ZBP1 regulates mRNA stability during cellular stress

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An essential constituent of the integrated stress response (ISR) is a reversible translational suppression. This mRNA silencing occurs in distinct cytoplasmic foci called stress granules (SGs), which transiently associate with processing bodies (PBs), typically serving as mRNA decay centers. How mRNAs are protected from degradation in these structures remains elusive. We identify that Zipcode-binding protein 1 (ZBP1) regulates the cytoplasmic fate of specific mRNAs in nonstressed cells and is a key regulator of mRNA turnover during the ISR. ZBP1 association with target mRNAs in SGs was not essential for mRNA targeting to SGs. However, ZBP1 knockdown induced a selective destabilization of target mRNAs during the ISR, whereas forced expression increased mRNA stability. Our results indicate that although targeting of mRNAs to SGs is nonspecific, the stabilization of mRNAs during cellular stress requires specific protein–mRNA interactions. These retain mRNAs in SGs and prevent premature decay in PBs. Hence, mRNA-binding proteins are essential for translational adaptation during cellular stress by modulating mRNA turnover.

Introduction

In response to environmental stress, cells reprogram their translational machinery and sort mRNAs that are released from polysomes to stress granules (SGs; Anderson and Kedersha, 2002; Kedersha et al., 2002). This translational arrest is initiated by the phosphorylation of the translation initiation factor eIF2α, which results in a limited availability of the eIF2–GTP–tRNA^{Met} complex (Kedersha et al., 1999, 2002). It is presumed that by this limiting of translation initiation, mRNAs become stalled in 48S complexes, which then aggregate in SGs (Anderson and Kedersha, 2006). Thus far, there is no evidence for a decay of mRNAs within SGs. Hence, by temporal "storage" of mRNAs in SGs, transcripts can be protected from decay via the exosome or from degradation in processing bodies (PBs) to provide a reservoir of silenced mRNAs available for resuming translation upon stress release (Decker and Parker, 2002; Meyer et al., 2004; Anderson and Kedersha, 2006). Recent studies identified an association of SGs with PBs, suggesting that mRNAs can become reprogrammed for further processing, including decay

in PBs (Kedersha et al., 2005). This model is supported by SG recruitment of several mRNA-binding proteins (RBPs), including TIA-1, TIAR, FMRP, Staufen, and CPEB, which, in nonstressed cells, regulate mRNA translation and/or mRNA turnover (Kedersha et al., 1999; Mazroui et al., 2002; Thomas et al., 2005; Wilczynska et al., 2005).

In recent studies, we have investigated the role of the Zipcode-binding protein (ZBP) family in posttranscriptional gene regulation. This family of oncofetal proteins comprises a group of three RBPs (Imp1-3 in human) that modulate the localization, translation or stability of their target mRNAs (Yisraeli, 2005). In primary neurons, ZBP1 regulates localized translation of the β-actin mRNA in growth cones under control of Src-family kinases (Huttelmaier et al., 2005). Translational control via ZBP proteins has also been identified for the insulin-like growth factor (IGF)-II mRNA (Nielsen et al., 1999; Liao et al., 2004). Besides the control of mRNA localization and translation, ZBP1 (CRD-BP in mouse) was identified as a regulator of c-Myc, CD44, and βTrCP1 mRNA stability (Leeds et al., 1997; Noubissi et al., 2006; Vikesaa et al., 2006). Although, to date, various RBPs have been identified as SG components in different cell lines, little is known about the requirement of these trans-acting factors for mRNA-specific processing in SGs (Anderson and Kedersha, 2006). We identify ZBP1 as a novel SG component that modulates the turnover of its target mRNAs during the integrated stress response (ISR).

Supplemental Material can be found at:
http://www.jcb.org/cgi/content/full/jcb.200608071/DC1

A

Merge
(ZBP, DAPI)   **TIAR**   **Merge**
(ZBP, TIAR)

anti-ZBP

GFP-ZBP1

arsenate

B

Merge
(ZBP, DAPI)   **TIAR**   **Merge**
(ZBP, TIAR)

anti-ZBP

heat shock

C

Merge
(G3BP, DAPI)   **ZBP**   **Merge**
(G3BP, ZBP)

anti-ZBP

G3BP

D

Merge
(ZBP, DAPI)   **RFP-DCP1**   **Merge**
(ZBP, DCP1)

anti-ZBP   arsenate

anti-ZBP   wo arsenate

Merge
(ZBP, DAPI)   **anti-DCP2**   **Merge**
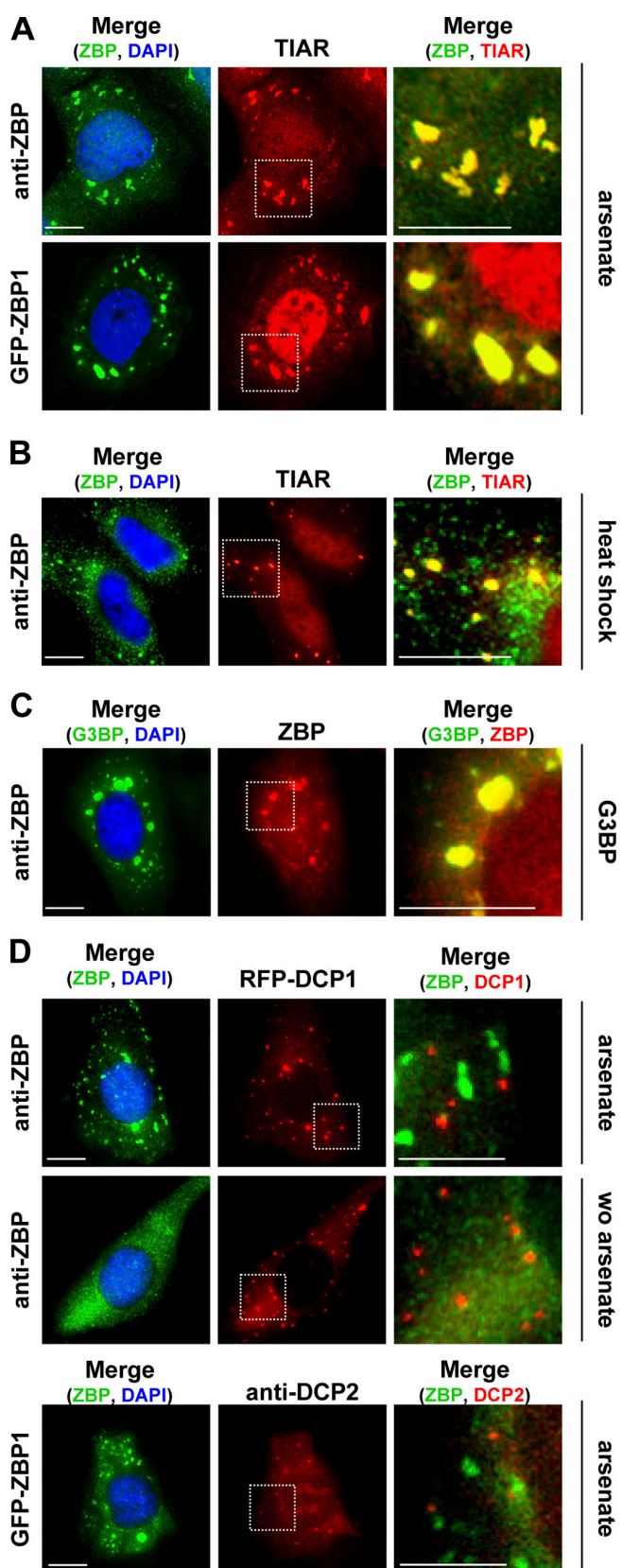(ZBP, DCP2)

GFP-ZBP1   arsenate

Figure 1. **ZBP1 is targeted to SGs, but not PBs, during cellular stress.** (A and B) Localization of endogenous ZBP1 or GFP-ZBP1 to SGs in cells either treated with arsenate (1 h) or exposed to heat shock (42°C). SGs were traced by staining for TIAR. (C) SG targeting of endogenous ZBP1 to SGs induced by the overexpression of GFP-G3BP. (D) ZBP1 is not targeted to PBs in U2OS cells. Cells transfected as indicated were stained for ZBP1 or DCP2 after arsenate (1 h) or buffer (wo arsenate) treatment. Merged enlargements of boxed regions are shown on the right. Bars, 5 μm.
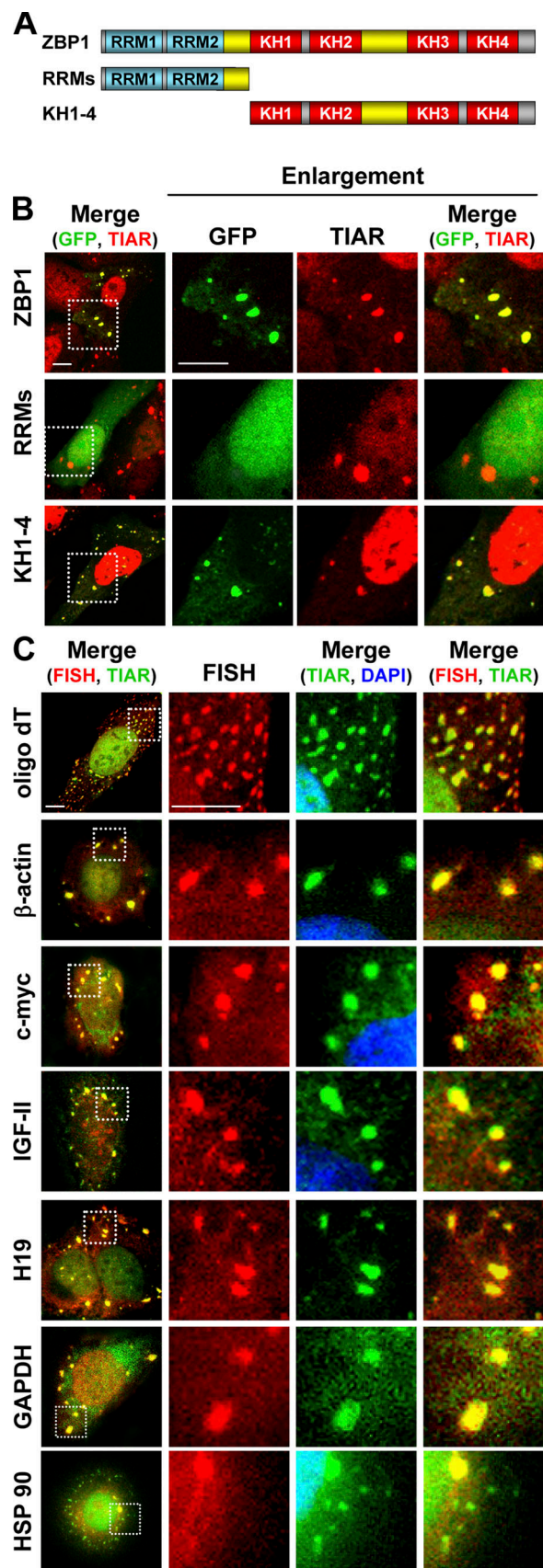
Results and discussion

ZBP1 is recruited to SGs, but not to PBs

To investigate the role of ZBP1 during the ISR, the subcellular distribution of ZBP1 was analyzed in response to oxidative stress or heat shock. Endogenous or GFP-fused ZBP1 were recruited to SGs traced by TIAR (TIA1-related protein; Fig. 1, A and B; and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200608071/DC1; Kedersha et al., 2005). ZBP1 was also identified as a component of G3BP-induced (Ras-Gap SH3 domain-binding protein) SGs, suggesting that the protein is a ubiquitous component of SGs (Fig. 1 C; Tourriere et al., 2003). However, in contrast to G3BP or TIA-1, which induce SG formation upon overexpression, forced expression of ZBP1 had no influence on the rate of SG assembly (Fig. S1, B and C).

Although ZBP1 was targeted to SGs, the protein remained absent from PBs traced by RFP-DCP1 (Decapping protein 1) or endogenous DCP2, which are both exclusively targeted to PBs in stressed and nonstressed cells (Fig. 1 D; Kedersha et al., 2005). In contrast, various RBPs tested for targeting to SGs or PBs, including PTB, Raver1, KSRP, and hnRNP-U, which, like TIAR, all reside mainly in the nucleus in nonstressed cells, were not localized to either SGs or PBs (Fig. S1 D). This indicated that ZBP1 is targeted exclusively to SGs during the ISR, a feature not shared by RBPs in general.

ZBP1 associates with target mRNAs in SGs

In nonstressed cells, ZBP1 regulates the fate of target transcripts via an interaction of these mRNAs with the C-terminal RNA-binding KH domains (Farina et al., 2003). The overexpression of GFP-fused deletion fragments revealed that the KH domains of ZBP1 are also required for SG targeting, suggesting that ZBP1 is associated with mRNA when localized to SGs (Fig. 2, A and B). Therefore, the subcellular distribution of various transcripts, including the ZBP1-associated RNAs IGF-II, c-Myc, β-actin, and H19, was analyzed by FISH during the ISR (Fig. 2 C). All ZBP1-specific RNA targets tested, including the nontranslated H19 RNA, were localized to SGs (Fig. 2 C; Li et al., 1998; Runge et al., 2000). Likewise, several non–ZBP1-associated mRNAs, including GAPDH, were recruited to SGs (Fig. 2 C; and Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200608071/DC1). The only tested mRNA that barely associated with SGs was the HSP90 mRNA, confirming that mRNAs translated during the ISR weakly associate or rapidly transit SGs (Fig. 2 C). These observations indicated that mRNA targeting to SGs is a nonspecific event affecting, presumably, most RNAs, whether actively translated or not. If so, one would expect that mRNA targeting to SGs is independent of regulatory cis-elements. This hypothesis was tested by exploring the SG recruitment of reporter mRNAs encoding a firefly luciferase equipped with MS2 repeats fused in proximity to the ZBP1-binding site of

Figure 2. **SG targeting of ZBP1 deletion fragments and mRNAs.** (A) Schematic of ZBP1 domains (linker regions in yellow). (B) Localization of GFP-ZBP1, GFP RNA recognition motif (GFP-RRM), or GFP-KH1-4 to SGs in transfected U2OS cells treated with arsenate before staining for TIAR.
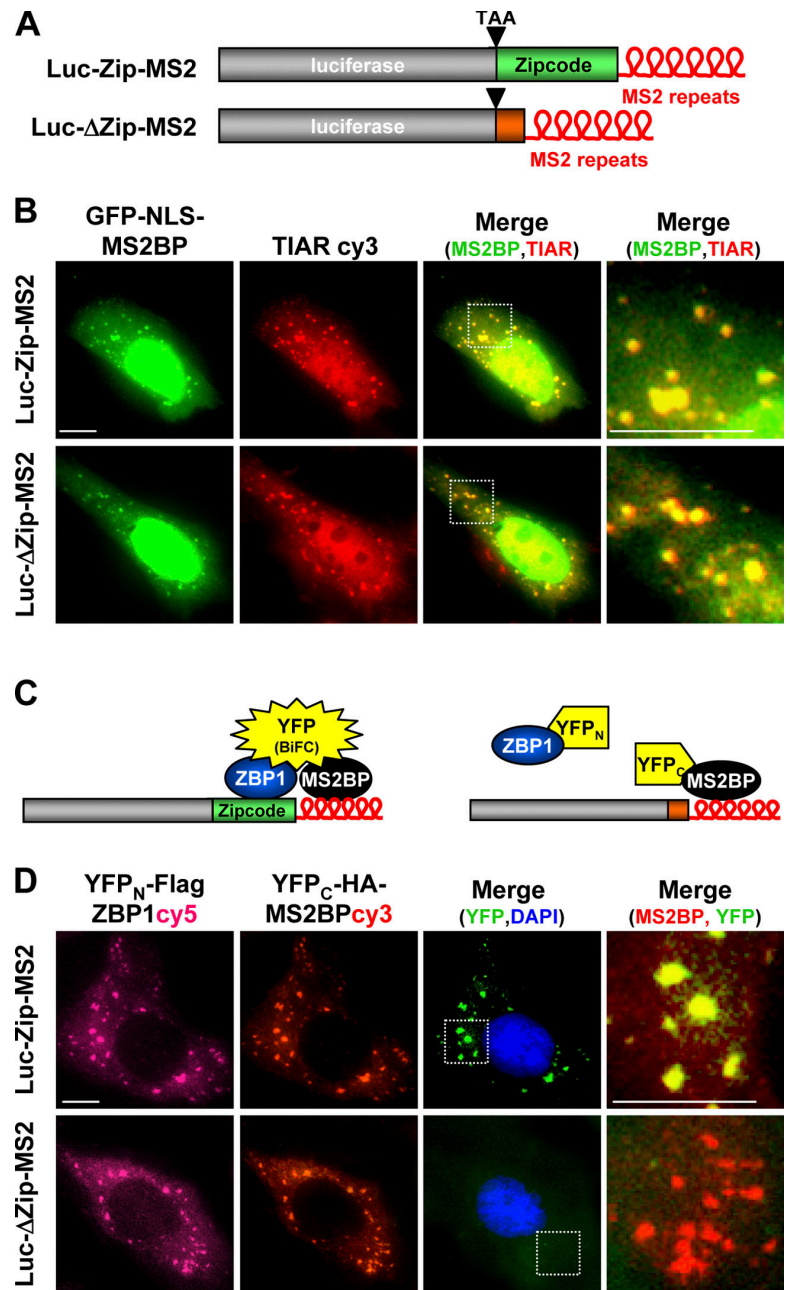
the β-actin mRNA ("Zipcode") or to a Zipcode deletion fragment (Fig. 3 A; Kislauskis et al., 1994). Both transcripts were targeted to SGs, as observed upon cotransfection of MS2-binding protein (MS2BP) fused to nuclear localization signal (NLS)–GFP (Fig. 3 B). Hence, mRNA targeting to SGs was independent of the cis-acting Zipcode. Therefore, we hypothesized that ZBP1 is not essential for SG recruitment of target mRNAs, but serves a function in regulating mRNA retention in SGs. Because this requires an association of ZBP1 with its target mRNAs in SGs, RNA binding was characterized by bimolecular fluorescent complementation (BiFC; Fig. 3 C; Rackham and Brown, 2004). YFP fluorescence in SGs was reconstituted from cotransfected YFP$_N$-fused ZBP1 and YFP$_C$-fused MS2BP only upon the expression of a reporter comprising Zipcode in proximity to the MS2 repeats (Fig. 3 D). This indicated that ZBP1 associated with the same transcript as the MS2BP in SGs when the Zipcode was present (Fig. 3, C and D). Thus, although SG targeting of reporter mRNAs appeared to be Zipcode independent, ZBP1 remained associated with its target mRNA in SGs.

ZBP1 regulates mRNA turnover during cellular stress

It appeared plausible that ZBP1 either was essential for the structural integrity of SGs or that it controlled the transit of specific mRNAs to PBs to regulate mRNA decay (Fig. 4 A). To investigate this, ZBP1 levels were reduced via siRNAs before stress induction (Fig. S3 E, available at http://www.jcb.org/cgi/content/full/jcb.200608071/DC1). The assembly and structure of SGs, as assessed by staining for TIAR, revealed no obvious defects upon ZBP1 knockdown (Fig. 4 B). In agreement with ZBP1 not being essential for SG targeting of mRNAs, the localization of associated mRNAs like c-Myc remained basically unaffected when ZBP1 was depleted (Fig. 4 C). To investigate a putative role of ZBP1 in preventing rapid SG transit, and thus mRNA degradation of associated mRNAs, decay rates of c-Myc mRNA that is stabilized by ZBP1 via a translation-coupled mechanism in nonstressed cells were tested during the ISR, when translation of c-Myc is prevented because of the sequestering of the mRNA to SGs (Lemm and Ross, 2002). Cellular mRNA levels of c-Myc were assessed by quantitative RT-PCR (qRT-PCR) upon stress induction and normalization to RPLP0 and 18S RNA ratios, respectively (Fig. S3, A and B). The ratios of various mRNAs tested, including c-Myc, increased after the onset of the ISR, indicating that these mRNAs are stabilized in SGs and that transcription continues during stress, possibly at elevated levels (Fig. S3, A and B). To explore mRNA turnover that was unbiased by transcriptional reorganization, the decay of c-Myc mRNA during stress was analyzed after blocking transcription by actinomycin D. Compared with nonstressed cells, the c-Myc mRNA decayed slower; ∼40–50% of the initial mRNA levels remained after 1 h of arsenate treatment. This indicated that at 1 h after stress-induction, most of the mRNA had

(C) SG targeting of polyadenylated mRNA (oligodT) and various mRNAs to SGs induced by arsenate. Cells were stained for TIAR before FISH with indicated probes. Merged enlargements of boxed regions are shown on the right. Bars, 5 μm.

Figure 3. **ZBP1 associates with mRNA targets in SGs.** (A) Schematic of reporter transcripts (from 5′–3′), as follows: Firefly luciferase ORF, Zipcode region of the β-actin 3′-UTR (Luc-ZIP-MS2), or Zipcode-deleted 3′-fragment (Luc-ΔZip-MS2), six MS2 repeats. (B) Localization of GFP-NLS-MS2BP traced mRNA to SGs. Arsenate-induced SGs were traced by TIAR staining. (C) Schematic of BiFC used to visualize protein–RNA interactions. YFP$_N$-Flag-ZBP1, ZBP1 fused to N-terminal Venus–YFP fragment; YFP$_C$-HA-MS2BP, MS2BP fused to C-terminal Venus–YFP fragment. (D) Reconstituted fluorescent Venus–YFP is targeted to SGs in U2OS cells transfected with the Zipcode-containing reporter. Cells were treated with arsenate before fixation and staining for YFP$_N$-Flag-ZBP1 and YFP$_C$-HA-MS2BP. Merged enlargements of boxed regions are shown on the right. Bars, 5 μm.

been targeted to SGs and only modest decay was observed after 1 h of stress induction, suggesting that SG transit of the transcript was slow (Fig. 4 D; Fig. S3 C).

Hence, we speculated that in SGs ZBP1 prevented the rapid decay of the c-Myc mRNA in a nontranslationally coupled fashion. Therefore, variations in cellular c-Myc and non–ZBP1-associated mRNAs (α-tubulin) were analyzed upon ZBP1 knockdown (Fig. 4 E). The c-Myc mRNA was selectively destabilized in stressed knockdown cells, whereas the stability of α-tubulin mRNA remained unaffected (Fig. 4 E). Notably, mRNA decay was normalized to mRNA ratios before stress induction to account for putative effects on mRNA stability upon ZBP1 knockdown. Thus, unbiased of "overall changes" in mRNA ratios, ZBP1 knockdown resulted in elevated c-Myc mRNA decay rates and increased total decay during the ISR (Fig. 4 F; and Fig. S3 D).

This selective stabilization of mRNAs by ZBP1 should also be observed for other mRNA targets, which, in nonstressed cells, are translationally regulated by ZBP1, as demonstrated for the β-actin and IGF-II mRNAs (Nielsen et al., 1999; Huttelmaier et al., 2005). Hence, various mRNAs ratios were analyzed in stressed knockdown cells treated with actinomycin D by normalization to input levels. In addition to c-Myc, all tested mRNA-targets of ZBP1, including IGF-II, CD44, and the β-actin mRNA, were selectively destabilized upon ZBP1 knockdown (Fig. 4 G). This was also observed in the absence of actinomycin D (Fig. S3 E). Because ZBP1 knockdown increased the decay of target transcripts, we predicted that overexpression of ZBP1 would result in increased stabilization. Therefore, mRNA levels of the β-actin and IGF-II mRNA were compared in cells transfected with either GFP alone or GFP-fused ZBP1.
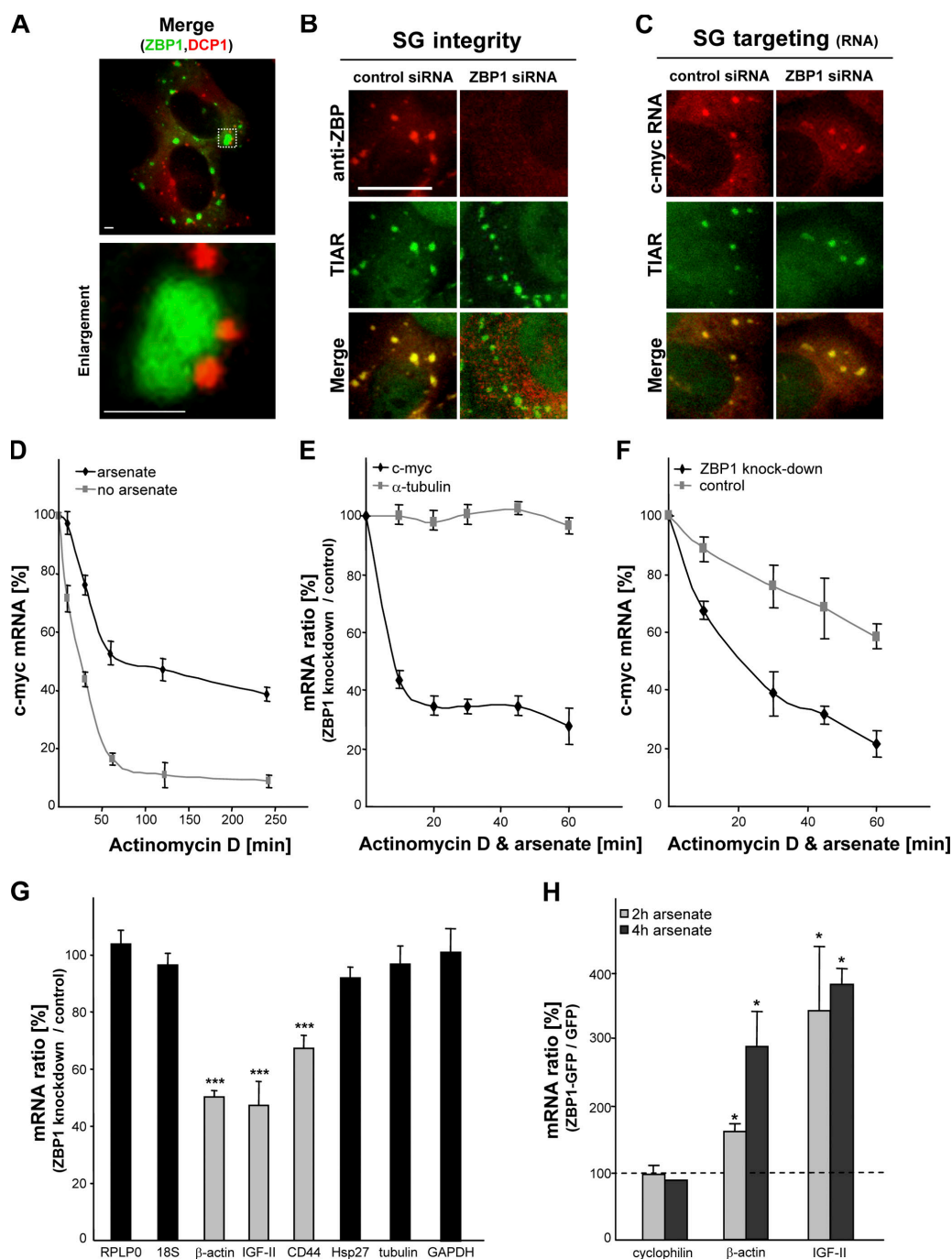
Figure 4. **ZBP1 regulates mRNA turnover during cellular stress.** (A) ZBP1-containing SGs associate with RFP-DCP1–traced PBs. Cells were transfected with RFP-DCP1 before arsenate treatment (1 h) and staining for ZBP1. (bottom) Enlargement of a boxed region is shown. (B) SG assembly remains unaffected by the knockdown of ZBP1. U2OS cells transfected with indicated siRNAs were treated with arsenate (1 h) before TIAR staining. (C) Targeting of mRNAs remained unaffected by ZBP1 knockdown. U2OS cells were treated as in B, before staining for TIAR and FISH for c-Myc mRNA. (D) The c-Myc mRNA is stabilized during the ISR. c-Myc mRNA levels were analyzed by qRT-PCR and cross-normalization to 18S and RPLP0 RNA in cells treated with arsenate and actinomycin D for the indicated time. (E) The c-Myc mRNA is selectively destabilized after ZBP1 knockdown in stressed cells. c-Myc and α-tubulin mRNA levels were analyzed by qRT-PCR and cross-normalized as in D. The relative mRNA ratios were determined between knockdown cells and controls after normalization to input levels before stress induction. (F) Decay of the c-Myc mRNA in cells treated with arsenate and analyzed as in E. (G) Knockdown of ZBP1 selectively destabilizes ZBP1-associated mRNAs during the ISR. The relative amount of indicated mRNAs was determined as in E. Cells were exposed to arsenate for 1 h. (H) Overexpression of ZBP1 results in the selective stabilization of target mRNA during the ISR. Cells were transfected with GFP-ZBP1 or GFP alone (control). The relative change of indicated mRNAs levels was analyzed by qRT-PCR, as described in E. Error bars indicate the SD. Bars: (A) 2 μm; (B and C) 10 μm. *, P ≤ 0.05; **, P ≤ 0.005; ***, P ≤ 0.0005.

The overexpression of GFP-ZBP1 resulted in selectively increased steady-state levels for both target mRNAs during the ISR (Fig. 4 H). In conclusion, these observations indicated that ZBP1 stabilized associated transcripts recruited to SGs, presumably by retaining target mRNAs in SGs. Thus, ZBP1 modified its function during the ISR; mRNAs such as IGF-II or
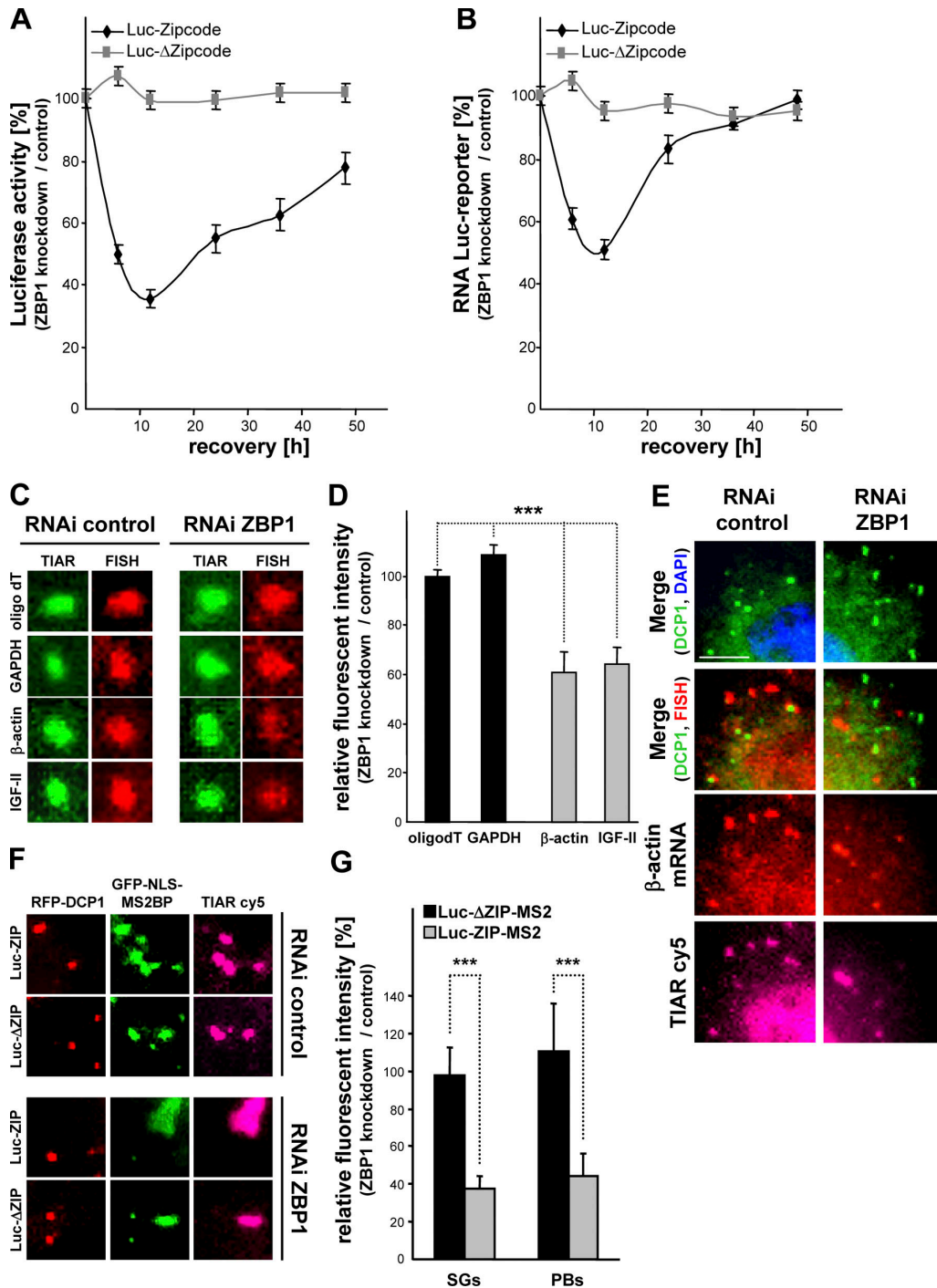
Figure 5. **ZBP1 regulates SG transit of target mRNAs during the ISR.** (A and B) Knockdown of ZBP1 leads to selective destabilization of associated mRNAs, resulting in reduced protein levels after stress release. Cells transfected with ZBP1-directed siRNAs or control siRNAs and Zipcode or Zipcode-deleted luciferase reporters were treated with arsenate (1 h) before medium replacement (stress release). The relative luciferase activity (A) and mRNA levels (B) of reporter transcripts were analyzed in ZBP1 knockdown cells and controls at indicated times after stress release. (C and D) ZBP1 knockdown induces a selective reduction of associated mRNAs in SGs. Cells treated with indicated siRNAs were stained for TIAR before FISH with indicated probes. (C) Enlargements of individual SGs are shown. The fluorescent intensities for indicated mRNAs (FISH) in SGs were normalized to TIAR fluorescent intensities and compared between ZBP1 knockdown cells and controls (D). A total of $n = 50$ SGs from various cells were analyzed for each hybridization. (E) The β-actin mRNA was barely detected in PBs by FISH in arsenate-treated (1 h) cells. PBs (GFP-DCP1) and SGs (TIAR) were traced in cells transfected with indicated siRNAs. (F and G) GFP-NLS-MS2BP–traced mRNA was reduced in a Zipcode-dependent fashion in cells exposed to arsenate after ZBP1 knockdown. After transfection of indicated siRNAs, cells were transfected with reporter plasmids indicated in Fig. 3 A, RFP-DCP1, and GFP-NLS–fused MS2BP. (F) Enlargements of regions containing PBs (RFP-DCP1) and SGs (TIAR) are shown. Upon normalization to either RFP-DCP1 (PBs) or TIAR (SGs) fluorescent intensities, GFP fluorescent intensities were compared between cells treated with indicated siRNAs (G). A total of at least 25 SGs and 40 PBs were analyzed for each transfection. Error bars indicate the SD. Bar, 5 μm. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$.

β-actin that are translationally regulated by ZBP1 without affecting mRNA turnover in nonstressed cells were stabilized by ZBP1 during the ISR.

ZBP1 controls SG transit of target mRNAs

The role of ZBP1 in regulating mRNA turnover during the ISR was further explored by analyzing the fate of SG-targeted reporter transcripts during a cycle of stress induction and stress release. Compared with controls, the recovery of luciferase activity expressed from reporter transcripts harboring Zipcode was reduced after stress release upon ZBP1 knockdown (Fig. 5 A). This decrease in luciferase activity correlated with a Zipcode-dependent destabilization of reporter transcripts (Fig. 5 B). Hence, specific interactions between ZBP1 and target mRNAs in SGs were required to retain, and thereby stabilize, mRNAs in SGs.

To validate these findings, the loss of ZBP1-associated mRNAs from SGs upon ZBP1 knockdown was quantified by FISH. As expected, the relative amount of β-actin and IGF-II mRNA in SGs was selectively reduced in cells treated with ZBP1-directed siRNAs (Fig. 5, C and D). Notably, this selective reduction of target mRNAs in SGs was not a result of an "overall decrease" of these mRNAs upon ZBP1 knockdown (Fig. S2 B). Hence, the stabilization of target-mRNAs by ZBP1 apparently required retention of these mRNAs in SGs to prevent rapid decay.

Aiming at investigating SG transit of mRNAs in further detail, the PB localization of ZBP1-associated mRNAs was analyzed by FISH. However, endogenous mRNAs were barely observed in PBs, suggesting that during the ISR, mRNAs shuttled to PBs were rapidly decayed and therefore could not be detected (Fig. 5 E). Hence, we traced exogenous mRNAs by MS2 tagging that was expected to transiently stabilize RNA, even in PBs. Quantification of the mRNA content in SGs and PBs based on GFP fluorescent intensities normalized to either TIAR or RFP-DCP1 signals revealed a Zipcode-dependent reduction of mRNA levels in both foci upon ZBP1 knockdown (Fig. 5, F and G). In contrast, the amount of transcripts lacking Zipcode remained unaffected. Although we cannot exclude that mRNAs lost from SGs were degraded in part by the exosomes, these findings indicated that mRNAs usually retained in SGs by ZBP1 were transferred to PBs once ZBP1 levels were reduced. Rapid decay then presumably prevented the accumulation of tested mRNAs in PBs, suggesting that these foci are not rate limiting for mRNA decay during the ISR.

In conclusion, this work presents experimental evidence demonstrating that interactions between mRNAs and transcript-specific RBPs like ZBP1 can determine the fate of an mRNA during cellular stress. ZBP1 associates with its target transcripts within SGs, and this binding is required for the stabilization of associated mRNAs. Although in nonstressed cells ZBP1 either controls mRNA turnover or translation; the protein adopts a solely stabilizing function in SGs, as it stabilizes transcripts exclusively regulated on the translational level in nonstressed cells, e.g., the β-actin or IGF-II mRNA. This selective stabilization correlates with increased mRNA levels in SGs, and upon

ZBP1 knockdown, ZBP1 target mRNAs are selectively reduced in SGs, whereas overall levels of the β-actin or IGF-II mRNAs in nonstressed cells remain unaffected by ZBP1 knockdown. Hence, stabilization of target mRNAs by ZBP1 requires retention of transcripts in SGs to prevent rapid translocation and subsequent decay. No accumulation of endogenous or reporter mRNAs was observed in PBs upon ZBP1 knockdown, suggesting that PBs are not rate limiting for mRNA decay during the ISR, and thus reflect the mRNA content observed in SGs. Therefore, one would assume that SGs serve as "filters" that regulate mRNA turnover during cellular stress. This function requires defined cis–trans interactions between mRNAs and RBPs like ZBP1. These retain, and thereby stabilize, target transcripts in SGs to prevent rapid decay by the exosome or, as previously suggested, in PBs (Kedersha et al., 2005).

Materials and methods

Plasmids
Full-length chicken ZBP1 or ZBP1 deletion fragments were generated by PCR, verified by sequencing, and inserted in pEGFP (CLONTECH Laboratories, Inc.) or mRFP plasmids, respectively. The human G3BP, Imp1, Imp2, or Imp3 cDNAs were generated by reverse-transcription of human total mRNA isolated from U2OS cells. Corresponding open reading frames were sequenced before subcloning in pEGFP.

Luciferase reporter constructs fused to MS2 repeats were generated by subcloning from previously described constructs (Bertrand et al., 1998; Huttelmaier et al., 2005). Plasmids used for BiFC were generated basically as previously described (Rackham and Brown, 2004).

Cell culture and transfection
U2OS cells were grown in DME (10% FBS). The assembly of SGs was induced by various stimuli, as follows: the application of sodium arsenate (final concentration of 2.5 mM) for indicated time, heat shock at 42°C for 1 h, or the overexpression of GFP-G3BP. Where indicated, transcription was blocked by 5 µM actinomycin D added 15 min before arsenate application. Plasmids and siRNAs were transfected with Lipofectamine 2000 (Invitrogen). ZBP1-specific siRNAs and controls were as previously described (Huttelmaier et al., 2005).

Immunofluorescence and FISH
U2OS cells grown on coverslips were treated with 2.5 mM sodium arsenate 48 h after transfection. Fixed cells were processed for immunostaining as previously described (Farina et al., 2003). For indirect immunofluorescence of ZBP1, cells were stained with the following indicated antibodies: anti-ZBP1 (Farina et al., 2003), anti-TIAR (BD Biosciences), anti–hnRNP U (Sigma-Aldrich), anti-HA (Sigma-Aldrich), anti-Flag (Sigma-Aldrich), and anti–human DCP-2 (raised in rabbit by peptide immunization).

FISH was essentially performed as previously described (Huttelmaier et al., 2005). Where indicated, cells were fixed after the addition of primary and secondary antibodies, before performing FISH. All probes used were designed, synthesized, and labeled with Cy3-dye according to Singerlab protocols.

All image acquisition and the quantification of fluorescent signal intensities was performed using standardized settings on a microscope (E600; Nikon) equipped with a camera (model C4742-95; Hamamatsu), a Lucia software module (Laboratory Imaging, Ltd.), and a 60× objective (Plan Fluor; Nikon). Mean integral fluorescence intensities for mRNA (FISH) were normalized to average integral fluorescence intensities for TIAR (immunostaining) and/or RFP-DCP1 before comparison between cell populations treated with the indicated siRNAs.

qRT-PCR and Western blotting
At ~72 h after transfection with siRNAs, U2OS cells were treated with 2.5 mM sodium arsenate, and 5 µM actinomycin D were stated. Total RNA was extracted using TRIZOL (Invitrogen). 2 µg RNA was used for reverse transcription with random primers and MMLV-RT (Promega). qRT-PCR was performed based on SYBRgreen technology using 2xTaq-Master mix (Promega) in a cycler (MX3000p; Stratagene). For all primer pairs, an annealing temperature of 60°C was used. For primer sequences, see the table

in Fig. S2 C. Relative changes of mRNA amounts were calculated based on the ΔC_t method, as described by Livak and Schmittgen (2001). For Western blotting, total protein extracts were analyzed by Western blotting for ZBP1 (anti-ZBP) and vinculin (hVin1; Sigma-Aldrich), as described previously (Huttelmaier et al., 2005).

Online supplemental material

Fig. S1 shows selective targeting of RBPs to SGs. Fig. S2 shows SG targeting of mRNAs not associated with ZBP1. Fig. S3 shows selective stabilization of mRNAs during the ISR. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200608071/DC1.

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