MicroRNA-1291-mediated silencing of IRE1 α enhances Glypican-3 expression

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ABSTRACT

MicroRNAs (miRNA) are generally described as negative regulators of gene expression. However, some evidence suggests that they may also play positive roles. As such, we reported that miR-1291 leads to a *GPC3* mRNA expression increase in hepatoma cells through a 3' untranslated region (UTR)-dependent mechanism. In the absence of any direct interaction between miR-1291 and *GPC3* mRNA, we hypothesized that miR-1291 could act by silencing a negative regulator of *GPC3* mRNA expression. Based on *in silico* predictions and experimental validation, we demonstrate herein that miR-1291 represses the expression of the mRNA encoding the endoplasmic reticulum (ER)-resident stress sensor IRE1 α by interacting with a specific site located in the 5' UTR. Moreover, we show, *in vitro* and in cultured cells, that IRE1 α cleaves *GPC3* mRNA at a 3' UTR consensus site independently of ER stress, thereby prompting *GPC3* mRNA degradation. Finally, we show that the expression of a miR-1291-resistant form of IRE1 α abrogates the positive effects of miR-1291 on *GPC3* mRNA expression. Collectively, our data demonstrate that miR-1291 is a biologically relevant regulator of *GPC3* expression in hepatoma cells and acts through silencing of the ER stress sensor IRE1 α .

Keywords: microRNA; post-transcriptional up-regulation; *GPC3*; *ERN1*; RIDD; FunREG

INTRODUCTION

MicroRNAs (miRNAs) are endogenous ~22-nt noncoding RNAs which regulate gene expression by controlling target mRNA translation and/or degradation (Bartel 2009; Fabian et al. 2010; Huntzinger and Izaurralde 2011). In most cases, miRNAs act as post-transcriptional repressors of gene expression through mechanisms involving sequence-specific mRNA:miRNA recognition and the regulated binding of miRNA-induced silencing complexes (miRISC) on target 3' untranslated regions (UTRs) (Pillai et al. 2007). In addition, some reports have also demonstrated the existence of miRNA-mediated target induction through molecular processes involving either the direct miRNA:target pairing or an indirect regulation through intermediary factors (Vasudevan 2012). Although the precise mechanisms underlying these phenomena remain elusive, recent evidence has provided some relevant information. For instance, some miRNAs positively regulate gene expression through a direct pairing

depending on the cellular context or the site location (e.g., 5' UTR) (Vasudevan et al. 2007; Orom et al. 2008; Vasudevan 2012). This was well illustrated with miR-10a which binds to ribosomal proteins RPS16, RPS6, and RPL9 encoding mRNA 5' UTR and consequently enhances their translation (Orom et al. 2008). miRNAs have also been implicated in gene up-regulation by targeting promoter elements. This is the case of miR-744 and miR-1186, which induce the transcription of mouse *Cyclin B1* (Huang et al. 2012). Other illustrations of miRNA-dependent gene induction were provided by recent discoveries showing that some miRNAs attenuate nonsense-mediated mRNA decay (NMD) (Bruno et al. 2011) and AU-rich-mediated decay (AMD) (Ma et al. 2010).

In a previous study, we showed that three miRNAs promote Glypican-3 (*GPC3*) expression in hepatoma cells by a mechanism dependent on the *GPC3* 3' UTR (Maurel et al. 2013). *GPC3* belongs to the heparan sulfate proteoglycan family and regulates the signaling pathways mediated by WNTs, Hedgehogs, fibroblast growth factors, and bone morphogenetic proteins (Fransson 2003; Filmus et al. 2008). *GPC3* is a glycosylphosphatidylinositol (GPI) membrane-anchored protein that uses the secretory pathway to reach the plasma membrane. *GPC3* is a gene involved in various human diseases including type 1 Simpson-Golabi-Behmel

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syndrome and Wilms tumors. Moreover, GPC3 is overexpressed in hepatocellular carcinoma (HCC) and hepatoblastoma (Jakubovic and Jothy 2007), in which its expression correlates with tumor aggressiveness and poor prognosis (Shirakawa et al. 2009). To characterize the miRNAs regulating *GPC3* expression in HCC-derived cells, we screened a library of 876 human mature miRNA mimics using the *GPC3* 3' UTR as a bait (Maurel et al. 2013). miR-129-1-3p, miR-1291, and miR-1303 promote the up-regulation of *GPC3* mRNA expression through uncharacterized mechanisms all depending on the *GPC3* 3' UTR. Interestingly, miR-1291 is more particularly up-regulated in HCC subgroups that express high levels of *GPC3* (Maurel et al. 2013).

In the present study, we investigated the molecular mechanisms by which miR-1291 may induce *GPC3* mRNA expression in hepatoma cells. To this end, an integrated approach combining in silico analyses, in vitro, and cell-based validations was undertaken. We demonstrate that miR-1291 represses the expression of the endoplasmic reticulum (ER)-resident endoribonuclease IRE1 α , which itself promotes *GPC3* mRNA decay. The latter regulation occurs through a mechanism which could be related to the regulated IRE1 α -dependent decay (RIDD) of mRNA (Hollien et al. 2009), therefore adding to the repertoire of miRNA-mediated decay mechanisms of repressive protein-associated machineries.

RESULTS

miR-1291 targets an intermediate factor that regulates *GPC3* mRNA expression

At first, to characterize the mechanisms involved in a miR-1291-mediated *GPC3* mRNA expression increase, we used our previously described FunREG fluorescence reporter system in HCC-derived HuH7 cells (Laloo et al. 2009; Maurel et al. 2013). The average number of lentiviral transgene copies per cell [transgene copy number (TCN)] was measured by quantitative PCR in HuH7 cells expressing the eGFP-*GPC3* 3' UTR transgene. Then, the cells were transfected with a mature miR-1291 mimic or a control RNA. Three days later, eGFP protein (P) and mRNA (M) expression levels were determined using FACS and RT-qPCR, respectively. Finally P/TCN, M/TCN, and P/M ratios, which, respectively, correspond to the global post-transcriptional regulation, the mRNA stability, and the translation efficiency, were calculated (Laloo et al. 2009, 2010). As previously reported (Maurel et al. 2013), FunREG ratios (Fig. 1) indicated that miR-1291 enhanced eGFP-*GPC3* 3' UTR expression by ~50%. This effect exclusively resulted from an increased mRNA stability, as the translation efficiency remained unchanged (Fig. 1). Because miR-1291 had no effect on expression of an eGFP transgene bearing a control 3' UTR in HuH7 cells (Maurel et al. 2013), we concluded that miR-1291 stabilizes *GPC3* mRNA through a mechanism involving the 3' UTR.

FIGURE 1. miR-1291 specifically enhances *GPC3* mRNA stability through its 3' UTR. *Top panel:* Schematic representation of the eGFP-*GPC3* 3' UTR transgene used in this study. *Bottom panel:* eGFP-*GPC3* 3' UTR-expressing HuH7 cells were transfected with a control RNA or miR-1291. Three days later, the transgene copy number (TCN) and the expression of eGFP protein (P) and mRNA (M) were measured. Finally, the FunREG ratios were calculated as described in Materials and Methods. (P/TCN) global post-transcriptional regulation, (M/TCN) mRNA stability, (P/M) translation efficiency. (ANOVA: $P < 0.0001$; $n = 5$) (**) $P < 0.01$.

In the absence of any direct interaction between miR-1291 and *GPC3* mRNA, we hypothesized that miR-1291 could act on *GPC3* expression by silencing a negative regulator. Bioinformatic analysis of the 83 miR-1291 targets, predicted using miRWalk (Dweep et al. 2011) and annotated as post-transcriptional regulators using Gene Ontology (Supplemental Fig. S1), revealed only seven candidate genes whose expression products were membrane-bound and only one of them which presented an endoribonuclease activity. A functional clustering analysis of those seven candidates that integrated the Gene Ontology biological process (Supplemental Fig. S1A), specific molecular properties, including the presence of a transmembrane domain or specific RNase activity (Supplemental Fig. S1B), and the Gene Ontology cellular compartment (Supplemental Fig. S1C), which all were parsed through a scoring analysis (Troyanskaya et al. 2003), revealed that *ERN1* branched out from the other candidates and presented the highest overall scores (Fig. 2A). *ERN1* displayed an endogenous RNase activity and represented the best candidate that could directly regulate *GPC3* mRNA stability. *ERN1*, also known as inositol-requiring enzyme 1 α (IRE1 α) is an endoplasmic reticulum-resident transmembrane protein and a site-specific endoribonuclease activated upon accumulation of misfolded protein in this cellular compartment (ER stress). It is a major sensor of the unfolded protein response (UPR), an adaptive mechanism activated upon ER stress (Calton et al. 2002; Schroder and Kaufman 2005). The cytoplasmic endoribonuclease domain of IRE1 α was first described to cleave *XBPI* mRNA in metazoans, yielding to its splicing and the production of an active transcription factor in response to ER stress (Walter and Ron 2011). In addition, Hollien and colleagues showed that, upon ER stress, IRE1 α

FIGURE 2. miR-1291 targets an intermediate factor that regulates *GPC3* mRNA expression. (A) Selection of miR-1291-predicted targets involved in mRNA destabilization. Using miRWalk, 2782 gene candidates were predicted as miR-1291 targets ($P < 0.01$). Among them, 83 are described as post-transcriptional regulators. A hierarchical clustering was performed for seven candidate genes that were identified as relevant to a membranous compartment, namely *ERN1*, *TIRAP*, *BICD1*, *CHERP*, *TLR7*, *SLC11A1*, and *TRMU*. Scale bar is indicated. (B) Identification of three sites homologous to the 5'-CUGCAG-3' IRE1 α consensus cleavage site in *GPC3* mRNA (red). (C) IRE1 α inhibits eGFP-*GPC3* 3' UTR mRNA stability. EGFP-*GPC3* 3' UTR-expressing HuH7 cells were transfected with an empty vector or IRE1 α vector. Three days later, the expression of eGFP mRNA was measured (ANOVA: $P < 0.0001$; $n = 3$). (*) $P < 0.1$. (D) Whereas miR-1291 increases *GPC3* expression, it decreases that of IRE1 α . HuH7 cells were transfected with a control miRNA and miR-1291. The relative expression of IRE1 α and *GPC3* mRNA was measured using RT-qPCR (ANOVA: $P < 0.0001$; $n = 10$). (***) $P < 0.001$. (E) HuH7 cells were transfected with increasing concentrations of anti-miR-1291 (AM1291). The relative expression of IRE1 α and *GPC3* mRNA was measured using RT-qPCR (ANOVA: $P < 0.0001$; $n = 3$). (***) $P < 0.001$.

also induces the decay of mRNAs encoding membrane and secreted proteins in fly and mammalian cells through the RIDD pathway (Hollien and Weissman 2006; Hollien et al. 2009; Gaddam et al. 2013). *GPC3* corresponds to the latter criteria as this GPI-membrane-anchored protein uses the secretory pathway to reach its final destination, i.e., the plasma membrane. Interestingly, three sites homologous to the 5'-CUGCAG-3' IRE1 α consensus cleavage site previously defined by Oikawa and colleagues (Oikawa et al. 2010) are present in *GPC3* mRNA, including one in the 3' UTR (Fig. 2B). Moreover, overexpression of IRE1 α in HuH7 cells significantly decreased eGFP-*GPC3* 3' UTR-mRNA stability (Fig. 2C), and this recombinant mRNA was found associated to the ER cytosolic surface (Supplemental Fig. S2), as previously observed for other cytosolic protein-encoding mRNAs (Lerner et al. 2003; Pyhtila et al. 2008). These observations led us to hypothesize that miR-1291 might target IRE1 α expression, thereby attenuating its IRE1 α endoribonuclease activity toward *GPC3* mRNA.

Based on the above data, we tested the impact of miR-1291 on IRE1 α and *GPC3* mRNA expression in HuH7 cells. Overexpression of miR-1291 led to down-regulation of IRE1 α mRNA expression by ~40% (Fig. 2D). The amplitude of this effect was consistent with that observed for most other functional miRNAs (Avraham and Yarden 2012) and was accompanied by the simultaneous increase in *GPC3* mRNA (Fig. 2D). Transfection of HuH7 cells with increasing amounts of a miR-1291 inhibitor (AM1291) led to an increase in endogenous IRE1 α mRNA expression and to a decrease of endogenous *GPC3* mRNA expression at concentrations above 90 nM (Fig. 2E). At lower concentrations (i.e., 12 to 30 nM) (Fig. 2E; Supplemental Fig. S3A), AM1291 efficiently depleted miR-1291 in HuH7 cells (Supplemental Fig. S3A). However, because the expression of miR-1291 in HuH7 cells is about 500 times higher than that of its IRE1 α mRNA target (Supplemental Fig. S3B), these concentrations were most likely too low to impact the regulatory effects of miR-1291 on the expression of IRE1 α and *GPC3* mRNAs. Collectively, these results indicate that miR-1291 regulates IRE1 α expression and support the first part of our hypothesis.

IRE1 α is a direct target of miR-1291

miRWalk (Dweep et al. 2011) predicted that IRE1 α mRNA contains a site pairing with miR-1291 in its 5' UTR, a miRNA:mRNA interaction that was further supported using RNAhybrid (Fig. 3A; Rehmsmeier et al. 2004). Using eGFP-expressing HuH7 cells bearing the IRE1 α 5' UTR (Fig. 3B), we found that miR-1291 decreased eGFP expression by ~50%, whereas AM1291 induced it (Fig. 3C). Moreover, deletion of the sequence complementary to the miR-1291 seed within the IRE1 α 5' UTR abolished the observed miR-1291-mediated regulation (Fig. 3C). Using FunREG, we further demonstrated that miR-1291 post-transcriptionally controlled IRE1 α expression by destabilizing its mRNA with no effect on translation (Fig. 3D). These data confirmed that miR-1291 regulates IRE1 α expression by targeting its 5' UTR and induces IRE1 α mRNA degradation through a classical miRNA-seed recognition process.

Our initial hypothesis raised the possibility that miR-1291 positively controls *GPC3* by repressing the expression of a negative regulator of *GPC3* mRNA stability. Our results

FIGURE 3. miR-1291 targets and destabilizes *IRE1 α* mRNA through its 5' UTR. (A) *IRE1 α* 5' UTR contains a potential miR-1291 binding site. Schematic representation of miR-1291/*IRE1 α* 5' UTR interaction using RNAhybrid. (B) *Top panel:* Schematic representation of *IRE1 α* 5' UTR-eGFP transgene. *Bottom panel:* Schematic representation of miR-1291 pairing with *IRE1 α* 5' UTR in its wild-type or mutated form. (C) HuH7 cells expressing the indicated transgenes were transfected with the indicated small RNAs. Three days later, eGFP protein expression was measured (ANOVA: $P < 0.0001$; $n = 3$). (***) $P < 0.001$. (D) HuH7 cells expressing the WT-*IRE1 α* 5' UTR-eGFP transgene were transfected with a control RNA or miR-1291. After 3 d, FunREG ratios were calculated as described in Figure 1 (ANOVA: $P < 0.0001$; $n = 4$). (***) $P < 0.001$. (E) Overexpression of *IRE1 α* lacking its mRNA 5' UTR counteracts the miR-1291-mediated increase in *GPC3* mRNA. HuH7 cells were transfected with the indicated plasmid and small RNA. Then, *GPC3* mRNA expression was measured using RT-qPCR (ANOVA: $P < 0.0001$; $n = 5$). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

pointed toward *IRE1 α* as being this protein. In this context, it would be predicted that the expression of a miR-1291-resistant form of *IRE1 α* mRNA (i.e., devoid of its 5' UTR) would prevent miR-1291-mediated *GPC3* mRNA up-regulation. As anticipated, overexpression of an *IRE1 α* transgene lacking its 5' UTR in HuH7 cells (Supplemental Fig. S4A) inhibited miR-1291-mediated up-regulation of *GPC3* mRNA expression and led to a decrease in *GPC3* mRNA expression, thereby confirming the functional relationship among those three genes (Fig. 3E).

IRE1 α regulates *GPC3* mRNA expression and stability

To further investigate the relationship between *IRE1 α* and *GPC3*, *IRE1 α* expression was silenced in HuH7 cells, and the cells were then exposed or not exposed to ER stress. Both dithiothreitol (DTT), which reduces disulfide bonds, and tunicamycin (Tun), an N-linked glycosylation inhibitor, were used to induce ER stress and to activate *IRE1 α* (Schroder and Kaufman 2005). Both drugs induced *XBPI* mRNA splicing, and these effects were attenuated in *IRE1 α* -silenced

cells, thus confirming the activation of the *IRE1 α* /*XBPI* signaling axis upon ER stress in our experimental system (Fig. 4A). Under the same experimental conditions, miR-1291 expression remained stable (Supplemental Fig. S5). Interestingly, *GPC3* expression increased at both mRNA (Fig. 4B) and protein (Fig. 4C) levels upon attenuation of *IRE1 α* expression, and this occurred independently of ER stress. HuH7 cells were then transfected with miR-1291 to evaluate the effects of miR-1291 toward the *IRE1 α* arm of the UPR. At first, the impact of miR-1291 was evaluated on the expression of *HERPUD*, *GRP94*, and *CHOP* mRNAs, genes whose transcription is, respectively, increased downstream from the ATF6 and PERK arms of the UPR upon ER stress (Schroder and Kaufman 2005). In parallel, we monitored *IRE1 α* -mediated *XBPI* mRNA splicing and the RIDD activity under the same conditions. As shown in Figure 5, miR-1291 had no influence on the signaling of the ATF6 (Fig. 5A) and PERK (Fig. 5B) arms of the UPR. Indeed, *HERPUD*, *GRP94*, and *CHOP* mRNA expression was not affected by a variation of miR-1291 expression under basal or ER stress conditions. In contrast, miR-1291 specifically affected *IRE1 α*

endoribonuclease activity as shown by the attenuation of *XBPI* mRNA splicing upon ER stress and by the inhibition of the RIDD activity as assessed with the expression of the well-characterized RIDD substrates *PDGFRB*, *COL6A1*, and *SCARA3* mRNAs (Fig. 5C). Using actinomycin D to inhibit transcription, we further demonstrated that *IRE1 α* silencing led to *GPC3* mRNA stabilization (Fig. 6A). To test whether *GPC3* mRNA is a genuine stress-independent target of *IRE1 α* , we measured the amount of *GPC3* mRNA in control or stressed cells in the presence or absence of actinomycin D. *GPC3* mRNA expression was similar in control and stressed cells in the presence of actinomycin D, thereby suggesting an absence of a transcriptional compensatory mechanism that could increase *GPC3* mRNA levels upon ER stress (Fig. 6B). Therefore, in our conditions, *GPC3* mRNA appears not to be a target of stress-activated *IRE1 α* . Interestingly, the analysis of *SCARA3* mRNA revealed that, although the cleavage of this RIDD substrate was enhanced upon ER stress, it was also observed under basal conditions (Fig. 5C). For the other RIDD substrates tested, *PDGFRB* and *COL6A1* mRNAs were selectively cleaved upon ER stress (Fig. 5C). These data demonstrate that *IRE1 α* controls *GPC3* mRNA stability

FIGURE 4. IRE1 α -silencing increases *GPC3* mRNA independently of ER stress. (A) IRE1 α mediates *XBP1* mRNA splicing upon ER stress. ER stress-induced splicing of *XBP1* mRNA yields a transcript with a 26-nt deletion in comparison to the unspliced transcript. HuH7 cells transfected with the indicated siRNAs were exposed to dithiothreitol (DTT) for 6 h or to tunicamycin (Tun) for 24 h. Then, *XBP1* mRNA splicing was monitored by RT-PCR. (B,C) IRE1 α -silencing increases *GPC3* expression. HuH7 cells were transfected with the indicated small RNA and then treated or not treated with DTT or Tun. Three days later, mRNA and protein expression (ANOVA: $P < 0.0001$; $n = 8$) was measured by qPCR (B) and Western blotting (C). GPC3 and nonglycosylated GPC3 (ngGPC3) proteins were as shown. (***) $P < 0.001$.

independently of ER stress and that miR-1291 selectively targets the IRE1 α arm of the UPR.

IRE1 α cleaves *GPC3* through a canonical site located in its 3' UTR

As mentioned above, *GPC3* mRNA sequence contains three sites homologous to the 5'-CUGCAG-3' IRE1 α consensus cleavage site and similar to those found in *XBP1* mRNA (Oikawa et al. 2010). In addition, these three sites form P-loop structures, thereby yielding potential cleavage sites for IRE1 α (Fig. 7A; Oikawa et al. 2010). To test whether IRE1 α cleaves *GPC3* mRNA and to identify these sites, total RNA from HuH7 cells was subjected to an in vitro IRE1 α -mediated cleavage assay (Bouchecareilh et al. 2010). RT-PCR was then carried out using primers flanking the three predicted consensus sites. Interestingly, only one site among the three predicted appeared sensitive to IRE1 α endoribonuclease activity in vitro (Fig. 7B). Indeed, the amount of RT-PCR product corresponding to the site located at nucleotides 2039/40 in the *GPC3* mRNA 3' UTR decreased by ~60%, whereas the amount of the four other *GPC3* fragments, as well as

the control *GAPDH* mRNA, did not change. This suggests that *GPC3* mRNA contains only one functional in vitro IRE1 α -mediated cleavage site located in its 3' UTR. To further demonstrate the relevance of this cleavage site, in vitro transcribed *GPC3* mRNA and its mutant counterpart deleted for the 2039/40 site were subjected to IRE1 α -mediated cleavage. In a model where IRE1 α would cleave *GPC3* mRNA at the 2039/40 site (Fig. 7C), two RNA cleavage products of 1700 nt and 300 nt, respectively, would be produced. As

FIGURE 5. miR-1291 specifically alters IRE1 α signaling independently of ER stress. (A,B) Overexpression of miR-1291 did not affect ATF6 and PERK signaling upon Tun treatment. HuH7 cells transfected with the indicated small RNA were exposed to Tun (24 h). (A) The relative expression of *HERPUD* and *GRP94* mRNAs was measured using RT-qPCR (ANOVA: $P < 0.0001$; $n = 5$). (*) $P < 0.05$. (B) *CHOP* mRNA expression was measured (ANOVA: $P < 0.0001$; $n = 5$). (***) $P < 0.001$. (C) miR-1291 selectively affects IRE1 α signaling. *Top* panel: HuH7 cells transfected with the indicated small RNA were exposed to DTT (6 h). Then, *XBP1* mRNA splicing was monitored. The results are representative of five independent experiments. *Bottom* panel: HuH7 cells transfected with the indicated small RNA were exposed to Tun (24 h). Then, the relative expression of *PDGFRB*, *COL1*, and *SCARA3* mRNAs was measured (ANOVA: $P < 0.0001$; $n = 5$). (**) $P < 0.01$, (***) $P < 0.001$.

FIGURE 6. IRE1 α -silencing leads to *GPC3* mRNA stabilization. (A) HuH7 cells were transfected with the indicated small RNA. Three days later, *GPC3* and *ALB* mRNA were measured at different times following transcription inhibition using actinomycin D (ANOVA: $P < 0.0001$; $n = 3$). (*) $P < 0.05$, (**) $P < 0.01$. (B) HuH7 cells were exposed or not exposed to Tun (24 h). Then, *GPC3* expression was measured following transcription inhibition using actinomycin D or not (ANOVA: $P < 0.0001$; $n = 5$).

anticipated, cleavage of wild-type *GPC3* mRNA generated two RNA fragments of the expected size (Fig. 7D, left). In contrast, deletion of the 2039/40 site in *GPC3* mRNA abrogated IRE1 α -mediated cleavage (Fig. 7D, right). These results demonstrated that, in vitro, IRE1 α cleaves *GPC3* in its 3' UTR at position 2039/40.

To test the physiological relevance of this observation, we evaluated the capacity of IRE1 α to cleave *GPC3* mRNA in cultured cells. Using targeted RT-qPCR (Iqbal et al. 2008), the amount of each *GPC3* mRNA fragment was measured in HuH7 cells upon siRNA-mediated silencing of 5'–3' exonucleases *XRN1/2* or RNA helicase *SKI2*, the latter mediating the 3'–5' degradation of mRNA through the cytoplasmic exosome (Fig. 8A). Compared to the control and as expected, *SKI2* silencing led to the accumulation of the *GPC3* mRNA fragment upstream of IRE1 α cleavage (Fragment 1) with no effect on the amount of the downstream fragment (Fragment 2) (Fig. 8B). In contrast, the silencing of *XRN1* or *XRN2* led to an increase in Fragment 2, with no effect on the amount of Fragment 1 (Fig. 8B). Therefore, the in vitro cleavage site present in *GPC3* mRNA also exists in cells. This result was further reinforced as IRE1 α silencing led to an increase of all fragments in all conditions, due to the absence of *GPC3* mRNA cleavage (Fig. 8B). To further assess whether miR-1291 up-regulates *GPC3* mRNA through the repression of IRE1 α expression and the subsequent decrease in RNase

activity, HuH7 cells were transfected with miR-1291 in the presence or absence of siSKI2. IRE1 α mRNA, *GPC3* mRNA Fragment 1, and the amplicon framing the IRE1 α cleavage site were then quantified (Fig. 8C,D; Supplemental Fig. S4B). As expected, miR-1291 down-regulated IRE1 α mRNA expression (Supplemental Fig. S4B) and subsequently up-regulated *GPC3* mRNA (Fig. 8C,D). *SKI2* silencing further increased the amount of Fragment 1 (Fig. 8C) but had no impact on IRE1 α mRNA expression (Supplemental Fig. S4B). Quantification of the amplicon framing the cleavage site revealed that cleavage was reduced upon transfection with miR-1291 both in the presence or absence of *SKI2* and that *SKI2* silencing alone did not impact on *GPC3* mRNA cleavage (Fig. 8D). Hence, our results demonstrated that miR-1291 targets IRE1 α through its 5' UTR, thereby attenuating IRE1 α -mediated decay of *GPC3* mRNA and consequently leading to increased *GPC3* expression in hepatoma cells.

DISCUSSION

In this work, we characterized the molecular mechanism by which miR-1291 post-transcriptionally controls the *GPC3* mRNA expression increase (Fig. 9). Using an integrated approach combining both in silico analyses and in vitro/cell-based validation, we report that miR-1291 binds to the IRE1 α 5' UTR, thus destabilizing its mRNA and leading to its degradation. We also demonstrate the presence of a functional IRE1 α cleavage site in the *GPC3* mRNA 3' UTR, thereby indicating that *GPC3* mRNA is a genuine RIDD substrate. Collectively, our data show that miR-1291 up-regulates *GPC3* mRNA expression by down-regulating IRE1 α mRNA expression. Therefore, our results point toward a novel miRNA-dependent gene expression control mechanism through IRE1 α silencing and RIDD attenuation.

miRNAs generally act as negative regulators of gene expression (Bartel 2009). However, some were also shown to act positively (Vasudevan 2012). Using a functional miRNA screening, we recently identified three miRNAs enhancing *GPC3* expression through a 3' UTR-dependent mechanism including miR-1291 (Maurel et al. 2013). Little is known about the functional properties of miR-1291 in cells. miRNA loci are located in different regions of the genome. Half are intragenic and encoded by protein-coding genes or noncoding RNA genes. This is the case with miR-1291, which is localized in the *SNORA34* gene (Scott et al. 2009; Brameier et al. 2011). miR-1291 expression is down-regulated in renal cell carcinoma (Hidaka et al. 2012) and in the peripheral blood of acute myocardial infarction patients (Meder et al. 2011). However, no functional or pathophysiological roles have been associated with these observations. As there is no predicted miR-1291 binding site on *GPC3* mRNA, we hypothesized that miR-1291 could enhance *GPC3* expression by an indirect mechanism involving an intermediate regulatory factor recognizing *GPC3* mRNA 3' UTR. Such a mechanism has, for instance, been illustrated with the targeting of

FIGURE 7. IRE1 α cleaves *GPC3* mRNA in vitro at a canonical site located in its 3' UTR. (A) Three potential IRE1 α -mediated cleavage sites were identified in *GPC3* mRNA. *Top*: Sequence alignment of IRE1 α cleavage sites in *XBP1* and *GPC3* mRNAs. *Bottom*: Two-dimensional M-fold RNA modeling of potential IRE1 α cleavage sites in *GPC3* mRNA. (B) IRE1 α cleaves *GPC3* mRNA within its 3' UTR in vitro. Total RNA extracted from HuH7 cells was incubated with heat-inactivated or not GST-IRE1 α^{cyto} . IRE1 α -mediated cleavage of *GPC3* mRNA was monitored by PCR at the indicated site. (C,D) IRE1 α directly targets the 2039/40 cleavage site in the *GPC3* 3' UTR. (C) Expected *GPC3* mRNA products following IRE1 α -mediated cleavage at the 2039/40 site. (D) In vitro-transcribed wild-type and IRE1 α -site-deleted *GPC3* RNAs were incubated with or without heat-inactivated or not GST-IRE1 α^{cyto} in the presence or absence of RNase H. Resulting reaction products were resolved on denaturing agarose gels.

genes involved in NMD (Bruno et al. 2011) or in the CCR4-NOT complex (Behm-Ansmant et al. 2006).

As one miRNA can target hundreds of different mRNAs, it is likely that many negative regulatory intermediates might be the target of miR-1291 to control *GPC3* expression. To restrict the number of candidates obtained in silico to an experimentally testable set, we focused on regulatory factors involved in RNA catabolism and likely acting on a sequence-specific site recognition basis. In silico data pointed toward IRE1 α as a potential candidate, as this gene fulfilled the above-mentioned criteria (Fig. 2A; Supplemental Fig. S1). The experimental validation revealed that IRE1 α was a direct target of miR-1291 (Fig. 3). Remarkably, miR-1291 specifically paired with a site located in the IRE1 α 5' UTR (Fig. 3). miRNA:5' UTR target interactions are currently poorly described, likely due to the fact that most of the current bioinformatics tools generate predictions on 3' UTR sequence and not on the full mRNA sequence, which, therefore, may introduce a prediction bias. In contrast to most available tools, miRWalk produces information on miRNA-targets interactions gathered on the complete mRNA sequence as well as on the gene promoter (Dweep et al. 2011). Finally, the identification of human miR-1291 orthologs in other mammalian species using miRBase (Griffiths-Jones et al. 2008; Kozomara and Griffiths-Jones 2011), together with the conservation of their binding sites in IRE1 α 5'

UTRs and the conserved IRE1 α target site in the 3' UTR of *GPC3* mRNA in several species, suggests the existence of a general regulatory mechanism in mammals (Supplemental Fig. S6).

In the past two years, several studies have identified miRNAs whose expression is regulated upon ER stress. Each one of the UPR sensors relays information on the protein folding status from the ER lumen to the nucleus, thereby controlling gene expression. The PERK branch was described to control the expression of miR-708 (Behrman et al. 2011), miR-106b-25 (Gupta et al. 2012), miR-30c-2* (Byrd et al. 2012), and miR-211 (Chitnis et al. 2012). In the same way, ATF6 signaling down-regulates the expression of miR-455 (Belmont et al. 2012), and IRE1 α signaling increases that of miR-346 (Bartoszewski et al. 2011). Recently, miR-122, the most abundant miRNA in the liver, whose expression is repressed in HCC, was found to inhibit CDK4, which interacts and induces accumulation of PSMD10, a proteasome component and an enhancer of the UPR (Yang et al. 2011). Herein, we describe

for the first time a miRNA acting as an upstream inhibitor of the UPR pathway by directly targeting IRE1 α expression. Intriguingly, this regulation did not depend on ER stress. As the same IRE1 α -dependent cleavage under unstressed conditions was also observed for *SCARA3* mRNA (this study) and *SPARC* mRNA (Dejeans et al. 2012), one might suggest potential roles for UPR signaling components in nonstressed conditions as also recently described for IRE1 and PERK (Tam et al. 2012).

Fine-tuning of the UPR plays a fundamental role in cancer cell fate decisions by determining adaptation and survival to ER stress and eliminating irreversibly damaged cells (Moenner et al. 2007; Tabas and Ron 2011; Woehlbier and Hetz 2011). In this context, IRE1 α has evolved a dual function to preserve ER homeostasis (Han et al. 2009). First, the IRE1 α /XBP1 axis is thought to favor tumor cell adaptation to stress by increasing the ability of these cells to synthesize and fold large amounts of transmembrane and secreted proteins. Second, prolonged activation of the RIDD pathway might decrease tumor growth in a cell-specific manner by degrading mRNAs encoding pro-oncogenic proteins, including PDGFR and SPARC (Hollien and Weissman 2006; Dejeans et al. 2012). In the present study, we identify *GPC3* mRNA as a novel RIDD substrate and demonstrate its cleavage by IRE1 α endoribonuclease at a consensus site located in *GPC3* mRNA 3' UTR (Figs. 7, 8). *GPC3* is known to promote

FIGURE 8. IRE1 α cleaves *GPC3* mRNA in cultured cells. (A) Schematic representation of the expected *GPC3* mRNA products following IRE1 α -mediated cleavage and their subsequent regulation by the exonucleases XRN1 and XRN2, or the RNA helicase *SKI2*. (B) HuH7 cells were transfected with the indicated small RNAs. Three days later, the presence of the *GPC3* mRNA Fragment 1 and 2 was monitored using RT-qPCR (ANOVA: $P < 0.0001$; $n = 6$). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. (C,D) HuH7 cells were transfected with the indicated small RNAs. Three days later, *GPC3* fragment 1 (C) and the *GPC3* amplicon framing the IRE1 α cleavage site (D) were monitored using RT-qPCR (ANOVA: $P < 0.0001$; $n = 3$). (*) $P < 0.05$, (**) $P < 0.01$.

HCC cell growth by stimulating the WNT/ β -catenin pathway (Capurro et al. 2005). Consequently, our results suggest that miR-1291 could act as an oncomiR by attenuating IRE1 α expression and RIDD, thereby leading to *GPC3* overexpression in liver tumors. This hypothesis should, however, be considered carefully as, in the meantime, miR-1291-mediated attenuation of IRE1 α expression would also reduce *XBPI* mRNA splicing. The integrated signaling outcomes resulting from this initial event would, therefore, determine the oncogenic potential of miR-1291. Finally, although it is currently believed that IRE1 α activation plays an instrumental role in tumor progression (Auf et al. 2010; Dejeans et al. 2012), somatic driver mutations in the IRE1 α gene were identified in cancers (Greenman et al. 2007) and recently associated with a loss-of-signaling function of this molecule (Xue et al. 2011), thereby also associating IRE1 α inactivation with cell transformation. This might be consistent with the

hypothesis of a pro-oncogenic miR-1291-mediated attenuation of IRE1 α signaling.

In conclusion, we report a novel mechanism of miRNA-mediated positive regulation of gene expression originating from the silencing of the endoribonuclease IRE1 α (Fig. 9). The physiological and pathophysiological consequences of such a mechanism still remain to be fully investigated, especially in liver cancers in which *GPC3* overexpression plays a master regulatory role. However, one can easily anticipate a significant contribution of IRE1 α down-regulation to cancer development through the overexpression of cancer-associated downstream gene targets.

MATERIALS AND METHODS

Plasmids and cloning

The pL-eGFP-*GPC3* 3' UTR lentiviral and pED-IRE1 α plasmids were as described previously (Nguyen et al. 2004; Maurel et al. 2013). The pEF-h*GPC3* plasmid was kindly provided by S. Mizushima and S. Nagata (Osaka Bioscience Institute, Japan) (Mizushima and Nagata 1990). The pcDNA3.1- Δ CUACAG-h*GPC3* was obtained as follows: A mutant *GPC3* gene devoid of the IRE1 α cleavage site was synthesized (Eurofins MWG Operon), digested by NotI, and cloned into the pcDNA3.1 vector (Invitrogen). The pL-wt-IRE1 α 5'-UTR-eGFP and pL-mut-IRE1 α 5'-UTR-eGFP plasmids were constructed as follows: IRE1 α 5' UTR

FIGURE 9. miR-1291 up-regulates *GPC3* through inhibition of IRE1 α expression. Schematic representation of miR-1291-mediated *GPC3* up-regulation through inhibition of IRE1 α expression. The gray frame indicates the original observation that miR-1291 up-regulates *GPC3* mRNA expression through its 3' UTR (Maurel et al. 2013). The green dashed arrow indicates a positive and indirect regulation of *GPC3* mRNA expression. Red signs indicate negative and direct regulations.

was amplified using 5'-CACGGATCCTGCCTAGTCAGTTCTGCG TC as the forward primer and either 5'-CACGGATCCGGCGAGG ACTCGGCCCT or 5'-CACGGATCCGGCGAGGACTCCGGCGGT GGCTCCGGGGG as the reverse primer, respectively. Each PCR product was digested by BamHI and sub-cloned into the pL-eGFP plasmid (Maurel et al. 2013). Sequence was verified by DNA sequencing.

Cell culture

HuH7 cells were grown in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (1000 units/mL). Enhanced green fluorescent protein (eGFP)-expressing HuH7 cells were established using lentiviral transduction (multiplicity of infection = 3) as previously described (Laloo et al. 2009; Jalvy-Delvaile et al. 2012). Tunicamycin (Tun; 10 µg/mL) was from Calbiochem (EMD Biosciences Inc.). Dithiothreitol (DTT; 2 mM) and actinomycin D (5 µg/mL) were obtained from Sigma.

Small RNA transfection

Small inhibitory RNAs (siRNAs) (Supplemental Table S1) were designed using Greg Hannon's web tool (<http://hannonlab.cshl.edu/>). The negative control RNA, mature miRNA mimics, and hairpin miRNA inhibitors were from Qiagen. Unless otherwise stated, small RNAs were transfected into the target cells at 12 nM by reverse-transfection using Lipofectamine RNAi Max (Invitrogen) following the manufacturer's instructions.

RNA isolation, reverse transcription, and qPCR analyses

Total RNA was prepared using the TRI Reagent (Sigma) following the manufacturer's instructions. Mature miRNA expression was quantified using the TaqMan microRNA Reverse Transcription Kit and TaqMan microRNA assays (Applied Biosystems). Messenger RNA expression was quantified using the SYBR Green Supermix (Quanta Biosciences). Quantitative PCR reactions were performed using the Step One Plus Quantitative PCR System (Applied Biosystems). For each data point, experiments were performed in triplicate. In all cases, each sample was normalized toward the expression of the 18S ribosomal RNA. Absolute quantification was performed using a standard curve-based approach. A standard curve was obtained using serial dilutions of plasmid or miRNA of known concentrations on a 10-fold basis. The copy number was calculated as follows: $X \text{ g/mol} / \text{Avogadro's number} = X \text{ g/molecule}$. Standard curves were obtained by plotting the crossing threshold (Ct) against the log number of molecules. The equation drawn from the graph was used to calculate the precise number of target molecules tested in the same reaction plate as the standard as well as in the sample. PCR products were resolved on 1% agarose TBE 0.5× electrophoresis gels or 4% for the *XBPI* splicing experiments. Primers used are as described in Supplemental Table S2. For ER-associated RNA enrichment, an immunoprecipitation approach was undertaken as previously described (Nguyen et al. 2004). Briefly, HuH7 cells were homogenized using a Teflon potter in the presence of RNasin, and homogenates were clarified by centrifugation at 1500g for 10 min. Clarified homogenates were incubated in the presence of anti-calnexin antibodies for 2 h, and then magnetic beads were added for an additional 20 min at 4°C. Beads were collected using a magnet and washed

extensively. Purified ER membranes were then treated with Trizol, RNA extracted, and reverse-transcribed.

Antibodies and Western blot analyses

Antibody against GPC3 was from Biomosaics (Burlington). Anti-IRE1α and anti-GAPDH were from Santa-Cruz Biotechnology. Protein extraction and Western blotting were performed as previously described (Jalvy-Delvaile et al. 2012). Signals were normalized to the amount of the housekeeping protein GAPDH.

FunREG analyses

FunREG analyses were performed 3 d after cell transfection as previously described (Laloo et al. 2009; Maurel et al. 2013). Cells were washed with PBS, detached with trypsin/EDTA, collected, and analyzed by FACS using the BD LSRFortessa cell analyzer and the BD FACSDiva software (BD Biosciences). In parallel, genomic DNA and total RNA were extracted by TRI Reagent (see above) from each cell population. Quantitative RT-PCR was performed as described above. The transgene copy number was measured by quantitative PCR using genomic DNA as described previously (Laloo et al. 2009; Jalvy-Delvaile et al. 2012).

Bioinformatic analyses

In silico analyses were performed using miRWalk (Dweep et al. 2011). This program identifies the longest consecutive complementarity between miRNA and gene sequences, produces information about miRNA:target interactions on the complete gene sequence (promoter, 5' UTR, coding sequence, and 3' UTR) of all known genes and compares the candidate miRNA binding sites with those established by eight miRNA-target prediction programs (i.e., DIANA-microT, miRanda, miRDB, PicTar, PITA, RNA22, RNAhybrid, and TargetScan/TargetScanS). Finally, miRWalk incorporates all the predicted miRNA binding sites produced by the miRWalk algorithm and the eight other programs into a relational database. The secondary structure of the *GPC3* mRNA:miR-1291 interaction was predicted using M-FOLD (Zuker et al. 1999). Functional classification was achieved by g:profiler (Reimand et al. 2007). Generated data were also manually sorted and integrated to generate quantitative and qualitative information on the Gene Ontology (cellular compartment and biological process components). Moreover, an additional piece of information concerning candidate miR-1291 targets was retrieved from databases (GeneCard, NCBI, miRBase, miRDB, HNGC) and from the literature to constitute a binary (No = 0; Yes = 1) annotation file. A scoring system was established based on the methodology previously described for integration of heterogeneous data for gene function prediction (Troyanskaya et al. 2003). All these data were then clustered and represented using the CLUSTER and TREEVIEW programs (Hoon et al. 2004; Saldanha 2004). All the clusters were built using Euclidian distances. Trees were generated using average linkage.

RNA cleavage assay

Ten µg of total RNA extracted from HuH7 were incubated at 37°C with the cytoplasmic domain of human IRE1α (5 µg) fused to GST

(GST-IRE1 α^{cyto}) for 4 h in a buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 mM MgCl₂, and 1 mM ATP, as previously described (Bouchecareilh et al. 2010, 2011). Heat-denatured GST-IRE1 α^{cyto} was used as a control. RNA fragments were detected by RT-PCR using specific primers (Supplemental Table S2). The pcDNA3.1- Δ CUACAG-hGPC3 and pEF-hGPC3 plasmids were used as templates for in vitro RNA transcription using the T7 polymerase (Promega). In vitro transcribed RNA was incubated at 37°C with 5 μ g of GST-IRE1 α^{cyto} and 1 mM ATP for 4 h. Reaction products were then denatured for 10 min at 65°C in an RNA sample buffer (56% formamide, 37% formaldehyde, 7% MOPS). Fragments resulting from the enzymatic reaction were resolved on 1% formaldehyde agarose gels and visualized by UV transillumination.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 software. Data are presented as the mean of the indicated number of independent experiments $\pm$ standard deviation (SD). When the experiment contained three groups of values or more, regular one-way analysis of variance (ANOVA) was used for the comparison of multiple means. Means were considered significantly different when $P < 0.05$. The ANOVA test was followed by a Bonferroni's multiple-comparison post-test, and selected pairs of data were compared. Significant variations are indicated with asterisks.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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