase A before hybridization with the oligo(dT) probe. Two adjacent sections were analysed and images were acquired using the same exposure time. Zoom (x 3.8) of the boxed region shows a strong oligo (dT) signal in the Unr-NR (no RNAse), with a heterogeneous sub-localization within the NR. Scale bars: 10 μm. (B-G) Translational factors localize to Unr-NRs. TGCs were double-stained with antibodies against Lamin A/C (LN, red) and: (B) eIF4E (green); (C) eEF2 (green); (D) PABPC1 (green); (E) PABPN1 (green); (F) NCBP2 (green); (G) SMN (green).
Zoons (x3.8) are enlargement (x3.8) of the boxed regions. Scale bars: 5 \( \mu \text{m} \). (H) TEM micrographs of a TGC showing ribosomes either free or as « rosettes » (polysomes, boxed region), localized in a nuclear invagination. The inset is a zoom image (x 2.3) of the boxed region.
Figure 5. Unr-NRs are found in mouse hepatocytes. (A) Immunostaining analyses of representative sections from an adult mouse liver. DAPI was used to visualize nuclei. Sections were double-stained with antibodies against Unr (green) and the nuclear pore (NP), red). The two right panels show red and blue fluorescence in gray, respectively, for better visualization. Zooms (x3.8) show enlarged views of the boxed region. Scale bar: 5 μm. (B) Left panel: poly (A) RNA detection by FISH (green); Middle and right panels: Confocal images of liver sections stained with antibodies against the KDEL peptide marking ER proteins or the translation factor eIF4E (middle panels, green) and the SMN protein as negative control (right panel, green). DAPI was used to visualize nuclei. Scale bars: 5 μm. (C) Immunostaining analyses showing that Unr-NRs visualized in hepatocytes form a tubular structure continuous with the nuclear membrane. Left and middle panels: Immunofluorescence analysis of a liver section co-stained with Unr (green) and lamin A/C (LN, red) antibodies. Left and middle panels show green, red fluorescences in gray, respectively, and overlay of the signals (constituting the 1 μm confocal section shown in the XY plane). 3D analysis: serial confocal sections (1 μm each) spanning the nucleus were used to reconstruct the Y-Z plane (right side) and X-Z planes (bottom). Scale bars: 5 μm. (D) Quantifications of hepatocytes exhibiting Unr-NRs. (Left panel) A representative field of a liver section immunostained with anti-Unr antibodies (green) and counterstained with Dapi (blue) is shown. (Middle panel) Distribution of the nuclear area of hepatocytes without (left, n= 60) or with (right, n=12) Unr-NRs. (Right panel) Histogram representing the quantification of DNA content in hepatocytes nuclei devoid (left) or containing (right) Unr-NRs. DNA content was evaluated by recording the intensity of Dapi integrated fluorescence. Data are the average of ≈ 100 nuclei per field x 30 fields ± SD. P< 0.0001. (E, F) Unr-NRs are formed in Hep3B cells treated with etoposide. (E) Confocal images of Hep3B cells co-stained with antibodies against Unr (green) and lamin A/C (red). DAPI (blue) was used to visualize nuclei. Representative fields of normal (left panel) and etoposide-treated (right panel) Hep3B cells are shown. Enlarged views (x5) of the boxed regions are shown. Scale bars: 10 μm. (F) Quantifications of Hep3B cells exhibiting Unr-NRs. Left panel: Histograms representing the quantification of Hep3B positive for Unr-NRs in control or etoposide-treated cells. Middle panel: Histogram representing the quantification of nuclear areas in control or etoposide-treated Hep3B cells. Right panel: Distribution of the nuclear area of etoposide-treated cells without (left, n= 60) or with (right, n=12) Unr-NRs.
Figure 6. Unr-NRs are formed in BeWo cells treated with taxol and represent sites of active mRNA translation. (A) BeWo cells were either cultured in the absence of drug (left panel) or treated with 0.5\(\mu\)M taxol for 48h (middle and right panels). Cells were stained with antibodies against Unr or eIF4E (green), LN (red) and counterstain with Dapi. An enlarged view of a nucleus shows Unr-NRs, co-stained with Unr (green) and Lamin A/C (red).
antibodies. DAPI was used to visualize nuclei and evaluate the average nuclear size. Scale bar: 5 μm. (B) Quantification of BeWo cells exhibiting Unr-NRs. Left, middle and right panels: see legend Fig. 5F). (C) In vitro formed Unr-NRs contain poly (A) RNA. Double-staining of taxol-treated BeWo cells for poly(A) RNA (green) and Unr (white), either without (Left panels) or with (right panels) RNAse treatment. Nuclei were counterstained with DAPI. Scale bars: 5 μm. (D, E) Nascent polypeptide chains are present within Unr-NRs. BeWo cells were pulsed with puromycin and emetin to label translating ribosomes. Cells were then fixed, permeabilized and double-stained for puromycin (anti-PMY, red) and Unr (anti-Unr, green). Right panels show, red fluorescence in grey for better visualization. Dapi was used to visualize nuclei. Scale bars: 5 μm. (D) Control Bewo cells were pretreated (right) or not with 40 nM anisomycin for 30 min before puromycin pulse, to validate the specificity of the antibody against puromycin. (E) Control (left panels) or taxol-treated Bewo (right panels) were pulsed with puromycin and emetin and double-stained for puromycin (red) and Unr (green).
Figure 7. Unr is required for Unr-NRs formation. (A-C) Unr-NRs are not formed in unr KO TGCs. Confocal images of TGCs co-stained with antibodies against Unr (green) and the nuclear pore (NP, red) are shown. (A) A wild-type TGC (positive control) with Unr-NRs is shown. Scale bar: 10 µm (B) Absence of nuclear envelope invaginations in unr\(^{-/-}\) TGCs. A representative field, an enlarge view of a TGC and a zoom (x5) of the boxed region (nuclear membrane) are shown. Scale bars: 50 µm (left panel), and 10 µm (middle panel). 3D analysis of a representative TGC from unr KO placenta, co-stained with anti-Unr and anti-lamin A/C antibodies. A unique 0.3 µm confocal section shows the XY plane; serial confocal sections (1 µm each) spanning the nucleus were used to reconstruct the Y-Z plane (right side) and X-Z planes (bottom). The white dashed lines represent the cutting position for the analysis. Scale bar: 5 µm. (C) Absence of ER resident proteins and translation factors in nuclei from unr\(^{-/-}\) TGCs. Confocal images of unr\(^{-/-}\) TGCs co-stained with antibodies against the nuclear pore (NP, red) and eIF4E, Calnexin (CNX), PABPC1 and PABPN1 (green). DAPI was used to visualize nuclei. Scale bars: 5 µm.
Figure 8. Schematic representations of a wild-type TGC exhibiting Unr-NRs. Left panels: schematic of a whole cell (top) and enlarged view of the nucleus (bottom). Right panel: Schematic representation of an Unr-NR, with the main components identified in this work.
Figure S1. (A) Delay in maturation of unr−/− hearts. Sections through the heart of E 10.5 litter-matched unr+/- and unr−/- embryos were H&E stained and imaged at 100x magnification. The unr−/- heart is delayed and presents a defect of ventricular trabeculation (arrows in A) and smaller atrioventricular cushions at the AVC (star in A). A: Atrium; AVC: atrioventricular canal; OFT: outflow tract; V: Ventricule. Scale bars: 150 mm left), 125 mm (right). (B) Lack of Unr does not alter yolk sac formation. Histological comparison of wild-type and unr−/- yolk sac at E 9.5. Scale bar: 100 mm. (C-F). Immunohistochemical staining for Unr (brown staining) of a murine tissue microarray and of a paraffin-embedded 10.5 embryo. Representative images from brain(20x), intestine (40x), Epididymis (40x) and E 10.5 embryo (4x) sections are shown. The counterstain was haematoxylin.
Figure S2. (A) Unr is concentrated inside the Unr-NRs. Left panel, confocal images of a TGC double-stained with antibodies against Unr (green) and the nuclear pore complex (NP, red). DAPI was used to visualize nuclei. Middle panel, zoomed (x4) image of the boxed region. Right panel: intensity profile of the region of interest (ROI, white line). Scale bar: 10 mm. (B) Unr-NRs from TGCs contain cytoplasmic cytoskeleton elements. Left panel, confocal images of TGCs double-stained with antibodies against Unr (green) and (left) tubulin (red) or (right) vimentin (red). DAPI was used to visualize nuclei. Middle panel: zooms (x 3.8) of the boxed regions. Note that the penetration of a microtubule into the Unr-NR is visible (white arrow). Scale bar: 10 mm. (C) Structural organization of an Unr-NR showing the heterogeneous distribution of Unr and poly (A) RNA within an Unr-NR. Right panel: A confocal image of TGC after triple staining for calnexin (red), nuclear pore (NPC, white) and poly (A) RNA (green), is shown. The three left panels show green, red and white fluorescence in grey, respectively, for better visualization. Scale bar: 5 mm. (D) Unr-NRs contain markers of mRNA storing granules. Confocal images of TGCs double-stained with antibodies against Lamin A/C (red) and: left, rck (green) or TIA-1 (green). Zooms (x 2.5 and x3.8) are enlarged views of the boxed regions in Fig.S2 C and S2 D. Scale bars: 10 mm.
Figure S3. Unr-NRs correspond to a novel class of NRs. (A) Confocal images of normal MDA-MB231 cells co-stained with antibodies against Unr (green) and the nuclear pore (NP, red). DAPI (blue) was used to visualize nuclei. A nuclear envelope invagination (left), devoid of DNA (blue fluorescence in grey, right panel), is clearly visible, but does not contain Unr not (zoom and green fluorescence in grey). Zoom (x4) is an enlarged view of the boxed region. Scale bar: 5 mm. (B-C) Confocal images of MDA-MB231 cells co-stained with antibodies against the nuclear pore (NP, red) and α-tubulin (green, B) or eIF4E (green, C), showing that these components are not detected into the nuclear envelope invaginations. The right panels show green fluorescence in gray for better visualization. Scale bar: 5 mm. (D) Confocal images of MDA-MB231 cells co-stained with antibodies...
against Unr (green) and lamin A/C (red). DAPI (blue) was used to visualize nuclei. Representative fields of normal (left panel) and etoposide-treated (right panel) MDA-MB231 cells are shown. Scale bars: 10 mm. Zooms (x 5) of normal and etoposide-treated MDA-MB231 cells are shown. (E) Quantifications of MDA-MB231 cells exhibiting Unr-NRs. Left panels: Histograms representing the quantification of MDA-MB231 cells positive for Unr-NRs in control or etoposide-treated cells. Middle panels: Histogram representing the quantification of nuclear areas in control or etoposide-treated MDA-MB231 cells. Right panels: Distribution of the nuclear area of etoposide-treated cells without (left, n= 60) or with (right, n=12) Unr-NRs. (F) The structure of an Unr-NR in MDA-MB231 cells, concentrating Unr in a nuclear membrane invagination, is clearly visible. Scale bar: 5 mm.
Figure S4. (A) Active and repressed mRNA translation in Unr-NRs. (A, B) Control (A) or taxol-treated (B) BeWo cells were pulsed with puromycin and emetin and triple-stained for puromycin (anti-PMY, red), Unr (anti-Unr, green) and TIA-1 (anti-TIA-1, white). DAPI was used to visualize nuclei. The three right panels show red, green, and white fluorescence in grey, respectively, for better visualization. Scale bars 10 mm. (C) mRNA translation co-localises with ER in BeWo cells. Control (left panels) or taxol-treated (right panels) BeWo cells were pulsed with puromycin and emetin and double-stained for puromycin (red) and Calnexin (CNX, green) antibodies. DAPI was used to visualize nuclei. Red (PMY) and green (CNX) fluorescences are also shown in grey for better visualization. Scale bars 10 mm.
Movie 1. The movie illustrates the 3D analysis of a representative TGC with Unr granules co-stained with anti-Unr and anti-lamin A/C (LN, red) antibodies, as shown in Fig. 3D.
Movie 2. The movie illustrates the 3D analysis of a representative hepatocyte with Unr granules co-stained with Unr (green) and lamin A/C (LN, red) antibodies, as shown in Fig. 5C.