Specific interaction of KIF11 with ZBP1 regulates the transport of β-actin mRNA and cell motility

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Abstract

ZBP1-modulated localization of β-actin mRNA enables a cell to establish polarity and structural asymmetry. While the mechanism of β-actin mRNA localization has been well revealed, the underlying mechanism of how a specific molecular motor contributes to transport of the ZBP1 complex in non-neuronal cells remains elusive. In this study, we report the isolation and identification of KIF11, a microtubule motor, which physically interacts with ZBP1 and is a component of β-actin mRNP. We show that KIF11 co-localizes with the β-actin mRNA and the ability of KIF11 to transport β-actin mRNA is ZBP1-dependent. We characterize the corresponding regions of ZBP1 and KIF11, which mediate the two protein’s interaction in vitro and in vivo. Disruption of the in vivo interaction of KIF11 with ZBP1 delocalizes β-actin mRNA and affects cell migration. Our study reveals a molecular mechanism that a particular microtubule motor mediates the transport of an mRNP through the direct interaction with an mRNA-binding protein.
Introduction

mRNA localization is a fundamental mechanism for spatial and temporal control of gene expression (Martin and Ephrussi 2009). A systematic investigation in *Drosophila* embryos reveals that more than 70% of endogenous transcripts are localized and the localization of mRNA corresponds to the localization of their encoded proteins (Lecuyer et al. 2007). In a variety of cell types and species, transport of messenger RNA to a specific cellular compartment enables localized translation, hence generating asymmetric distribution of proteins that are essential for the establishment and maintenance of cellular polarity, and structural asymmetry within the cell (Holt and Bullock 2009; Mili and Macara 2009).

Several recent studies in yeast and *Drosophila* have illuminated the roles that molecular motors play in the process of RNA localization. These studies have revealed complex mechanisms in which one motor protein or the coordinated action of a few motor proteins acts to direct transport and localization of RNAs to their final destination (Gagnon and Mowry 2011). Both dynein and kinesin motors have been implicated in RNA localization in *Drosophila* oocytes, while a type V myosin motor is required for transport of *ASH1* mRNA in budding yeast (Long et al. 1997; Brendza et al. 2000; Schorrer et al. 2000; Cha et al. 2002; Duncan and Warrior 2002; Januschke et al. 2002; St Johnston 2005). A general model suggests that, to localize RNAs, RNA binding proteins recognize localization elements of their target mRNAs while directly or indirectly connecting to molecular motors. Yeast *ASH1* and *Drosophila* pair-rule mRNAs have provided valuable evidence for this model, in which the unique interactions between RNA binding proteins and the motors are necessary in order to assemble an mRNP that is fully competent for transport and localization (Darzacq et al. 2003; St Johnston 2005).
Localization of β-actin mRNAs to the leading edge of migrating cells and to neuronal growth cones of extending axons associate with cell polarity, cell invasion and neuronal plasticity (Zhang et al. 1999; Condeelis and Singer 2005; Lapidus et al. 2007). The localization process relies on a trans-acting RNA-binding protein, ZBP1, which contains a unique combination of two RNA recognition motifs (RRMs) and four hnRNP K homology (KH) domains, and specifically recognizes a cis-acting zipcode within the 3’ untranslated region (UTR) of β-actin mRNA (Ross et al. 1997; Farina et al. 2003; Huttelmaier et al. 2005; Chao et al. 2010). Biochemical characterization of the ZBP1 recognition motif reveals that the ZBP1 KH34 region functions as a single unit to interact with the zipcode of β-actin mRNA (Chao et al., 2010). Knockdown of ZBP1 by siRNA impairs cellular adhesion, motility, and invadopodia formation (Vikesaa et al. 2006; Gu et al. 2012; Katz et al. 2012). Orthologues of the ZBP1 family include IMP1 (human), CRD-BP (mouse) and Vg1 RBP/Vera (Drosophila) (Yaniv and Yisraeli 2002).

Although the majority of localized RNAs are transported via the microtubule cytoskeleton (Bassell et al. 1998; Wilkie and Davis 2001; Singer 2008), transport of the ZBP1/β-actin mRNP seems to rely on both microtubules and/or actin filaments (Fusco et al. 2003; Oleynikov and Singer 2003). Recently, myosin Va and KIF5A have been shown to play roles in dendritic and axonal transport of β-actin mRNA (Ma et al. 2011; Nalavadi et al. 2012), and Rho mediated signaling pathway operating through a myosin IIB motor was responsible for the sorting of β-actin mRNA in fibroblasts (Latham et al. 2001). It could be hypothesized therefore that in order to properly transport β-actin mRNA, a specific recognition is required for a microtubule or actin motor with ZBP1 that acts as an adaptor protein to associate with the mRNA cargoes. Here, we report the isolation and identification of a kinesin motor, KIF11, which physically associates with ZBP1 in vivo to regulate the transport of β-actin mRNA. We
characterized the corresponding regions of ZBP1 and KIF11 through which the two proteins interact. Either inhibition of the motor activity of KIF11 or blocking the in vivo interaction of ZBP1 with KIF11 delocalizes β-actin mRNA at the cell leading edge and hence alters the cell migration ability. Our study demonstrates a novel mechanism that KIF11, through the direct interaction with ZBP1, regulates the transport of β-actin mRNP and leads to cell motility.
Results

*KIF11 associates with ZBP1 and is a structural component of β-actin mRNP*

In mobile cells, ZBP1 binds to β-actin mRNA and mediates its localized translation. In order to identify potential motor proteins that directly associate with ZBP1 and contribute to the transport process of β-actin mRNA, we prepared extracts from MDA231 breast carcinoma cells in which a FLAG-GFP-tagged human ZBP1 gene (IMP1) was genetically inserted (Gu et al. 2012), treated the cell extracts with RNase I and used anti-FLAG IgG-coated Sepharose to pull down proteins associated with ZBP1. Purified proteins were resolved by 10% SDS-PAGE and visualized by Coomassie blue staining (Fig. 1A). In contrast to the control precipitation (Fig 1A, lane 5), two distinctive protein bands of about 90 kDa and 110 kDa (Fig 1A, lane 4) were isolated. The faster migrating band was identified as FLAG-GFP-ZBP1 (data not shown). Mass spectrometry analysis of the slower migrating band revealed the identity to be KIF11, a motor protein of the kinesin-5 family (Fig. 1B). To determine whether KIF11 was specifically associated with ZBP1 and β-actin mRNA, we performed another pull-down assay. We prepared primary fibroblasts from embryos of a 14-day-old knock-in mouse strain (MBS strain), in which an MS2 binding site cassette was targeted to the 3’UTR of the β-actin gene (cells expressing β-actin-MS2 mRNA) (Lionnet et al. 2011). We then used the MS2-binding coat protein (MCP) of the RNA bacteriophage to affinity precipitate the β-actin-MS2 mRNA and its associated proteins. The ability to pull down β-actin-MS2 mRNA by MCP affinity precipitation was assessed by RT-PCR (Fig. 1C). The corresponding precipitates were then evaluated by immunoblotting analysis with anti-KIF11 and anti-ZBP1 antibodies (Fig. 1D). Both ZBP1 and KIF11 were co-precipitated with β-actin-MS2 mRNA (Fig. 1D, Prec.). The specificity of the results was tested by the control experiments in which MCP binding protein was not present (Fig. 1D, Prec.(c)).
**KIF11 colocalizes with β-actin mRNA in fibroblasts**

To investigate the subcellular localization of KIF11 and whether KIF11 was colocalized with β-actin mRNA, we isolated and cultured primary fibroblasts from an MBS mouse (MEF) and performed a combined assays using IF (immune-fluorescence) analysis with antibodies for KIF11 and in situ hybridization with fluorescently labeled MS2 oligonucleotide probes for β-actin mRNA. Distinctive β-actin mRNA were detected in the cell leading edge, owing to the presence of 24 MS2 binding sites at the 3’ UTR of the mRNA (Fig. 1E, left panel). KIF11 was also observed as granules and distribute in the cytoplasm of the MEF cell (Fig. 1E, middle panel). We superimposed the cell images where substantial amount of KIF11 and β-actin mRNA were colocalized (Right panel of Fig. 1E).

**Association of KIF11 with β-actin mRNA depends on ZBP1**

We then tested the relationship between KIF11, ZBP1 and β-actin mRNA. We used two stable MEF lines isolated from MBS mouse strain. In one line, ZBP1 was normally expressed and in another cell line, the ZBP1 gene was deleted (Katz et al. 2012). Affinity precipitation of β-actin-MS2 mRNA showed that KIF11 was co-precipitated in the presence of ZBP1, but not co-precipitated when the ZBP1 gene was not expressed (Fig. 2A, lower panel, right two lanes). This suggests that the association of KIF11 with β-actin mRNP was ZBP1-dependent.

**Inhibition of KIF11 activity or knocking-down KIF11 expression impairs the localization of β-actin mRNA**

The observation that KIF11 associated with ZBP1 and β-actin mRNA raised the possibility that KIF11 could play a role in the asymmetric transport and localization of the mRNA. To test this assumption, we treated primary fibroblasts with monastrol, a drug that specifically inhibits the motor activity of KIF11 (Mayer et al. 1999), and analyzed the endogenous β-actin mRNA
distribution by FISH experiments. Compared with untreated cells, in which about 34% of the
cells showed localized β-actin mRNA at the cell leading edge, localization was decreased to less
than 19% in monastrol-treated cells (Fig. 2B and 2C). A diminution of β-actin mRNA
localization to 17% was also observed in colchicine treated cells, where the microtubule structure
was destroyed (Fig. 2C). To determine the effect of monastrol treatment on cytoskeleton, we
immunostained the cells with anti-tubulin antibody and examined levels of β-actin mRNA using
RT-PCR, which indicated that both microtubule structure and β-actin mRNA expression were
not altered (Supplemental Fig. 1A and 1B). To further assess the involvement of KIF11 in β-
actin mRNA localization, we established two MDA231-ZBP1 stable cell lines in which KIF11
expression was knocked down using shRNA method. Normalized to the internal control of β-
actin, KIF11 in shRNA-1 cells was down-regulated to 44% and in shRNA-2 was to 41% (Fig.
2D). Experiments indicated that, in comparison to control cells, down-regulation of KIF11
delocalized β-actin mRNA from 29% to 20% or 16%, respectively (Fig. 2E). Thus, either
inhibition of KIF11 activity or down-regulation of KIF11 expression impaired β-actin mRNA
localization. We have previously reported that ZBP1, in addition to localize β-actin mRNA, is
also plays roles in localizing Arp-16 and α-actinin mRNAs, which are important for focal
adhesion dynamics (Gu et al. 2012). To address whether KIF11 has the same effect on the
localization of these two mRNAs, we performed FISH assays in KIF11 knock-down cells.
Results showed that localization of both Arp-16 and α-actinin mRNAs at the cell leading edge
was significantly reduced (Supplemental Fig. 2). These results suggest the importance of KIF11
on mRNA transport and localization.

*In vitro identification of the ZBP1 domain that binds to KIF11*
ZBP1 is an RNA-binding protein that contains six canonical domains with two RNA recognition motifs (RRM12) followed by four hnRNP K homology (KH1234) domains (Fig. 3A). Previous reports indicate that the KH34 domain of ZBP1 binds to the zipcode of β-actin mRNA (Chao et al. 2010). In order to determine the putative region of ZBP1 that was required for interaction with KIF11, we expressed, as maltose-binding fusion proteins (MBP), the full-length ZBP1 and ZBP1 truncates that contained ZBP1-RRM12, ZBP1-RRM12+KH12, ZBP1-KH1234, ZBP1-KH12 and ZBP1-KH34 domains in E.coli. (Fig. 3B) and affinity purified the fusion proteins with amylose resin (Supplemental Fig. 3A). We then attached equal amount of fusion proteins into amylose resin and tested the ability of the truncated ZBP1 protein(s) to pull-down endogenous KIF11 from a cellular extract (Fig. 3C). The experiments showed a strong interaction between the full length ZBP1 and KIF11. Truncated ZBP1 proteins that contained individual KH12, KH34 or KH1234 regions did not bind KIF11. However, the truncated ZBP1-RRM12 and ZBP1-RRM12+KH12 were co-precipitated with KIF11 (Fig. 3C), demonstrating that the RRM12 motifs of ZBP1 specifically recognized KIF11.

The tail domain of KIF11 binds to ZBP1 in vitro

KIF11 is composed of an N-terminal head domain (motor, residues 1-360), a stalk domain (residues 361-761) and a C-terminal tail domain (residues 762-1056) (Replay J. 2008; Fig. 4A). In addition, three coiled-coil regions within KIF11 were predicted by PAIRCOIL (Berger et al. 1995). In order to identify the potential regions of KIF11 that recognize and interact with ZBP1, we dissected KIF11 protein structure, generated various truncated KIF11 cDNAs by PCR and subcloned the PCR fragments into plasmid pMalc for expressing MBP-KIF11 fusion proteins (Fig. 4B). After identifying the purified fusion proteins by SDS-PAGE (Supplemental Fig. 3B), we used equal amount of proteins for in vitro pull-down analyses. We first tested whether the
head and stalk domain interact with ZBP1 in vitro (Fig. 4C, upper panel). Using extracts prepared from 293T cells, we found that endogenous ZBP1 was efficiently pulled down with the full length KIF11 and the KIF11 truncate (272-1056) containing both stalk and tail domains. However, the KIF11 stalk domain (362-761) alone showed a relatively weak binding to ZBP1, and no binding was seen with the head domain (residues 1-300). These data suggested that the regions other than head and stalk domains interacted with ZBP1. We then focused on the C-terminal region of KIF11 to assess the potential of the tail domain to interact with ZBP1 (Fig. 4C, lower panel). Consistent with the former experiments, full length KIF11 has strongest ability to pull down ZBP1 in a cell extract. Two tail-containing truncates of KIF11 with amino acid residues of 762-1056 and 557-1056, respectively, were able to effectively pull down ZBP1, while the 762-1056 tail fragment lacking the 40 amino acid residues at the N-terminus (802-1056) significantly decreased the binding affinity to ZBP1. These results indicated that the tail domain of KIF11 (762-1056) was sufficient and necessary to interact with ZBP1.

**RRM12 domain of ZBP1 directly interacts with the tail domain of KIF11**

Based on the above results, we anticipated that the recognition of KIF11 with ZBP1 could be through the direct interaction of the ZBP1-RRM12 domain with the KIF11 tail domain. To test this, we coupled MBP-tagged ZBP1-RRM12 to amylose beads, incubated with purified recombinant KIF11 tail fragment, and performed co-precipitation experiments. These experiments allowed for detecting the ability of proteins of interest to form stable complexes. As expected, the tail fragment of KIF11 was able to efficiently bind to immobilized ZBP1-RRM12 to form complexes (Fig. 5A, left panel). We next performed reciprocal experiments in which His-tagged KIF11 tail fragment was coupled to Ni-Sepharose beads and mixed with the recombinant ZBP1-RRM12 fragment. Consistently, the His-tagged KIF11 tail fragment formed
the complexes with ZBP1-RRM12 (Fig. 5A, right panel). These results indicate the mutual and direct recognition of the tail domain of KIF11 with the RRM12 domain of ZBP1 in vitro.

The KIF11 tail and the RRM12 domain of ZBP1 interact in vivo

To determine the in vivo interaction of the tail fragment of KIF11 with ZBP1, and the RRM12 domain of ZBP1 with KIF11, we infected 293T cells with lentivirus expressing MBP-fused truncates of KIF11 or His-tagged ZBP1 fragments, respectively. Meanwhile, we also infected cells with a lentivirus expressing tail domain of KIF5A, which has been shown to play a role in β-actin mRNA localization in neuronal cells (Ma et al. 2011). After analyzing the expression of fusion protein in infected cells (Supplemental Fig. 4A and 4B), we used amylose beads or Ni-Sepharose beads for co-precipitation experiments to analyze their in vivo mutual interactions. We observed that the tail region (Fig. 5B, lane 4), but not the the 1-300 and the 1-361 head regions of KIF11 (Fig. 5B, lane 2 and 3), was able to co-precipitate of endogenous ZBP1. In contrast to the tail domain of KIF11 (Fig. 5C, lane 2), the KIF5A-tail domain had no ability to co-precipitate with endogenous ZBP1 (Fig. 5C, lane 3). We also detected that the RRM12 domain was able to complex with endogenous KIF11 (Fig. 5D, lane 2), while the KH12 domain of ZBP1 did not precipitate KIF11 (Fig. 5D, lane 3). These data were consistent with the in vitro binding results, suggesting the in vivo physical association of ZBP1 with KIF11 through their corresponding domains.

Affecting the in vivo interaction of ZBP1-KIF11 delocalizes β-actin mRNA

We postulated that the in vivo competition, between KIF11 tail and endogenous KIF11, for binding to endogenous ZBP1 should interfere β-actin mRNA localization. To determine this, primary fibroblasts isolated from an MBS mouse were separately infected with lentivirus expressing mCherry, or Flag-tagged mCherry fused to the tail (762-1056) of KIF11, KIF5A-tail
or ZBP1-RRM12. Infected fibroblasts could be visualized by the mCherry signal and the expression of the protein was analyzed by Western blots (Supplemental Fig. 4C). Localization of β-actin mRNA was visualized by fluorescence in situ hybridization. In contrast to the control cells expressing mCherry (Fig. 6A), localization of β-actin mRNA to the cell leading edge was affected in the cells that expressed the mCherry-KIF11 tail or mCherry-ZBP1 RRM12 fusion protein (Fig. 6B and 6C). The percentage of localized β-actin mRNA was decreased roughly from 30% to about 17% in cells expressing the KIF11-tail (762-1056) or to about 18% in cells expressing ZBP1-RRM12 domain. While the cells expressing the mCherry-KIF5A tail (806-1032) fusion protein, showed little effect on β-actin mRNA localization (Fig. 6D). We then analyzed the pattern of cytoplasmic distribution of β-actin mRNAs in infected cells, which was determined as a polarization index using a custom MATLAB script (Gu et al. 2012). Bar graphs (Supplemental Fig. 4D) show that polarization index of β-actin mRNA in KIF11-tail or ZBP1-RRM12 cells are all lower than those in control and KIF5A-tail cells, indicating that cells expressing KIF11-tail or ZBP1-RRM12 leads to less polarized distributions of the mRNAs. These data suggested that the dominant-negative expression of the KIF11 tail or the ZBP1-RRM12 fragment impairs the in vivo interaction of endogenous ZBP1 and KIF11, resulting in decreased β-actin mRNA localization.

*Blocking in vivo interaction of ZBP1 with KIF11 alters fibroblast motility and increased invasiveness of breast carcinoma cells*

To determine the effect of KIF11-ZBP1 interaction on cell migration ability, we used MDA231/ZBP1 as parental cells to generate stable cell lines expressing mCherry (WT/mCherry), mCherry fusion proteins of KIF11-tail, ZBP1 RRM12 or KIF5A-tail, respectively. Western blots were utilized to evaluate the expression of the Mcherry fusion proteins (Fig 7A) and a pull-down
assay was used to confirm the ability of KIF11-tail fusion protein to interact with endogenous ZBP1 in vitro (Fig. 7B). We then selected two stable cell lines, one cell line expressing mCherry and the other expressing mCherry/KIF11-tail fusion protein, to test cell motility by using live-cell imaging. This provided an assessment of the differences in motility between genetically identical cell populations that differed only in their level of KIF11-tail expression. The experiments showed that, although the random velocity in two cell lines was not substantially changed (Fig. 7C, upper panel), KIF11-tail expression led to decreased directionality in cell motility (Fig. 7C, lower panel). These results suggest that dominant-negative expression of KIF11-tail could affect directional migration of these cancer cells, caused by the impairing of endogenous KIF11-ZBP1 interaction.

Gain of ZBP1 function in ZBP1 non-expressing MDA231 cells increased localization of β-actin mRNA and decreases cell invasion (Gu et al. 2012). To determine whether the in vivo interaction of ZBP1-KIF11 might also influence the invasive ability of carcinoma cell, we performed invasion analyses using Matrigel coated-transwells in the presence of a 10% FBS. The invasive ability of control MDA231-ZBP1/mCherry cells was not changed compared with MDA231 parental cells. However, cells expressing KIF11-tail or ZBP1-RRM12 fusion proteins exhibited near 40% increase of invasion through Matrigel. The observed invasiveness was not seen in the cells that express the KIF5A-tail (806-1032) (Fig. 7D), suggesting the importance of endogenous communication of ZBP1-KIF11 in invasiveness of cancer cells.
Discussion

KIF11 (also called Eg5 or kinesin-5) is a plus end kinesin motor with a primary function in assemble and maintenance of mitotic spindle (Kashina et al. 1997). The protein has a catalytic motor/ATPase domain that mediates its interaction with ATP and microtubules (Valentine and Gilbert 2007). Loss of KIF11 in mice results in abnormal spindle structure, rested cell cycle and lethality in embryonic pre-implantation (Castillo and Justice 2007). Overexpression of KIF11 slows neuronal migration and affects leading process development (Falnikar et al. 2011). In fibroblasts, KIF11 was shown to be involved in microtubule-dependent cell migration in the absence of myosin IIA (Even-Ram et al. 2007). We identified a novel role for KIF11 to regulate the transport of β-actin mRNA and cell motility through the direct interaction with ZBP1.

Our studies show that both KIF11 and ZBP1 are components of the β-actin mRNP complex. However, the ability of KIF11 to associate with β-actin mRNP depends on the presence of ZBP1. This provides evidence that ZBP1 is a connector protein for the molecular motor KIF11 and β-actin mRNA cargoes. Previously, it has been shown that the KH34 domain of ZBP1 is required for β-actin mRNA binding (Chao et al. 2008). We reveal the ZBP1-RRM12 motif that is responsible for interacting with KIF11. Moreover, we characterized the tail domain (762-1056) of KIF11 that is necessary for ZBP1 binding. The specificity and the biological significance of the mutual interaction of KIF11 and ZBP1 were further supported by the dominant-negative experiments in which overexpression of the KIF11 tail domain or ZBP1 RRM12 motif markedly delocalized β-actin mRNA and affected cell motility. These results strongly suggest that during the process of RNA localization, ZBP1 enables β-actin mRNP cargoes to be transported along microtubules by simultaneously binding, via its RRM12 domain, to KIF11 and binding, via its KH34 domain, to the zipcode of β-actin mRNA.
Previous studies have shown that ZBP1 is able to mediate directional motility of cells and to repress the invasion of breast cancer cells through regulating the localized expression of many adhesion- and motility-related mRNAs, including β-actin, Arp-16 and α-actinin mRNAs (Shestakova et al. 2001; Condeelis and Singer 2005; Jonson et al. 2007; Gu et al. 2012). These phenotypes have also been observed in our studies presented here, indicating the role for KIF11-ZBP1 interaction in maintaining directionality in cell motility and suppressing invasiveness of breast carcinoma cells. Based on the findings that KIF11 is responsible for transport of mRNAs important for cell migration and adhesion and that overexpression of KIF11-tail or ZBP1-RRM12 truncate affects cell motility and invasiveness, we conclude that the in vivo physical interaction of KIF11-ZBP1 contributes to the transport of mRNAs bound to ZBP1, causing a broader impact on cell polarity and motility.

It is well documented that kinesin motors are required for transporting mRNAs and mRNA binding proteins in neurons. Hirokawa and colleagues isolated a detergent-resistant RNase-sensitive granule from mouse brain that associated with KIF5 and showed that transport of RNA-containing granules in dendrites required the C-terminal tail of KIF5 (Kanai et al. 2004). KIF5A was also reported to co-localize with β-actin mRNP in the dendrites of rat cortical neurons (Ma et al. 2011). The involvement of particular kinesin motors to regulate the transport dynamics of β-actin mRNA in non-neuronal cells has not been previously shown. While we identified the requirement of KIF11 for the transport of β-actin mRNP in fibroblasts and carcinoma cells, KIF5A was not shown to be involved in this cellular process. This is not surprising because KIF5A is a neuronal specific kinesin motor and it suggests that neuronal and non-neuronal cells could use different mechanisms to transport β-actin mRNA.
Localization of oskar RNA in Drosophila oocytes not only requires kinesin-1 motor activity, but also needs myosin-V for short-range transport and to mediate the tight anchoring of the mRNA at the posterior pole (Brendza et al. 2000; Krauss et al. 2009). In neuronal cells, both actin-based motor myosin Va and/or microtubule-based motor KIF5A were required for the dynamic transport of β-actin mRNA granules (Ma et al. 2011; Nalavadi et al. 2012). In chicken fibroblasts, myosin IIB played a role in localizing β-actin mRNA regulated by a signal transduction pathway (Latham et al. 2001). β-actin mRNA was delocalized in the fibroblasts of myosin Va-null mice (Salerno et al. 2008). Our findings provide the first evidence that microtubule motor KIF11 is required for transporting ZBP1 cargoes to the leading edge of the cell. Combining these data, it is most likely that ZBP1 is as an adaptor protein for different motor machineries to direct RNA localization. The next challenge lies in determining how the microtubule motor is coordinated with myosin motor to transport and localize ZBP1/β-actin mRNA cargoes.

In summary, our study provides a novel molecular mechanism of how in mammalian cells, a particular microtubule motor contributes to mRNP transport through the recognition of a specific RNA-binding protein. These data begin to elucidate a sequence of events involved in β-actin mRNA transport: (1) RNA-binding protein ZBP1 binds to the zipcode of β-actin mRNA via its C-terminal KH34 domain (Farina et al. 2003; Chao et al. 2010), (2) other complementary proteins or translational factors assemble with ZBP1/β-actin mRNA to form a fully functional mRNP complex (locasome) (Huttelmaier et al. 2005; Pan et al. 2007), (3) the locasome is recognized by a motor through the interaction of KIF11 with the N-terminal RRM12 domain of ZBP1 to form a transport complex or granule, (4) KIF11 transports the locasome along
microtubules to the programmed destination and (5) the mRNA is released from the locosome for local translation (Buxbaum et al. 2014).
Materials and Methods

Isolation and identification of ZBP1-associated proteins

Cultured MDA231/GFP and MDA231/FLAG-GFP-ZBP1 (MDA231 stable cell line constitutively expressing FLAG-tagged GFP-ZBP1 fusion protein) cells were lysed in ice-cold lysis buffer (10 mM HEPES [pH 7.8], 100 mM NaCl, 10 mM KCl, 0.5% NP-40, with a protease inhibitor mixture (Roche). After centrifugation at 3,500 rpm for 5 min, cell debris was removed and supernatants were subjected to an additional step of high-speed centrifugation (14,000 rpm for 30 min at 4°C). After adding RNase A (Roche) to a final concentration of 1 µg/ml, the supernatants were incubated with agarose beads immobilized with FLAG-specific monoclonal antibody M2 (Sigma) with gentle rotation for 6 hrs at 4°C. The beads coupled with ZBP1 and its associated proteins were extensively washed in lysis buffer, eluted and separated on a 10% SDS–polyacrylamide gel. Protein bands, after Coomassie blue staining, were excised from the gel and subjected to MALDI-TOF at Rockefeller University.

Isolation of β-actin mRNA complexes

We used a knock-in transgenic mouse line in which a cassette of 24 MS2 binding sites (MBS) was targeted to the 3’UTR of the β-actin gene (Lionnet et al. 2011). β-actin mRNA complexes were purified from cytoplasmic extracts of embryonic fibroblasts of the knock-in mice using amylose resin-attached MBP-MCP (MBS binding protein) or amylose resin-attached MBP, respectively. An aliquot of precipitates was used for extraction of total RNAs for RT-PCR, and the remainder was used for SDS-PAGE and Western blots using ZBP1 (Santa Cruz Biotechnology, Cat# SC-166344) and KIF11 (Novus Biologicals, Cat# NB100-78467) antibodies.
Primary cell preparation, fluorescence in situ hybridization (FISH), immunofluorescence (IF) and microscopy

Primary 14-day-old mouse embryo fibroblasts (MEFs) were isolated from an MBS mouse. The MBS cassette provides a means for high sensitivity fluorescence in situ hybridization (FISH), allowing detection and localization of β-actin mRNA molecules in mouse cells (Lionnet, T et al., 2011). Cover slips were coated with fibronectin (Sigma, working solution 10 μg/ml, stock solution 1 mg/ml) for 30 min and washed with PBS. Approximately 2x10^4 cells were inoculated (12-well plates) on the fibronectin-coated cover slips. For serum-starvation, MEF cells were washed in Hank’s balanced saline solution and incubated overnight in Dulbecco’s modified Eagle’s medium (DMEM) with 0.5% BSA. Cells were serum stimulated with fresh DMEM plus 10% FBS for 30 min, followed by fixation in 4% formaldehyde. In some experiments, cells were treated with 10% FBS culture medium containing monastrol (working solution 100 μm; stock solution 10 mm in DMSO) or with 10% FBS culture medium containing colchicine (working solution 10 μg/ml, stock solution 10 mg/ml in ethanol), respectively, for 30 min and fixed in 4% formaldehyde. Coverslips containing treated cells were then processed for FISH and IF assays as previously described (Lionnet et al. 2011; Gu et al. 2012). For IF/FISH combination assays, cells were first incubated with primary and secondary antibodies, fixed in 4% formaldehyde for 5 min and then hybridized with Cy3-labelled oligonucleotides. β-actin mRNA signal was visualized using an Olympus BX61 microscope with a UPlanApo 1003, 1.35NA objective (Olympus) coupled to an X-Cite 120 PC metal halide light source (EXFO Life Science). Images were captured using MATLAB software. β-actin mRNA was judged to be localized when most of the colored in situ signal was asymmetrically distributed in the leading edge (Kislauskis et al. 1997). Analysis of cytoplasmic β-actin mRNA distribution
The intracellular polarization and localization of β-actin mRNA were quantified using a custom MATLAB code (Gu et al, 2012). Briefly, the cells were segmented from the FISH images, and the centroid of the nucleus and the intensity-weighted centroid of the cytoplasmic RNA were located. To avoid potential effects that might result from cell shape and size, the polarization index was defined as the distance between the two centroids divided by the radius of gyration of the cell. The radius of gyration was calculated as the root mean square distance of the pixels within the cell area from the centroid of the cell. A higher polarization index indicates a more asymmetric distribution of β-actin mRNA within the cells.

Cell transfection and infection

For transfection assays, truncates of KIF11 and ZBP1 constructs were transfected, using FuGENE 6 (Roche) or Lipofectamine 2000 (Invitrogen), into MEFs or MDA231 cells that were inoculated onto coverslips. After transfection, the MEFs were incubated at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 24 h prior to washing with PBS and fixation using 4% formaldehyde in PBS/5 mM MgCl₂ for 20 min. For cell infection assays, lentiviral vectors were constructed by subcloning cDNAs encoding mCherry fusion KIF11 or ZBP1 truncates into the pUBC vector. A pGIPZ lentiviral vector encoding an shRNA for KIF11 was purchased from Open Biosystems. Lentiviruses were generated by co-transfecting 20 μg of lentiviral vector and 1 μg of five individual packaging vectors (coding for Gag, Pol, Tat, Rev and VSVG) in HEK 293T cells by using Fugene 6 Transfection Reagent (Roche). Supernatants were collected three times for every 24 hours after transfection and filtered through a 0.4-μm membrane. Viral particles were pelleted by centrifugation at 4,000g using Lenti-X concentrator (Clontech). The pellets were resuspended in 200 μl serum-free medium. 50 μl of the viral suspension was mixed with 0.5 ml culture medium
(serum-free) and used to infect MDA231, MEF or T47D cells that were seeded in a six-well dish with 30% confluence in the presence of 3 μg/ml polybrene (Sigma). Stably infected cell clones were selected by puromycin or sorted by flow cytometry at Einstein College of Medicine.

Recombinant protein expression and purification

cDNA fragments coding for KIF11 and ZBP1 truncates were PCR amplified and were subcloned into a pMalc plasmid (New England Biolabs, NEB) after the MBP gene or in some cases, into a pET21 vector (Novagen). Recombinant protein expressed from pET21 vectors contained a His<sub>6</sub> tag at the C-terminus. The constructs were transformed into the Rosetta2 E. coli strain (NEB), and expression of the recombinant proteins was induced with 0.5 mM IPTG for 4.5 hours.

Purification of recombinant proteins was performed according to manufacturer’s protocol with slight modifications. Briefly, cells were harvested by centrifugation and were resuspended in lysis buffer (50 mM Tris-HCl, 1.5 M NaCl, 1 mM EDTA, pH7.5) supplemented with protease inhibitors. After sonication and clearing of the cell lysate at 12,000 rpm, the cell extracts that contained MBP-fusion proteins were incubated with amylose beads on ice overnight with gentle stirring. After the beads were extensively washed with lysis buffer, the affinity-attached MBP-fusion proteins were eluted with a gradient of maltose ranging from 0.5 to 10 mM. Purity of the proteins was determined by SDS-PAGE and Coomassie blue staining. Fractions containing pure proteins were pooled and extensively dialyzed against lysis buffer to remove maltose residues.

In Vitro pull down experiments

Cell extracts were prepared from cultured 293T or MDA231-IMP1 cells. Pull down experiments were performed using MBP-KIF11 or MBP-ZBP1 fusion proteins. Briefly, equal amount of recombinant MBP fusion proteins (0.1-0.5 μg) were attached to 50 μl amylose beads and washed twice with 200 μl of lysis buffer. Beads were incubated with 200 μl cell extract on ice for 4 h.
with gentle agitation. After centrifugation, the amylose beads were washed five times with 200 µl of lysis buffer, followed by a final wash with 50 µl of lysis buffer. Bound proteins were eluted with 30 µl of 20 mM maltose. Eluted fractions were analyzed by SDS-PAGE and western blots using monoclonal anti-ZBP1 (kindly provided by Dr. Huttelmaier) or anti-KIF11 antibodies (Sigma Inc), respectively.

*In vitro detection of direct protein-protein interactions*

His-tagged KIF11 truncates or MBP-ZBP1 fusion proteins were expressed in E. coli and purified. About 5 µg His-tagged tail domain of KIF11 was attached into Ni-beads (Qiagen) and separately incubated with 5 µg purified MBP1-RRM12 of ZBP1 for 6 hours at 4°C with continuous stirring. Reciprocally, 5 µg MBP-RRM12 was attached into maltose beads and incubated with 5 µg purified tail domain of KIF11 for 6 hours at 4°C with continuous stirring. The beads were then pelleted and washed 5 times with binding buffer. Bound proteins were eluted from Ni-beads with 400 mM imidazole or eluted from maltose beads with 20 mM maltose, respectively. Eluted proteins were analyzed by SDS-PAGE and Coomassie blue staining.

*Cell motility assays*

Cells were cultured in wells of a glass bottomed 24-well plate (MatTek) in DMEM medium containing 10% FBS. Before imaging, media was changed to L-15 (Gibco) containing 10% FBS. Cell motility imaging was performed on an IX71 inverted Olympus microscope at 20X using multifield acquisition driven by MetaMorph software. Environmental conditions were controlled for the entirety of the 4 hour motility experiment through the use of a heated chamber. Motility analysis was performed on images at 2 minutes intervals over the 4-hour period as a way of fully analyzing a path of cell movement. Tracking was performed in ImageJ using the Manual Tracking and Chemotaxis Tool Plugins freely available on the ImageJ website. Detailed
explanation of motility parameters described in Soll (Soll 1995). Centroid coordinates were user-determined and later used to calculate the motility statistics presented in the results.

*Cell invasion assays*

Cell invasion assays were performed using transwells (BD Biosciences) that were pre-coated with Matrigel. Briefly, serum-starved cells were suspended in 400 μl DMEM medium supplemented with 0.5% bovine serum albumin (BSA) and placed into the transwell chambers (2×10⁴ cells). The chambers were inserted into 24-well culture dishes containing 500 μl DMEM medium with 10% FBS. Cells were allowed to invade through the Matrigel for 16 hours. The invasive cells underneath the chamber were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes and stained with 0.2% crystal violet in 2% ethanol for 10 minutes. Noninvasive cells were scraped from the top chambers. The level of invasion was quantified by visual counting of the cells on the underside of the membrane. Each experiment was performed three times, and the results were expressed as means ± s.e.m.
Legends:

Figure 1: KIF11 binds to ZBP1 and is a component of the β-actin mRNA complex.

(A) Co-immunoprecipitation was performed to identify the proteins directly associated with ZBP1 in extracts of MDA231/GFP-FLAG-ZBP1 (lane 2) and MDA231/GFP (lane 3) cells. Precipitates were resolved on 4-12% SDS-PAGE and stained with Coomassie blue. Two protein bands with the molecular weight of about 90 kD and 110 kD, respectively, were identified in the precipitates of the MDA231/FLAG-GFP-ZBP1 cell extract (lane 4, * labeled), but not in the MDA231/GFP cell extract (lane 5). Western blots showed that the faster migrating band was the FLAG-GFP-ZBP1 (not shown). (B) The upper migrating protein band was excised and sent for peptide sequence analysis. Red sequences indicate sequenced peptides that completely match with the sequences of human KIF11. (C) β-actin mRNP was purified from cytoplasmic extracts of MEF cells expressing the β-actin-3’UTR-MBS24, using amylose/MBP resin or amylose/MBP-MCP resin. Total RNAs were isolated from starting extracts (Ext), flow-through of amylose/MBP-MCP resin (FT) and amylose/MBP resin (FT(c)), and the precipitates of amylose/MBP-MCP (Prec.) and amylose/MBP resin control (Prec.(c)). RT-PCR was performed to detect the presence of β-actin mRNA in the RNA samples. Lane L: DNA ladder. (D) Western blots to analyze the presence of KIF11 using rabbit anti-KIF11 antibody and ZBP1 using mouse anti-ZBP1 antibodies in the precipitates. KIF11 and ZBP1 were co-precipitated with β-actin mRNA using amylose/MBP-MCP resin (Prec.), but not precipitated in the control sample using amylose/MBP resin control (Prec.(c)). Ext: total extracts. (E) Primary 14-day-old mouse embryo fibroblasts (MEF) of an MBS mouse were cultured on fibronectin-coated coverslips, fixed and processed for immunofluorescence using antibodies against KIF11 (green) and followed by FISH experiments using Cy3-labelled oligonucleotides for β-actin mRNA (red). Blue color
indicates the nuclear position. KIF11 was mostly cytoplasmic and colocalized with β-actin mRNA. Scale bar: 10µm.

Figure 2: Inhibition of KIF11 motor function or Knocking-down KIF11 expression impairs the localization of β-actin mRNA in MEF cells.

(A) Association of KIF11 with β-actin mRNA is ZBP1-dependent. Cytoplasmic extracts of MEF/MBS cells (lane Ext (w)) and ZBP1 gene-deleted MEF/MBS cells (lane Ext (△ZBP1)) were prepared. β-actin mRNP was purified from the extracts using amylose resin-attached MBP-MCP (lanes Prec.(w) and Prec.(△ZBP1), respectively). Total RNAs were isolated from starting extracts and the precipitates of amylose/MBP-MCP. RT-PCR was performed to detect the presence of β-actin mRNA in the RNA samples. The negative control for RT-PCR was indicated as Cont. (upper panel). Western blots were utilized to detect co-precipitated ZBP1 (middle panel) or KIF11 (lower panel) with β-actin mRNA. (B) Primary MEF cells were cultured and serum stimulated for 30 min. Fluorescence in situ hybridization (FISH) was performed to determine β-actin mRNA localization in MEF cells treated with monastrol. Blue color indicates the position of nuclei. Scale bar: 10 µm. (C) Localization of β-actin mRNA to the leading edge of the cell was decreased in monastrol- or colchicine-treated cells. An average of 60 cells was counted from three independent experiments. (D) Western blots showing the expression levels of KIF11 protein in MDA231-ZBP1/KIF11 shRNA cells. Numbers below the bands reflect the relative levels of KIF11 normalized to β-actin. The arrows indicate the detected proteins. (E) Graph showing the percentages of cells with localized β-actin mRNA was significantly reduced in MDA231-ZBP1/KIF11 shRNA-1 cells and MDA231-ZBP1/KIF11 shRNA-2 cells, which represent the data from three independent experiments.
Figure 3: The RRM12 motif of ZBP1 binds to KIF11 in vitro.

(A) A schematic diagram of ZBP1 showing conserved domain organization. (B) Representative drawing of recombinant proteins for full-length ZBP1, ZBP1-RRM12+KH12, ZBP1-KH34, ZBP1-KH1234, ZBP1-RRM12 and ZBP1-KH12 fragments used in this study. (C) Pull down assays and western blots were performed to detect co-precipitated KIF11. Results indicate that the full-length ZBP1, ZBP1-RRM12+KH12 and ZBP1-RRM12 fragments co-precipitated with KIF11 in the cell extracts.

Figure 4: Identification of the ZBP1-interacting domain of KIF11.

(A) A schematic diagram of KIF11 showing domain organization. The predicted regions that form coiled-coil structures are indicated. (B) Representative drawing of recombinant fragments of KIF11 used in the experiments to identify regions responsible for binding to ZBP1. (C) MBP-KIF11 fusion proteins were attached to amylose beads and incubated with extracts of 293T cells. Co-precipitation and western blots for ZBP1 indicate that the head domain of KIF11 does not bind to ZBP1, the stalk domain shows a weak interaction with ZBP1, while the tail domain (762-1056) containing KIF11 fragments were effectively bound to ZBP1. Ext: cell extracts; C: control co-precipitation experiment with MBP protein coupled amylose beads.

Figure 5: Direct interaction of the ZBP1 RRM12 motif with KIF11 tail domain.

(A) ZBP1-RRM12 directly interacts with KIF11 tail domain (762-1056). Left panel: lane 1, maltose beads without attached proteins were incubated with his-tagged KIF11 tail. Lanes 2 and 3, MBP-RRM12 was attached to maltose beads and incubated with the tail domain of KIF11 or buffer containing BSA, respectively. Right panel: lane 1, Ni-beads without attached proteins were incubated with ZBP1-RRM12. Lanes 2 and 3, Ni-beads attached with His-tagged KIF11 tail fragment were incubated with buffer containing BSA or the ZBP1-RRM12 motif,
respectively. Staining after SDS-PAGE was performed with Coomassie blue. **(C)** KIF11 tail domain interacted with endogenous ZBP1. Pull-down experiments were performed with extracts of 293T cells that were infected with lentivirus expressing MBP fusion truncates of KIF11 (1-300) (lane 2), KIF11 (1-363) (lane 3) or the KIF11 tail domain (762-1056) (lane 4), respectively, using amylose affinity chromatography and analyzed by western blots using ZBP1 antibodies. Lane 1, total cell extracts of 293T cells. **(D)** KIF5A tail domain was not bound to ZBP1 in vivo. Pull down experiments were performed with the extracts of 293T cells infected with lentivirus expressing MBP fusion truncates of KIF11 tail domain (762-1056) (lane 2) or KIF5A tail domain (806-1032) (lane 3), and analyzed by western blots using ZBP1 antibodies. Lane 1, total cell extracts of 293T cells. **(E)** Analyzing the *in vivo* interaction of the RRM12 domain of ZBP1 with endogenous KIF11. 293T cells were infected with lentivirus expressing His-tagged ZBP1-RRM12 (lane 2) or ZBP1-KH12 (lane 3), respectively. Pull-down assays were performed from the extracts of infected cells using Ni-Sepharose beads. The precipitates were analyzed by western blots using antibodies against KIF11. Lane 1: control pull-down assay with extracts of uninfected 293T cells.

**Figure 6: Impairing the *in vivo* interaction of ZBP1 with KIF11 delocalizes β-actin mRNA to the cell leading edge.**

MEF cells were infected with an mCherry lentivirus construct, and the constructs expressing the mCherry-KIF11 (702-1056) tail domain, mCherry-KIF5A (806-1032) tail domain, or the Mcherry-RRM12 motif of ZBP1, respectively. After fixation and permeabilization, FISH experiments were performed to detect localization of β-actin mRNA. **(A) and (B)** Representative images showing β-actin mRNA localization in cells expressing mCherry or mCherry-KIF11 tail fusion protein, respectively. **(C)** Bars show the percentage of infected cells counted with
localized β-actin mRNA at the leading edge. An average of 50 cells were counted blind per coverslip in three experiments each.

**Figure 7: Expression of the dominant-negative KIF11-tail fragment affects carcinoma cell motility and increases cell invasive ability.**

(A) Western blots were performed to detect protein expression in the stable cells expressing mCherry (not Flag-tagged) (lane 1), Flag-tagged mCherry/KIF11-tail fusion protein (lane 2), Flag-tagged mCherry/ZBP1-RRM12 fusion protein (lane 3) and Flag-tagged mCherry/KIF5A-tail fusion protein (lane 4) using anti-Flag antibodies. The arrow indicates the position of β-actin.

(B) Pull-down experiments were performed with extracts of MDA231-ZBP1 cells infected with lentivirus expressing Flag-tagged Mcherry/KIF11 tail (lane 2) or KIF5A/tail (lane 3), respectively, using anti-Flag argrose beads. The precipitates were analyzed by western blots using ZBP1 antibodies. Lane 1, total cell extracts of MDA231-ZBP1 cells. (C) Motility analysis was performed by tracking stable cells expression mCherry or mCherry-fused KIF11 tail domain under regular growth conditions at 2-minute intervals over a 4-hour period. Expression of the KIF11-tail domain did not change the random velocity (P=0.0561), but decreased directionality in cell motility (cells expressing mCherry versus cells expressing mCherry KIF11-tail fusion protein, n=31 and 32. P=0.027). (D) Overexpression of KIF11-tail or ZBP1-RRM12 increases invasive ability of carcinoma cells. Stable cells expressing different mCherry fusion proteins were seeded in serum-free medium into the upper chamber of 8 μm pore Matrigel-coated transwell filters. The lower chamber contained medium with 10% serum. Cells that had invaded to the underside of the filter were stained and counted 16 hours later. Invasion was quantified by visual counting of the total cells on the underside of the filter. The relative numbers of invading cells from each assay are normalized to parental MDA231-ZBP1 clone and are represented as a
fold change to the MDA231-ZBP1. Data shown in the figure represent the means ± s.e.m. of data from three experiments.
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Author contributions:

Tingting Song and Yi Zheng designed and carried out major biochemical experiments.

Zachary Katz and Yarong Wang designed, carried out and interpreted life cell imaging and cell motility experiments.

Robert H. Singer coordinated the study and participated in revising the manuscript.

Wei Gu designed research plan, coordinated the study, carried out cell biologic experiments and prepared manuscript.

Competing interests

The authors have declared that no competing interests exist.

Abbreviations

ZBP1, zipcode binding protein 1; KIF11, kinesin family protein 11; MEF, mouse embryo fibroblast; GFP, green fluorescent protein; TIRF, total internal reflection fluorescence.
References


Tingting Song_ Fig. 1

B

\begin{align*}
1 & \text{NASQFNNSSAK} \quad \text{KKEERGKNIQ} \quad \text{VVVRCRFNPL} \quad \text{AERKASAHI} \quad \text{VECDPVRKEV} \\
51 & \text{SVRTGGLADK} \quad \text{SSRTYTFDM} \quad \text{VFGASTIQID} \quad \text{VRASVCIFIL} \quad \text{DEVMSYNTC} \\
101 & \text{FAFYQGTGTG} \quad \text{KTTMEGERS} \quad \text{PMKUYTMEED} \quad \text{PLAGIIIPRTL} \quad \text{BQIFKBLDN} \\
151 & \text{GTEFSVRKIL} \quad \text{LEYNNHEELD} \quad \text{LNNPSEEDSE} \quad \text{RJQMDPPDRN} \quad \text{KPGYVLKOL} \\
201 & \text{KTVNIDKKE} \quad \text{YQILKEKAK} \quad \text{RTAAATLWMA} \quad \text{YSSRSHFSYPS} \quad \text{VTIPHNRETIT} \\
251 & \text{DOEELVKGK} \quad \text{LNLVLDASGE} \quad \text{NIOUGSDAVKD} \quad \text{RAREAIGNIQ} \quad \text{SLLTSLQIIT} \\
301 & \text{ALVERTPHNP} \quad \text{YRESKLTRIL} \quad \text{QDSLOGRTAP} \quad \text{SIIATASPA} \quad \text{LMLSTTLSTL} \\
351 & \text{EYARHAKKIL} \quad \text{NPKFVQNLKT} \quad \text{KKALIKYIEZ} \quad \text{ERIELKKBIA} \quad \text{AAREKKNVYI} \\
401 & \text{SEENFRVMSG} \quad \text{KTVQSERQIV} \quad \text{ELIEKIGAVE} \quad \text{EELNRTVEFL} \quad \text{MDGNSEGQNC} \\
451 & \text{KSDLQNKJQ} \quad \text{LETTRQRHLQ} \quad \text{TQLQILKVEEE} \quad \text{ITSALESTE} \quad \text{KILHDAASKL} \\
501 & \text{NTVZETTRDV} \quad \text{SGHSLKLRDK} \quad \text{KAVQHNMAEA} \quad \text{QDIFQIKNMS} \quad \text{LPNNBHEELIR} \\
551 & \text{DGSEQKHAL} \quad \text{EVPHTLTGPP} \quad \text{LSSSSALDTI} \quad \text{ITTVGERSTI} \quad \text{HIPVSTHTV} \\
601 & \text{SQIFRMILKE} \quad \text{QSLAASEKTV} \quad \text{LQELINLVKL} \quad \text{DLLSSEMLIL} \quad \text{SPTVSILKI} \\
651 & \text{NSQLRHKFLKT} \quad \text{SLTVADKED} \quad \text{QQKELDGLFLS} \quad \text{ILCNSNHLEILQ} \quad \text{ENTICSLIVES} \\
701 & \text{QQCQONLED} \quad \text{LQCTQTHGQ} \quad \text{ELCQGMLWFT} \quad \text{BRFCAEEKRC} \quad \text{ENIQKPDLSY} \\
751 & \text{QSHEIQQRKID} \quad \text{TVHMTMPHSQ} \quad \text{KPCADSDQDS} \quad \text{QELMNFIQEG} \quad \text{TKLVEEEVKN} \\
801 & \text{SDKLNQNLNEK} \quad \text{IAORTEQRCEQ} \quad \text{SLNRTTVFYS} \quad \text{RQWVSSLER} \quad \text{EQELLNLEVL} \\
851 & \text{VSCQCEASSS} \quad \text{DITEKSGDRK} \quad \text{AAILEKQNHIF} \quad \text{LDQMTIDEDK} \quad \text{LIAQNLNLENE} \\
901 & \text{TIIKGLTLKN} \quad \text{CFLEQDLKLD} \quad \text{IPTGTIPQRK} \quad \text{SYLYPSTLVR} \quad \text{TEFREHLDQ} \\
951 & \text{LLEKQPELM} \quad \text{MLNCRRHEH} \quad \text{ETIPDVVDEE} \quad \text{AVLQGTYEEP} \quad \text{LSQEPVSVDAG} \\
1001 & \text{VDCCSISGGGP} \quad \text{FPQHHKSHGR} \quad \text{DKEHNGINTL} \quad \text{ERSKVEETTE} \quad \text{HLVTKSRPL} \\
1051 & \text{RAQINL} \\
\end{align*}

C

\(\beta\)-actin

D

\(\beta\)-actin mRNA
KIF11

Merge