LETTERS

Spatial regulation of β-actin translation by Src-dependent phosphorylation of ZBP1

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Localization of β-actin messenger RNA to sites of active actin polymerization modulates cell migration during embryogenesis, differentiation and possibly carcinogenesis[1–5]. This localization requires the oncofetal protein ZBP1 (Zipcode binding protein 1), which binds to a conserved 54-nucleotide element in the 3′-untranslated region of the β-actin mRNA known as the 'zipcode'. ZBP1 promotes translocation of the β-actin transcript to actin-rich protrusions in primary fibroblasts and neurons[6,7]. It is not known how the ZBP1–RNA complex achieves asymmetric protein sorting by localizing β-actin mRNA. Here we show that chicken ZBP1 modulates the translation of β-actin mRNA. ZBP1 associates with the β-actin transcript in the nucleus and prevents premature translation in the cytoplasm by blocking translation initiation. Translation only occurs when the ZBP1–RNA complex reaches its destination at the periphery of the cell. At the endpoint of mRNA transport, the protein kinase Src promotes translation by phosphorylating a key tyrosine residue in ZBP1 that is required for binding to RNA. These sequential events provide both temporal and spatial control over β-actin mRNA translation, which is important for cell migration and neurite outgrowth.

In order to characterize how the ZBP1–RNA complex achieves asymmetric protein sorting, we established an *in vitro* cell model using the NG108-15 neuroblastoma cell line (NG). As in primary embryonic fibroblast or neuronal cells, ZBP1 colocalized with β-actin mRNA at the leading edge, in growth cone filopodia and along the neurites of differentiated NG cells[8] (Fig. 1a, b, d, e). At the cell periphery, sites of colocalization were interspersed with regions where β-actin mRNA and ZBP1 were separated, suggesting release of the mRNA transcript (Supplementary Fig. 1a–c). In the nucleus, ZBP1 was found in discrete foci, coinciding with the sites of β-actin mRNA transcription and indicating that ZBP1 associates with β-actin mRNA co-transcriptionally[9,10] (Fig. 1c, f and Supplementary Fig. 1d–f).

A well-accepted paradigm of mRNA localization is that it precedes translation. We propose that assembly of a localized mRNA–protein complex occurs in the nucleoplasm, priming the mRNA to be translationally silenced when it reaches the cytoplasm. On the basis of this hypothesis, we tested recombinant ZBP1 for a role in regulating the translation of β-actin mRNA.

In rabbit reticulocyte lysate, ZBP1 impaired translation of the β-actin transcript in a zipcode-dependent manner, without affecting RNA stability (Fig. 1g and Supplementary Fig. 1g, h). Hence, the zipcode sequence seems to be essential for controlling both mRNA localization and translation. Similar to the RNA-binding protein hnRNP-K, ZBP1 impaired the formation of 80S ribosomal complexes on regulated transcripts in rabbit reticulocyte lysate, but the amount and positioning of the 48S complex remained unaffected[11] (Supplementary Fig. 2a–d). These findings confirm ZBP1 as a bona fide translational regulator that interferes with translation by blocking translation initiation.

We then attempted to validate the role of ZBP1 as a zipcode-dependent translational regulator *in vivo*. Knockdown of endogenous *ZBP1* by short interfering (si)RNA enhanced translation from reporter transcripts in a zipcode-dependent manner (Fig. 1h, i and Supplementary Fig. 1i). Specificity was further confirmed by transfecting an siRNA-insensitive chicken *ZBP1* (*ZBP1*$_{insens}$) into NG cells. ZBP1$_{insens}$ restored the translational repression of transcripts

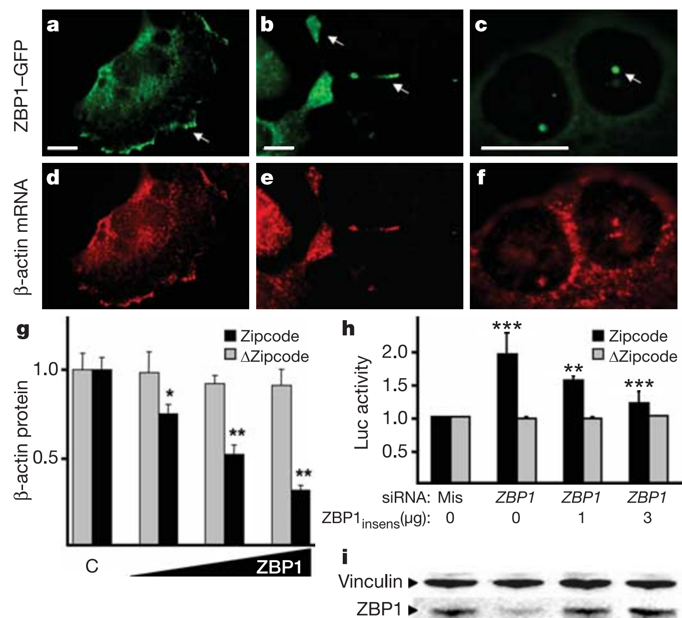

Figure 1 | ZBP1 associates with β-actin mRNA in NG cells and modulates its translation. a–f, Colocalization of ZBP1–GFP and β-actin mRNA in differentiated NG cells: leading edge (arrow in **a**, **d**), neurite extension (arrow in **b**, **e**) and discrete nuclear foci (arrow in **c**, **f**). Scale bars, 10 μm. **g**, Quantification of β-actin translation in rabbit reticulocyte lysate, normalized by *Renilla* luciferase in the presence of increasing ZBP1 concentrations or buffer (C). **h**, Normalized luciferase (luc) activity from cells transfected with the indicated siRNA (Mis, missense control) and plasmids encoding increasing amounts of siRNA-insensitive ZBP1 (ZBP1$_{insens}$). **i**, Western blot analysis of protein extracts from **i**, using the indicated antibodies. Error bars indicate s.d. Asterisk, $P \leq 0.05$; two asterisks, $P \leq 0.005$; three asterisks, $P \leq 0.0005$.

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containing the human β-actin mRNA zipcode (Fig. 1h, i). Hence, ZBP1 is a conserved *trans*-acting factor that controls translation of β-actin mRNA *in vivo*.

To achieve asymmetric protein sorting in neurites, a regulatory mechanism must exist to abrogate translational repression by ZBP1 once the mRNA has been localized. Sequence analysis of the ZBP1 protein reveals a possible SH3-binding motif (VP_4SS) in close proximity to a KH-domain essential for binding to the zipcode[7]. The identical motif is found in the cell-adhesion protein paxillin, where it serves as a docking site for Src kinase[12]. Like paxillin, we confirmed that ZBP1 is a substrate for Src *in vivo*. Exogenous ZBP1 was tyrosine-phosphorylated by endogenous Src. Overexpression of constitutively active Src (KP, which contains a K249Q and P250G mutation) increased ZBP1 phosphorylation, whereas inactivated Src (containing a Y416F mutation) had no effect (Fig. 2a). *In vitro* and *in vivo* phosphorylation experiments identified Tyr 396 as the ZBP1 residue phosphorylated by Src (Fig. 2b, Supplementary Fig. 3 and Supplementary Fig. 4a, b). Phosphorylation of endogenous ZBP1 *in vivo* and the recombinant protein *in vitro* was verified with a specific anti-peptide antibody directed against phosphorylated Tyr 396 (Supplementary Fig. 4c, d).

Phosphorylation of tyrosine residues outside the RNA-binding domain has been suggested to decrease RNA binding of the KH-domain-containing proteins hnRNP-K and Sam68 (refs 13, 14). To address the role of Src in regulating ZBP1 function, phosphorylated ZBP1 and non-phosphorylatable ZBP1 (containing a Y396F mutation) were probed for subcellular localization and zipcode binding. No obvious differences between the wild-type and mutant protein were found (data not shown). However, zipcode binding was significantly decreased as a result of ZBP1 tyrosine phosphorylation (Supplementary Fig. 5a–c). In contrast to the wild-type protein, ZBP1-Y396F retained significantly higher binding efficiency, suggesting that phosphorylation of Tyr 396 interferes with RNA binding (Fig. 2c). ZBP1–RNA complexes isolated from cells and analysed by quantitative polymerase chain reaction with reverse transcription (RT–PCR) revealed that the association of endogenous β-actin mRNA with ZBP1 was unaffected by the Y396F mutation. However, replacement of Tyr 396 with glutamate significantly impaired the association of ZBP1 with endogenous β-actin mRNA (Fig. 2d). This indicates that tyrosine phosphorylation or the mimicking of phosphorylation by glutamate substitution interferes with the binding of β-actin mRNA both *in vitro* and *in vivo*.

The control of β-actin mRNA binding through tyrosine phosphorylation suggests that Src might also regulate translational repression through ZBP1. This was supported by our finding that increased Src activity leads to increased translation only from zipcode reporter mRNA *in vivo* (Fig. 2e). Cells that were depleted of endogenous *ZBP1* by siRNA showed significantly increased translation, whereas replacement with $ZBP1_{insens}$ or ZBP1-Y396F, which are both insensitive to siRNA, decreased translation (Fig. 2f). Notably, the mutant was slightly more efficient at reducing translation. Amino acid replacement of Tyr 396 apparently counteracts the release of β-actin mRNA, as RNA binding was unaffected by the Y396F mutation both *in vitro* and *in vivo*. Together, our data suggest that Src phosphorylation of Tyr 396 abolishes translational repression by decreasing ZBP1 binding to β-actin mRNA.

In order for translational repression to be spatially regulated, Src should associate with and phosphorylate ZBP1 at the endpoint of β-actin mRNA transport. We therefore investigated the interaction between Src and ZBP1 using immunoprecipitation experiments (Supplementary Fig. 6a). This association was impaired by introducing a negative charge C-terminal to the conserved poly-proline motif, which has been shown to interfere with SH3-mediated binding[15] (Supplementary Fig. 6b–d).

We next analysed the spatial organization of the ZBP1–Src interaction by fluorescence resonance energy transfer (FRET; Fig. 3a–e). Acceptor photobleaching using ZBP1–cyan fluorescent protein (CFP) and Src–yellow fluorescent protein (YFP) fusion proteins as donor and acceptor molecules, respectively, revealed a significant energy transfer and thus an association between Src and ZBP1 in close proximity to filopodia and in the growth cones of differentiated NG cells (Fig. 3e, arrows and arrowhead, respectively). FRET signal was not detected outside the photobleached region or when using free YFP (Supplementary Fig. 7a–f). The spatial organization of the Src–ZBP1 interaction resembled the colocalization of β-actin mRNA and ZBP1 along dendrites and within the growth cone filopodia of primary hippocampal neurons[3,16].

We next investigated whether disruption of translational regulation by ZBP1 had a phenotypic consequence. Previous work has suggested that β-actin mRNA localization might regulate the density of F-actin in dendritic protrusions, thereby implying that the spatial regulation of translation can modulate neuronal outgrowth by providing monomeric actin and other actin-related proteins[8,17]. As predicted, the scoring of differentiated NG cells on the basis of cellular extension length showed that knockdown of *ZBP1* significantly impaired neurite outgrowth (Fig. 3f–h and Supplementary Fig. 8a–f). Neuronal outgrowth recovered upon expression of ZBP1 that was insensitive to siRNA. In contrast, expression of non-phosphorylatable ZBP1-Y396F, which is impaired in releasing the mRNA, did not restore neuronal outgrowth. Similarly, overexpression of ZBP1-Y396F interfered with neuronal outgrowth in primary hippocampal neurons, supporting the physiological relevance of the observed phenotype (Fig. 3i–k).

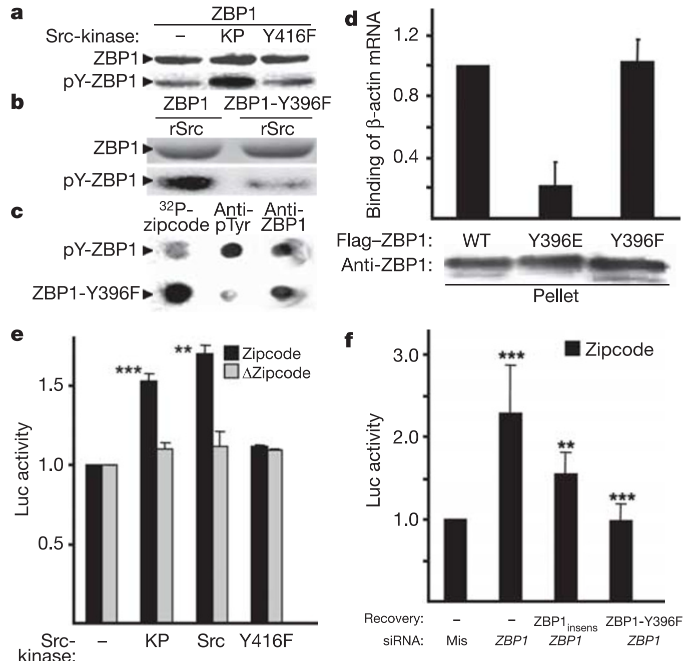

Figure 2 | Phosphorylation of ZBP1 by Src interferes with RNA binding and relieves translational repression. **a**, Western blot of Flag–ZBP1 from cells transfected with pcDNA3.1 (−), constitutively active (KP) or inactive (Y416F) Src, using the indicated antibodies. **b**, **c**, ZBP1 and ZBP1-Y396F phosphorylation by recombinant Src (rSrc), assayed by Coomassie staining (ZBP1), autoradiography (pY-ZBP1), zipcode-binding (^{32}P-zipcode) and immunoblotting (anti-pTyr). Anti-ZBP1 staining used as a loading control. **d**, β-actin mRNA binding to precipitated ZBP1 proteins (western blot showing precipitated ZBP1 levels), normalized to wild-type (WT) ZBP1. **e**, Luciferase activities of cells co-transfected with the indicated reporters and Src constructs, normalized relative to controls without kinase transfection. **f**, Luciferase activities relative to controls (Mis); co-transfected with the indicated siRNAs, WT, siRNA-insensitive $ZBP1_{insens}$ or mutant ZBP1-Y396F. Error bars indicate s.d. Two asterisks, $P \leq 0.005$; three asterisks, $P \leq 0.0005$.

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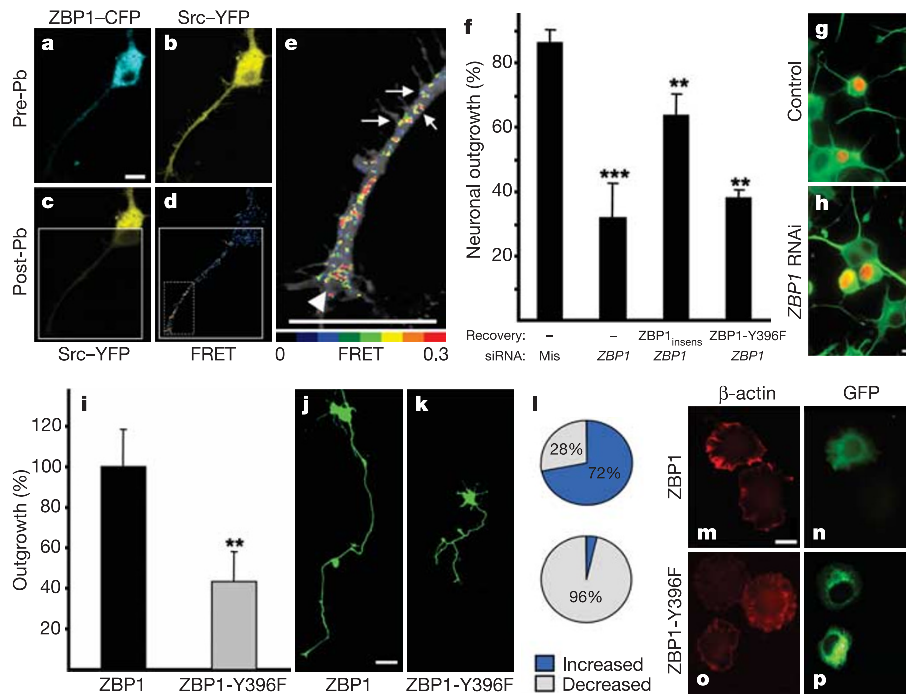

Figure 3 | Regulation of ZBP1 by Src modulates neuronal outgrowth. **a**–**d**, FRET CFP signals before (**a**, **b**) and after YFP-photobleaching (Pb) (box in **c** and **d**). **e**, Increased FRET intensity (dashed boxed in **d**) at filopodia (arrows) and growth cone (arrowhead). **f**, Neuronal outgrowth in NG cells transfected with indicated siRNAs, siRNA-insensitive ZBP1 or ZBP1-Y396F and polypyrimidine tract binding protein (PTB)–CFP transfection marker ($n \geq 350$). **g**, **h**, PTB–CFP (red) and tubulin (green) from NG cells transfected with *ZBP1* siRNA. **i**–**k**, Neuronal outgrowth in primary hippocampal neurons overexpressing ZBP1-Y396F (**k**) was normalized to wild-type ZBP1 expression (**j**, $n = 40$). GFP was used as a marker for transfection. **l**–**p**, Per cent NG cells transfected with ZBP1–GFP (**m**, **n**) or ZBP1-Y396F–GFP (**o**, **p**), showing increased or decreased peripheral β-actin protein. β-actin (red; **m**, **o**) and GFP signal (green; **n**, **p**) signals are shown. See Supplementary Fig. 9. Scale bars, 10 μm. Error bars indicate s.d. Asterisk, $P \leq 0.05$; two asterisks, $P \leq 0.005$; three asterisks, $P \leq 0.0005$.

We would expect that this suppression of outgrowth results directly from the inability to locally translate β-actin mRNA, as the non-phosphorylatable mutant should act as a dominant negative by failing to release translational repression at the endpoint of mRNA transport. In order to test this prediction, we assessed the levels of peripheral β-actin protein in spreading NG cells upon overexpression of ZBP1–GFP or ZBP1-Y396F–GFP (Fig. 3l–p and Supplementary Fig. 9a–h). β-Actin protein at the cell periphery was detected by indirect immunofluorescence using a β-actin-specific antibody (see Supplementary Fig. 9a–h). Enhanced expression of wild-type ZBP1 increased the actin signal at the leading edge of most spreading cells (~70% of scored cells). In contrast, exogenous expression of ZBP1-Y396F reduced peripheral actin intensity in over 90% of the scored cells. These observations indicate that the cellular amount of peripheral β-actin protein is regulated through tyrosine phosphorylation of ZBP1. Therefore, spatial control of β-actin protein levels results in proper neuronal differentiation.

Our findings emphasize the temporal and spatial relationship between mRNA localization and translation. If β-actin mRNA translation were not silenced, polysome formation would initiate immediately upon nuclear egress. This would make translocation of the mRNA problematic, as polysomes are essentially immobile within the isotropic network of cytoskeletal structures[18]. Although the association with nuclear factors may be important for preventing β-actin translation in the cytoplasm[19–23], equally important is the mechanism that relieves this repression at the right time and place. Hence, ZBP1 must be removed from β-actin mRNA at the endpoint of transport. We propose that this occurs when ZBP1 comes into proximity with Src, as Src phosphorylation of ZBP1 disrupts RNA binding and activates translation. Given that Src activity is restricted to the cell periphery, local activation of β-actin translation can be modulated solely through the spatially restricted activity of the kinase[24,25]. Our findings indicate that a sequence of regulatory events including (1) 'nuclear priming', (2) translational silencing that allows cytoplasmic sorting of transcripts, and (3) spatially controlled translational de-repression of a localized transcript have evolved as a common mechanism that can have profound physiological consequences for cellular asymmetry.

METHODS

Cell culture, transfection and antibodies. NG108-15 cells and 293 cells were cultured in DMEM medium (with 10% fetal bovine serum), containing 1% HAT (hypoxanthine, aminopterine, thymidine) supplement for the culturing of NG-hybridomas. NG cells were differentiated by reducing serum levels (to 1%) and plating on a poly-L-lysine/laminin matrix. For details see Supplementary Methods.

Protein purification and *in vitro* phosphorylation. Recombinant protein was purified essentially as described[7]. Purified proteins were phosphorylated with Src (p60c-Src, Upstate) for 10–30 min at 30 °C, according to the manufacturer's instructions. After phosphorylation, proteins were immobilized on a nitrocellulose membrane before probing for RNA binding using radiolabelled zipcode, comprising the first 233 nucleotides of the human β-actin 3′-UTR.

Immunoprecipitation. Phosphoprotein or protein–RNA complexes were isolated using anti-Flag beads (Sigma) before western blotting or quantitative RT–PCR, respectively. For details see Supplementary Methods.

Molecular cloning and siRNA. The human β-actin sequence (accession number CR625492, NCBI database) or a Δzipcode mutant (with nucleotides 1209–1441 removed) were cloned into pcDNA3.1 for *in vitro* transcription. For the luciferase reporter, the 3′ region of the β-actin cDNA sequence (zipcode, nucleotides 877–1808; Δzipcode, nucleotides 877–1208) was fused 3′ of the firefly luciferase coding sequence in pcDNA3.1. *ZBP1*-specific siRNAs were directed against a target sequence consisting of nucleotides 393–411 of human *ZBP1* (also known as *IMP-1* in human, accession number AF117106, NCBI database). For siRNA-insensitive ZBP1 mutants ($ZBP1_{insens}$), wobble bases were

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converted using site-directed mutagenesis without affecting the primary protein sequence.

FRET analysis. FRET measurements were made by acceptor photobleaching and imaging with a confocal laser scanning microscope (TCS SP2 AOBS, Leica). See Supplementary Methods for details.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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