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Review

How and why does β-actin mRNA target?

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Abstract

β-Actin mRNA is localized near the leading edge in several cell types where actin polymerization is actively promoting forward protrusion. The localization of the β-actin mRNA near the leading edge is facilitated by a short sequence in the 3′UTR (untranslated region), the 'zipcode'. Localization of the mRNA at this region is important physiologically. Treatment of chicken embryo fibroblasts with antisense oligonucleotides complementary to the localization sequence (zipcode) in the 3′UTR leads to delocalization of β-actin mRNA, alteration of cell phenotype and a decrease in cell motility. The dynamic image analysis system (DIAS) used to quantify movement of cells in the presence of sense and antisense oligonucleotides to the zipcode showed that net pathlength and average speed of antisense-treated cells were significantly lower than in sense-treated cells. This suggests that a decrease in persistence of direction of movement and not in velocity results from treatment of cells with zipcode-directed antisense oligonucleotides. We postulate that delocalization of β-actin mRNA results in delocalization of nucleation sites and β-actin protein from the leading edge followed by loss of cell polarity and directional movement. Hence the physiological consequences of β-actin mRNA delocalization affect the stability of the cell phenotype.

Introduction

In diverse cell types, asymmetric localization of specific mRNAs generates cell polarity by controlling sites of translation and restricting target proteins to specific subcellular compartments (Bashirullah et al., 1998; Zhang et al., 2001). During oogenesis in *Drosophila* and *Xenopus*, polarized localization of maternal mRNAs establish embryonic patterning (Ephrussi et al., 1991; Bashirullah et al., 1998; Deshler et al., 1998). In *Saccharomyces cerevisiae*, mating type switching is regulated by targeting ASH1 mRNA to the bud tip (Long et al., 1997). Asymmetric segregation of mRNAs in subcellular locations is also observed in somatic cells. Examples are tau mRNA in neurons, myelin basic protein (MBP) mRNA in oligodendrocytes (Kleiman et al., 1994; Behar et al., 1995; Ainger et al., 1997) and β-actin mRNA in crawling cells. β-Actin mRNA is localized near the leading edge of chick embryo fibroblasts (Sundell and Singer, 1991), 3T3 fibroblasts (Hill et al., 1994), endothelial cells (Hoock et al., 1991), myoblasts (Hill and Gunning, 1993), and non-metastatic (but not metastatic) adenocarcinoma cells (Shestakova et al., 1999). Localized mRNA maintains cellular asymmetry, presumably by restricting the synthesis of its protein product to a specific compartment of the cell. This creates a micro-environment in which the newly synthesized protein would be available in much greater amounts than elsewhere, and be regulated by other locally available macromolecules.

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Key words: actin polymerization, cell motility, cell polarity, mRNA localization.

Abbreviations used: DIAS, dynamic image analysis system; EBS, EF1α-binding site; EF1α, elongation factor 1α; FH, formin homology; MBP, myelin basic protein; NFAP, non-filamentous actin-containing particles; UTR, untranslated region; ZBP1, zipcode binding protein 1.

The importance of targeting β-actin mRNA for the assembly of β-actin-specific actin compartments

In situ hybridization has shown that β-actin mRNA is localized at the front of cells near the leading edge of lamellipods of crawling cells (Hill and Gunning, 1993; Sundell and Singer, 1990). Furthermore, the localization of β-actin mRNA is correlated with the localization of β-actin protein to apical structures such as filaments in microvilli of epithelia and auditory hair cells (Hofer et al., 1997), and the leading edge of lamellipodia and filopodia (needle-shaped protrusions that may have a pathfinding function) of crawling cells (Shestakova et al., 2001). This is in contrast with other actin isoforms, α and γ, whose mRNAs are not targeted to the cell front, and at the protein level are located perinuclearly and in stress fibres (Hill and Gunning, 1993; Kislauskis et al., 1993). The specific targeting of β-actin mRNA near apical structures suggests that local protein synthesis contributes to the localization of β-actin protein in apical structures. Based on the estimated 2500 β-actin mRNAs per crawling cell, at the established translation rate of 1.5 actins per second per mRNA molecule, the cell would synthesize 3900 actin molecules per second, or 2.4×10^5 per min (Kislauskis et al., 1997). In a crawling cell, the polymerization of actin at the leading edge uses about 3.6×10^6 actin molecules per minute (Chan et al., 1998). Thus, translation could supply 7% of the actin for polymerization. If the mRNA is scattered throughout the cell it is unlikely that this 7% would significantly alter the rate of polymerization. However, if the mRNA is targeted to a single location, and the β-actin protein is translated in this restricted volume, the local contribution of newly synthesized actin to polymerization could be substantial. Furthermore, if the locally synthesized actin is of a single isoform that has isoform-specific interactions with actin-binding proteins, then the effects of mRNA targeting and local translation could have profound effects on the actin cytoskeleton.

Targeting of mRNA and its protein product to specific compartments may involve multiple steps including transport, anchoring and local translation. Hence, mRNA transport, anchoring, local translation and isoform-specific sorting at the protein level may all contribute to the localization of β-actin to apical structures. The contribution of local translation has not been evaluated because the translation of targeted mRNA has not been observed *in vivo*. Thus, the timing and location of translation relative to the appearance of β-actin in apical structures has not been determined. However, the mechanisms of transport, anchoring and β-actin isoform-specific sorting have been studied at the molecular level.

There could be several reasons for suppression of cell motility upon delocalization of β-actin mRNA. Cell motility requires actin polymerization in the leading edge. Cells with delocalized β-actin mRNA may not polymerize actin filaments at the same rate if β-actin is not synthesized at sites of polymerization. As a result the cells would have a lower velocity of protrusion which is driven by actin polymerization. Alternatively, the rate of actin polymerization may be unaffected by actin synthesis. Instead, the site of actin synthesis may affect the location of nucleation of actin polymerization that would define the direction of protrusion and, therefore, polarity of movement.

The 3′UTR (untranslated region) of β-actin mRNA contains a zipcode that targets mRNA *in vivo*

In somatic cells the most common mechanism of mRNA localization is targeted transport, which involves attachment and vectoral translocation on the cytoskeleton, mediated by a specific 3′UTR localization sequence (Sundell and Singer, 1991; Ainger et al., 1993; Knowles et al., 1996; Carson et al., 1997; Long et al., 1997; Takizawa et al., 1997). The 3′UTR sequence required for β-actin mRNA localization is well defined. Analysis of the 3′UTR showed that a 54 nucleotide segment, just 3′ of the stop codon, could direct the localization of the entire β-actin mRNA. This segment was termed the zipcode (Kislauskis et al., 1993; Kislauskis et al., 1994). The β-actin zipcode sequence is absolutely essential for correct targeting of β-actin mRNA, and can be used to target unrelated marker mRNAs ectopically (Kislauskis et al., 1994). Localization of mRNA can be inhibited by antisense oligonucleotides directed against

Lamellipod: Flat protrusion parallel to the substratum of crawling cells.

the zipcode sequence (Kislauskis et al., 1994) or by disrupting the secondary structure of the zipcode (St Johnston, 1995; Ross et al., 1997).

In somatic cells, sequence analysis has revealed several regions in the zipcode that are conserved among β-actin mRNAs of several species, in particular the sequence ACACCC. A 68 kDa protein was shown to bind specifically and with highest affinity to β-actin zipcode oligonucleotides attached to chromatography beads and was therefore named zipcode binding protein 1, or ZBP1. Mutations of the ACACCC motif in the zipcode of β-actin mRNA disrupts mRNA localization *in vivo* and binding of ZBP1 to the zipcode of β-actin mRNA *in vitro* (Ross et al., 1997). ZBP1 is essential for the localization of β-actin mRNA (Oleynikov and Singer, 2003). Fragments of ZBP1, a KH-domain RNA-binding protein, that bind β-actin mRNA act as dominant negative for localization, presumably because the RNA-binding region blocks the full-length endogenous protein from assembling a transport particle around the RNA (Farina et al., 2003). Significantly, this dominant-negative fragment also inhibits fibroblast cell motility and polarity. In addition to fibroblasts, the same ZBP1 is essential for the formation of dendritic filopodia and filopodial synapses (Eom et al., 2003). All of this evidence points to an important contribution of β-actin mRNA localization to cell structure and function.

mRNAs are transported on microtubules and actin filaments

The development of high resolution *in situ* hybridization has revealed that many localized mRNAs are present as granules *in vivo* (Sundell and Singer, 1991). RNA labelled with the vital dye SYTO 14 demonstrates that granules containing ribosomal proteins, EF1α (elongation factor 1α) and poly(A) mRNA are transported on microtubule tracks at a rate of 0.1 μm/sec in neurons (Knowles et al., 1996). Similar results were obtained upon microinjection of fluorescently-labelled MBP mRNA into the cell body of oligodendrocytes (Ainger et al., 1993; Barbarese et al., 1995). Both results demonstrate that mRNA transport particles contain some components required for protein synthesis. Furthermore, translocation of MBP mRNA in oligodendrocytes is proposed to require microtubules and kinesin since nocodazole (a microtubule depolymerizing drug) and kinesin antisense oligonucleotides inhibit transport of microinjected MBP mRNA (Carson et al., 1997). In addition, Staufen, the mRNA-binding protein that is required for Oskar and Bicoid mRNA localization in *Drosophila* (St Johnston et al., 1991), induces the formation of granules when microinjected into *Drosophila* ooctyes (Ferrandon et al., 1994) and these particles subsequently are transported on microtubules *in vivo* (Clark et al., 1994).

In smaller cells such as fibroblasts (Bassell et al., 1994; Sundell and Singer, 1991), yeast cells (Long et al., 1997; Takizawa et al., 1997), and COS cells (Fusco et al., 2003), mRNAs are transported on both microtubules and actin filaments. In fibroblasts, β-actin mRNA appears to be transported as granules on actin filaments (Sundell and Singer, 1991). ZBP1 is essential for both granule formation and association with the cytoskeleton (Farina et al., 2003). In budding yeast the mRNA for the determinant for mating type switching, ASH1, is localized to the daughter cell in a step that requires actin filaments and myosin V (Long et al., 1997; Takizawa et al., 1997). The use of MS2 stem-loops (RNA stem-loops that bind the phage coat protein MS2) in the RNA that bind MS2–GFP fusion protein, allowing single marker mRNA molecules to be tracked (reviewed by Gonsalvez et al., 2005) demonstrates that both microtubules and actin filaments are involved in rapid linear transport of mRNA in the same cell. Furthermore, the presence of the β-actin mRNA zipcode in the marker mRNA increased both the frequency and length of transport (Fusco et al., 2003). This study also demonstrated that transport and anchoring could be distinguished as separate steps in the targeting of single mRNA molecules. In general, the requirement for an active transport process for large molecules like mRNA is predicted, from biophysical measurements of the diffusion constraints placed on large particles by cytoplasm (Janson et al., 1996).

mRNA is anchored, and may be translated, on actin filaments *in vivo*

Measurement of polysome association with the cytoskeleton after detergent, cytochalasin, DNase I and

Cytochalasin: A drug that binds to actin monomers and prevents polymerization.

phalloidin treatments demonstrated that the association of polysomes with the cytoskeleton depends on the integrity of actin filaments (Dancker et al., 1975; Lenk and Penman, 1979; Adams et al., 1983; Ornelles et al., 1986; Yisraeli and Melton, 1988; Yisraeli et al., 1990; Hesketh and Pryme, 1991). Furthermore, mRNA, EF2, EF1α and ribosomes colocalize with each other and with actin filaments *in situ.* Direct binding between actin and EF1α indicates that the components essential for the assembly of the translation apparatus and polypeptide elongation can associate with actin filaments in the same non-membrane-bound polysome compartment (Dharmawardhane et al., 1991; Shestakova et al., 1991; Bassell et al., 1994). At the electron-microscope level, polysomes are observed in association with actin filaments in a variety of cell types (Heuser and Kirschner, 1980; Ramaekers et al., 1983; Bassell et al., 1994). Biochemical fractionation of mRNAs from cells supports the concept of a particulate mRNA and its association with the cytoskeleton (Grossi de Sa et al., 1988; Hesketh and Pryme, 1991; Hamill et al., 1994).

EF1α is involved in anchoring mRNA to actin filaments

EF1α is a highly conserved protein family (> 75% sequence identity in eukaryotes) well known for catalysing the GTP-dependent binding of aminoacyl-tRNA to ribosomes, thereby regulating the fidelity and rate of polypeptide elongation during translation (Sprinzl, 1994). Numerous studies have demonstrated the binding of EF1α to actin filaments *in vitro* and *in vivo* in a variety of cell types (Yang et al., 1990; Edmonds et al., 1996; Liu et al., 1996). The concentration of EF1α in the cytoplasm of vertebrate cells is about 40 μM (Edmonds et al., 1996). Direct measurement of EF1α content in detergent cytoskeletons indicate that about 50% of EF1α is bound to the cytoskeleton (Edmonds et al., 1996). EF1α contains two actin-binding sites and these sites are highly conserved suggesting that all eukaryotic EF1α s can bind to actin. EF1α has been shown to colocalize with actin filaments in *Dictyostelium* amoebae (Dharmawardhane et al., 1991), mammary adenocarcinoma cells (Edmonds et al., 1996), and in fibroblasts (Bassell et al., 1994). The EF1α implicated in β-actin binding and bundling (Yang et al., 1990; Edmonds et al., 1995) is the translationally competent form of EF1α based on enzymic activity *in vitro* (Yang et al., 1993).

EF1α associates with actin filaments in regions where β-actin mRNA is anchored in crawling cells such as cultured chicken embryo fibroblasts (Liu et al., 2002) and rat adenocarcinoma cells (Shestakova et al., 1999). EF1α is associated with actin filaments at the front of the cell and with small filament bundles that are distinct from stress fibres at the base of lamellipods (Liu et al., 2002; Edmonds et al., 1996). In serum starved cells both β-actin mRNA and EF1α are diffusely distributed. The kinetics of recruitment of EF1α and mRNA to the front of the cell and the cytoskeleton after stimulation with serum or growth factors shows that EF1α precedes the recruitment of β-actin mRNA to the leading edge of the lamellipod (Edmonds et al., 1996; Shestakova et al., 1999; Shestakova et al., 2001). Visualization of EF1α at the electron-microscope level in cultured cells demonstrates that EF1α is bound along actin filaments and colocalizes with mRNA on the filaments (Bassell et al., 1994; Liu et al., 1996). The general conclusion from these studies is that in crawling cells that target mRNAs, EF1α associates with a population of actin filaments to which mRNA becomes localized. Therefore, EF1α is a candidate for binding mRNA to actin filaments at the site of mRNA anchoring.

The biochemical properties of EF1α are consistent with the participation of EF1α in assembling actin filaments into a compartment to which mRNA becomes anchored and translated. The evidence supporting this is summarized as follows.

(1) There are two actin-binding sites on EF1α, one in the N-terminus of domain I (amino acids 1–49) and one in the C-terminus of domain III (amino acids 403–456). This is consistent with an atomic resolution model of EF1α (Liu et al., 2002). The two binding sites can cross-link F-actin to form bundles (Edmonds et al., 1995; Edmonds et al., 1996; Murray et al., 1996). (2) EF1α crosslinks actin filaments by a unique square-pack bonding rule (where the filaments are in the corners of a square) resulting

Phalloidin: A drug that bundles actin filaments.

in bundles that exclude other actin-binding proteins that tend to follow a default hexagonal-bonding rule (where the filaments are in the interstices of a hexagon). This could create an EF1α-rich microenvironment composed of a unique population of actin filaments (Owen et al., 1992). (3) β-Actin tends to bind the GTP form of EF1α under physiological conditions. This GTP–EF1α can bind aminoacyl-tRNA to domain I and is translationally competent (Liu et al., 1996). (4) The binding of β-Actin mRNA to actin filaments *in vitro* and *in vivo* is EF1α-specific. β-Actin mRNA binds to domain III of EF1α, and domain III can act as a dominant-negative inhibitor of β-actin mRNA targeting *in vivo* (Liu et al., 2002).

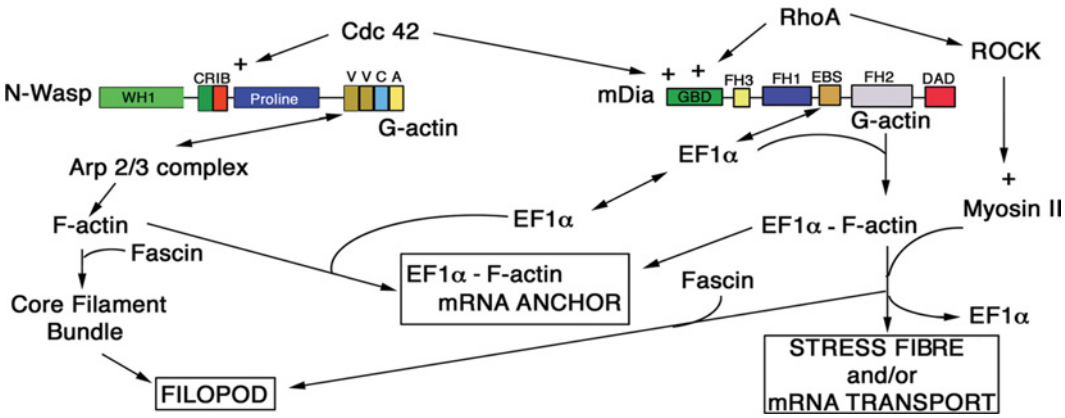
EF1α-binding site (EBS) of Diaphanous-related formins may regulate the EF1α–β-actin complex and mRNA targeting

Downstream targets of RhoA and Cdc42 include Bnilp in yeast, mDia1–3 in mammals and Diaphanous in *Drosophila* (Imamura et al., 1997; Watanabe et al., 1997; Peng et al., 2003). These proteins are members of the Diaphanous-related formin family, sharing conserved formin homology (FH) domains that mediate interactions with signalling kinases and actin-binding proteins (Edmonds et al., 1995; Wasserman, 1998). The functions of these proteins are related to the regulation of cell motility and polarity. Diaphanous (Castrillon and Wasserman, 1994) is required for cytokinesis, Bni1p is concentrated at the tips of mating projections and can nucleate actin polymerization *in vitro* (Pruyne et al., 2002; Sagot et al., 2002) and is required for yeast bud formation (Evangelista et al., 1997; Imamura et al., 1997), and mDia1–3 are enriched in the cell cortex of crawling cells with actin bundles including filopodia (Watanabe et al., 1997; Peng et al., 2003). Structural comparisons indicate that mDia, Bni1p and Diaphanous are the most highly related formins with more distantly-related members including cappuccino and formin (Watanabe et al., 1997). All contain FH1 and FH2 domains, and mDia3 and Bnilp contain a RhoA and Cdc42 binding site (Peng et al., 2003). The FH1 domains of mDia (Watanabe et al., 1997) and Bnilp (Evangelista et al., 1997; Imamura et al., 1997; Umikawa et al., 1998) are polyproline rich and bind profilin *in vitro* and may mediate co-localization of formins with profilin *in vivo* (Watanabe et al., 1997). Binding to profilin is not regulated by Rho family proteins.

Bni1p has been shown to bind to EF1α and inhibit its actin binding *in vitro* (Umikawa et al., 1998). In Bni1p, the EF1α binding site called EBS has been localized to a short sequence (amino acids 1328–1513) between the FH1 and FH2 domains (Umikawa et al., 1998; Liu et al., 2002). The sequence is sufficient to block F-actin bundling *in vitro* (Liu et al., 2002). Over-expression in yeast of the Bni1p truncate (amino acids 452–1953), but not full-length Bni1p, disrupts the usual distribution of F-actin in cells and polarized budding (Evangelista et al., 1997). This suggests that the EBS of Bni1p may regulate binding of EF1α to F-actin, since EF1α contributes to actin bundling *in vivo* (Edmonds et al., 1996). In mammalian cells, expression of the EBS (YEK-R)G mutant of mDia3 that lacks EF1α binding, inhibits bundling of actin filaments only in the cell cortex and in filopodia without inhibiting stress fibre formation (Peng et al., 2003).

BNI1 deletion mutants show temperature-sensitive growth, abnormal morphology and unpolarized distribution of cortical actin patches (Evangelista et al., 1997; Imamura et al., 1997). Deletion of the EBS from Bni1p (*BNI1Δ1328–1513*) results in a protein that still binds to profilin but fails to suppress the *BNI1* null phenotype (i.e. loss of cell polarity) indicating that the interaction between EF1α and Bni1p is important for the function of Bni1p in cell polarity (Umikawa et al., 1998). These results also indicate that changes in the actin cytoskeleton resulting from deletion of the EBS in Bni1p are independent of the interaction between Bni1p and profilin. Furthermore, the *BNI1* mutation, *SHE5*, prevents daughter cell localization of the repressor of the *HO* gene, Ash1p and ASH1 mRNA localization in daughter cells (Jansen et al., 1996; Long et al., 1997). These results indicate that the EF1α–Bni1p interaction may be important for ASH1 mRNA localization and cell polarity. Finally, transfection of mammalian cells with active but not with inactive RhoA and Cdc42 affects targeting of β-actin mRNA to the front of crawling cells (Latham et al., 2001). Since RhoA–C and Cdc42 regulate the binding of mDias to actin and EF1α (Peng et al., 2003), these results suggest that mDias might regulate mRNA targeting to the actin cytoskeleton by regulating the EF1α–F-actin interaction.

Scheme 1 | A schematic model for the relationship of the actin remodelling complex to the localization of β-actin mRNA



The EBS of Bni1p inhibits the binding of β-actin mRNA to EF1α–F-actin bundles *in vitro* and β-actin mRNA targeting *in vivo*

The biochemical properties of EF1α predict an anchoring role for EF1α in β-actin mRNA targeting through the simultaneous binding to both F-actin and mRNA. Recombinant EBS inhibits the binding of EF1α to F-actin and the binding of mRNA to EF1α–F-actin bundles *in vitro* by dissolving the bundles (Liu et al., 2002). In addition, expression of GFP–EBS fusion protein inhibits the targeting of β-actin mRNA to the front of crawling cells (Liu et al., 2002). These results indicate that an intact EF1α–F-actin complex is needed for binding of mRNA to F-actin and are consistent with the hypotheses that EF1α–F-actin is the anchor for β-actin mRNA, and that mDias regulate the formation of EF1α–F-actin bundles. The model shown in Scheme 1 summarizes these results and proposes a mechanism by which RhoA, Cdc42 and Bni1p/mDia regulate mRNA targeting by regulating the assembly of the EF1α–F-actin bundles and, therefore, the scaffold required for mRNA localization at the cell front.

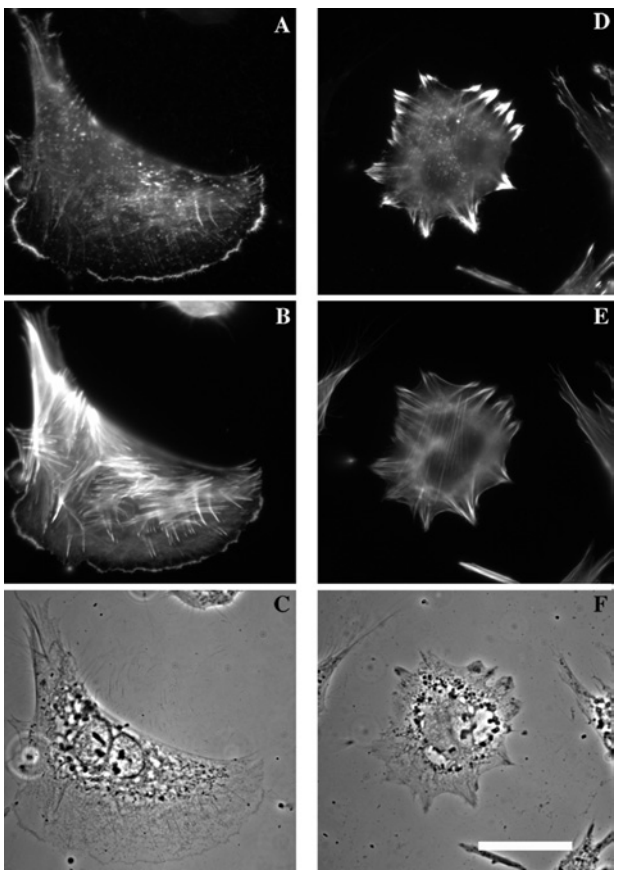
The consequences of targeting β-actin mRNA on cell polarity

The best example of the consequence of mRNA localization in crawling cells is the effect of the localization of β-actin mRNA on cell behaviour. It has been shown previously that crawling cells with high intrinsic polarity localize β-actin mRNA to the leading edge (Kislauskis et al., 1994), and that mRNA localization, cell polarity and movement are disrupted experimentally by treating cells with antisense oligonucleotides against the zipcode of β-actin mRNA (Kislauskis et al., 1997; Ross et al., 1997).

The effect of zipcode-directed antisense oligonucleotides on the movement of chicken embryo fibroblasts has been studied using dynamic image analysis software (DIAS). This revealed that these cells exhibit high intrinsic polarity as reflected by their protrusion of a lamellipod at only one side of the cell, resulting in polarized directionality of locomotion. Treatment of cells with antisense oligonucleotides, but not sense oligonucleotides, corresponding to the zipcode sequence, delocalized β-actin mRNA from the leading edge and caused a significant decrease in 'directionality' of movement (Shestakova et al., 2001). In particular, lamellipods protruded during locomotion, but protrusion direction was random and inconsistent. The underlying cause of this change in cell polarity was investigated by localizing β-actin protein and free barbed ends in cells before and after treatment with zipcode-directed antisense oligonucleotides to delocalize β-actin mRNA. The results in Figure 1 show that antisense but not control (sense) oligonucleotides caused a delocalization of both

Directionality: A parameter measured by DIAS morphometry software quantifying the ability to hold to a single direction of locomotion.

Figure 1 | Delocalization of β-actin mRNA causes loss of polarized addition to actin filaments
Sites of rhodamine actin incorporation in zip code sense-treated (Col1) and antisense treated (Col2) cells. Sense treatment (**A**–**C**); antisense treatment (**D**–**F**). Rhodamine actin incorporation showing the location of freebarbed ends on actin filaments (**A** and **D**). FITC-phalloidin-labelling of all actin filaments (**B** and **E**). Phase-contrast image (**C** and **F**). Bar, 10 μm. Note that rhodamine actin incorporation sites are unpolarized in antisense-treated cells. Reprinted from Shestakova et al. (2001) Proc. Natl. Acad. Sci. U.S.A. **98**, 7045–7050 [Copyright (2001) National Academy of Sciences, U.S.A.].



β-actin protein and barbed ends from the leading edge in fibroblasts, resulting in a random distribution of both. This was reversible upon removal of oligonucleotides (Shestakova et al., 2001). This suggests that the site of translation of β-actin protein defines the location of nucleation of actin polymerization and, therefore, cell polarity.

Cell polarity, metastatic potential and mRNA targeting

The relationship between β-actin mRNA targeting and cancer invasion was investigated in a pair of cell lines cloned from a breast adenocarcinoma primary tumour representing the most metastatic and least metastatic cells (MTLn3 and MTC, respectively) in the primary tumour (Shestakova et al., 1999; Wang et al., 2002). The presence of intrinsic polarity in MTC cells and tumours is correlated with the stable polarization of actin polymerization at one end of the cell only, resulting in polarized locomotion. Intrinsic cell polarity such as this is believed to cause chemotactic inefficiency because a polarized cell cannot extend protrusions from any side toward a source of chemoattractant, but must turn the entire cell toward the source (Condeelis et al., 2003; Condeelis and Segall, 2003; Wang et al., 2003). In contrast, carcinoma cells in MTLn3 tumours are unpolarized, except when they are near blood vessels where they become chemotactically polarized toward the blood space (Wyckoff et al., 2000; Condeelis and Segall, 2003). These results suggest that cells that have proceeded through the epithelial mesenchymal transition (EMT) to the point where all remnants of the intrinsic cell polarity of the original epithelium are lost, such as MTLn3 cells, may be more sensitive to external chemotactic signals and more attracted to blood vessels in the primary tumour. A key difference between metastatic and non-metastatic cells that may explain the inverse correlation between intrinsic cell polarity and metastasis is loss of the ability by metastatic cells to localize mRNA and proteins that define cell polarity (Shestakova et al., 1999; Condeelis and Segall, 2003). Highly metastatic cells have lost the ability to target mRNA for β-actin, which is required to maintain a stable leading edge. Without a stable leading edge, the intrinsic polarity of the metastatic cell is lost and cell direction is determined by signals from blood vessels, resulting in chemotaxis toward blood vessels and intravasation (Wyckoff et al., 2000; Condeelis and Segall, 2003).

Epithelial mesenchymal transition (EMT): The point where the epithelial cells are no longer connected by junctions and move as free, mesenchymal-like cells.

Molecular profiling of MTLn3 and MTC cells and tumours using both cDNA arrays and quantitative real-time PCR demonstrates that non-metastatic MTC cells and tumours express much higher levels of ZBP1 than the metastatic MTLn3 cells and tumours (Wang et al., 2002).

Low levels of ZBP1 expression in MTLn3 cells results in the inability of MTLn3 cells to target β-actin mRNA to the leading edge (Wang et al., 2002). Furthermore, invasive tumour cells isolated from primary mammary tumours using chemotaxis (Wyckoff et al., 2000) express 10-fold lower levels of ZBP1 than cells that remain behind in the primary tumour even though the cells were derived from the same progenitor cell. Decreased ZBP1 expression is strongly correlated with random cell motility and heightened chemotaxis, resulting in a more invasive and metastatic phenotype which is a consequence of the loss of intrinsic cell polarity (Wang et al., 2002). Invasive carcinoma cells expressing experimentally increased levels of ZBP1 after transfection with ZBP1 expression vectors exhibit increased cell polarity, suppression of chemotaxis, and inhibited invasion. Additionally, the tumours made from cell grafts of these ZBP1-expressing cells are much less metastatic. Therefore, ZBP1 is a candidate for a 'metastasis repressor', and, together with mRNA targeting and analysis of tumour cell polarity around blood vessels, might be used in prognosis.

β-Actin isoform-specific sorting

A case can be made for β-actin isoform-specific sorting that would capitalize on the targeting of β-actin mRNA. There are many actin-binding proteins that have a preferred interaction for one isoform of actin over another. Profilin (Schutt et al., 1993; Kinosian et al., 2002), thymosin β4 (Weber et al., 1992), L-plastin (Namba et al., 1992; Hofer et al., 1997), and βcap73 (Welch and Herman, 2002) prefer to bind to β-actin while ezrin forms an indirect complex exclusively with β-actin (Shuster and Herman, 1995), and annexin V (Tzima et al., 2000) preferentially binds to β-actin. Since profilin, ezrin and βcap73 are associated with the plasma membrane in apical structures, their preference for β-actin might create an apical zone where β-actin would be accumulated. While localized translation of β-actin might initiate the site of accumulation of β-actin protein, the special properties resulting from the interaction of β-actin with these and other actin-binding proteins would determine the function of β-actin-rich apical structures such as the leading edge. In this regard, profilin is of particular interest. Profilin exchanges bound nucleotide faster on β-actin than γ-actin, making β-actin more suited to the rapid monomer recycling known to occur at the leading edge in crawling cells (Kinosian et al., 2000). Profilin can form a high affinity complex with β-actin *in vitro* (Kinosian et al., 2000) and *in vivo* (Segura and Lindberg, 1984) where the actin is highly stable and polymerizable. Microinjection of the high affinity profilin–actin complex into cells causes actin polymerization and protrusion activity while microinjection of profilin alone has the opposite effects (Cao et al., 1992). Shuttling of the profilin–actin complex to polymerizing barbed ends by VASP (Bear et al., 2002) and N-WASP (Suetsugu et al., 1998) is required to antagonize capping of free barbed ends, of an actin filament preferred for polymerization, in lamellipods and to elongate filopods respectively. These results indicate that β-actin has special properties that contribute to the positioning of free barbed ends and the rapid elongation of actin filaments in the leading edge of protrusions in crawling cells, and that profilin may play an important role in β-actin function. This is important because in crawling cells the size and location of the initial protrusion determines subsequent steps in the motility cycle, and the directionality and speed of locomotion.

β-Actin protein appears to be accumulated and stored just behind the leading edge in a non-filamentous form to supply monomers for polymerization at the leading edge. In vertebrate cells, non-filamentous actin-containing particles (NFAP) have been identified by comparing the localization patterns of actin stained with vitamin D-binding protein (DBP), which binds exclusively to G-actin with 5 nM K_d affinity, various actin antibodies, and phalloidin which binds to F-actin (Hoock et al., 1991; Cao et al., 1993). NFAPs are found in lamellipods just behind the actin nucleation zone in the leading edge. Fluorescently labelled profilin (Tarachandani and Wang, 1996) and actin (Cao et al., 1993), both with wild-type activity, eventually localize to NFAPs after microinjection, suggesting the formation of a profilin–actin complex in NFAPs. This is an important observation given the properties of the

profilin–actin complex. Profilin depolymerizes actin filaments by a combination of blocking addition of monomers at the barbed end (Kinosian et al., 2002), uncapping filaments, and monomer sequestering (Bubb et al., 2003). Profilin–actin complex, on the other hand, increases the rate of elongation of actin filaments at the barbed end both *in vitro* (Kinosian et al., 2000; Loisel et al., 1999) and *in vivo* in filopods (Cao et al., 1992) and at the leading edge of lamellipods (Bear et al., 2002). These results are consistent with the microinjection into cells of profilin–actin complex causing the polymerization of actin and lamellipod protrusion while the microinjection of profilin alone has the opposite effects (Suetsugu et al., 1998). Furthermore, profilin–actin complex is essential for *Listeria* motility (Dickinson et al., 2002; Grenklo et al., 2003) where the rate of elongation of actin filaments and *Listeria* speed are dependent on positioning profilin at sites of polymerization (Loisel et al., 1999; Geese et al., 2000). The work in vertebrate cells follows a literature documenting NFAPs in gametes of marine invertebrates (Tilney et al., 1973; Tilney, 1976; Spudich et al., 1988; Bonder et al., 1989). In *Thyone* sperm, the nonfilamentous actin in NFAPs is complexed with profilin (Tilney, 1976; Tilney et al., 1983). The NFAPs in gametes support rapid polymerization of actin during fertilization.

The proposed mechanism for how the profilin–actin complex specifically affects elongation of barbed ends is that profilin binds to polyproline sequences on Ena/VASP family proteins that are associated with barbed ends, resulting in very high local concentrations of profilin–actin complex (Kang et al., 1997). This increases both the rate of polymerization from, and longevity of, the barbed ends (Bear et al., 2002). Therefore, the localization of profilin and actin containing NFAPs near sites of actin polymerization in the leading edge could increase the rate and extent of polymerization there. If β-actin is the isoform accumulated in NFAPs, and NFAPs supply profilin–β-actin for polymerization, this would result in the observed concentration of β-actin in leading edge filaments. Therefore we must ask: is β-actin protein preferentially accumulated in NFAPs, if so, is the β-actin protein in NFAPs dependent on β-actin mRNA targeting to NFAPs, is β-actin in NFAPs bound to profilin, and is β-actin in NFAPs preferred for barbed-end elongation at the leading edge?

A model: cell movement and β-actin mRNA localization

The molecular mechanism by which polarity of cell crawling is affected by β-actin mRNA localization could depend on several interdependent events: (1) localized synthesis of β-actin drives lamellipod protrusion; (2) specific proteins requiring the β-actin isoform are responsible for protrusion; (3) localization to the leading edge of the mRNAs for an actin-nucleating complex; and (4) β-actin mRNA localization is important not for the protrusion event, but rather for the assembly of the focal-adhesion complex. A discussion of the evidence for each of these is detailed below (see also Shestakova et al., 2001).

The localized synthesis of β-actin could supply at most 100 000 β-actin monomers per min (Kislauskis et al., 1997); however, the protrusion of a lamellipod requires a minimum of fifty times this amount (Chan et al., 1998). Therefore, it is unlikely that newly synthesized β-actin could contribute to the rate of actin polymerization at the leading edge in the short term. Since β-actin mRNA is restricted to the leading edge, however, all of the β-actin that is synthesized is done so in a very small volume, the thin lamellipod. Therefore the effective concentration of β-actin is increased and might influence actin polymerization in this restricted volume. Furthermore, newly synthesized actin could have a faster rate of polymerization, as actin is modified by post-translational enzymes (Solomon and Rubinstein, 1987). Thus 'new' actin may have a higher affinity for a nucleation complex than 'old' actin. For instance, interaction of a chaperone with the β-actin nascent chain (Hansen et al., 1999) could promote assembly of a nucleation complex near the site of synthesis. Alternatively, the synthesis of β-actin away from proteins that may prevent its polymerization, such as profilin or thymosin β-4, could sequester monomers into compartments primed for addition to barbed ends, unimpeded by inhibitory proteins.

Another explanation is related to the specific properties of the β-actin isoform, which is the only one to be localized to the leading edge in fibroblasts and myoblasts (Kislauskis et al., 1993), or neurons (Zhang et al., 1999). β-Actin monomers may be the only isoform that could be preferentially stored and hence allowed to 'build up' in preparation for polymerization at the leading edge. In this scenario, the local accumulations of G-actin could be released suddenly

upon stimulation, and their location would determine sites of actin polymerization. Potential storage compartments of non-filamentous actin have been identified using vitamin D-binding protein (DBP), which binds to β-actin monomers with high affinity, and comparing this to phalloidin, which binds only to filamentous actin (Cao et al., 1993). Stores of non-filamentous actin have been found at the leading edge and were located adjacent to sites of actin polymerization. This is the region of the cell where the β-actin mRNA is present, so it is feasible that translation of the mRNA gives rise to these localized pools.

β-Actin has unique properties that support the hypothesis that it is particularly suited for cell motility. First of all, it is found preferentially at the leading edge of crawling cells. β-Actin does not substitute for α-actin in either the formation of stress fibres (Cao et al., 1993) or myofibrils in cardiomyocytes (von Arx et al., 1995). It also has a higher affinity for actin-binding proteins that are believed to function at the leading edge of crawling cells, e.g. ezrin (Shuster and Herman, 1995), profilin (Segura and Lindberg, 1984), thymosin β4 (Weber et al., 1992) and L-plastin (Namba et al., 1992). The barbed end may be capped in an isoform-specific manner, for instance by β-cap 73 (Shuster et al., 1996). The Arp2/3 complex is required for nucleation of actin filaments at the leading edge (Machesky et al., 1997; Bailly et al., 1998; Mullins et al., 1998; Welch et al., 1998). Therefore, preference for the β-actin isoform by the Arp2/3 complex would require local synthesis of β-actin to supply the preferred monomer for polymerization, and the capping of the barbed ends would increase the branching, and hence the force needed for protrusion. Newly formed actin filaments and their interaction with β-actin isoform-specific binding proteins, would provide stability for cell polarity and this would provide directional motility for the cell (Liu, 1996).

β-Actin mRNA is not the only mRNA that can be localized. Many of the mRNAs for motility-related proteins contain sequence homology to the β-actin mRNA zipcode. The sequence GACUX$_{7\text{-}38}$ACACC is found in β-actin mRNAs known to target to the leading edge from all vertebrates. Besides β-actin mRNA and the mRNAs for all seven Arp2/3 subunits (G. Lui, personal communication), some other examples of mRNA with this zipcode are myosin IIB, zyxin, α-actinin, tropomyosin 1, and myosin VI (A. Wells, personal communication). Therefore we predict that they will be recognized by the same localization mechanism that targets β-actin mRNA to the leading edge. Mutation of the zipcode consensus sequence ACACCC, in a reporter mRNA introduced into fibroblasts, resulted in a failure to localize to the leading edge (Kislauskis et al., 1997; Ross et al., 1997). This occurs even if the β-actin coding sequence is used as the reporter mRNA. Localization of β-actin mRNA occurs within minutes in response to serum stimulation (Latham et al., 1994). Signalling mechanisms operating through the Rho kinase pathway may be involved in the localization of motility-related mRNAs, thereby coordinating their temporal and spatial distribution and expression (Latham et al., 2001).

A rationale for the asymmetric localization of mRNAs is that any polarized cell must be composed of asymmetrically distributed proteins. It stands to reason that proteins besides β-actin are synthesized nearby and would provide an increased likelihood of interactions among proteins relevant to the structural and functional identity of the lamellipod. All of these considerations lead to the conclusion that a complexity of RNA sequences in some way contributes to cell motility. These sequences probably contain a common motif that creates a recognizable structure that serves as a nucleating platform for mRNA localization and translational regulation. Conversely, if one of the mRNAs is localized to the wrong place, the cell structure is severely disrupted. For instance, when the zipcode for β-actin mRNA was transferred to vimentin, a protein that is not supposed to be at the leading edge, the leading edge became branched and attenuated (Morris et al., 2000). This also supports the argument that synthesis of the correct protein in the correct place (near the leading edge) is an important requirement for cell structure and polarity. Further investigation will clarify the mechanism by which RNA localization leads to cell asymmetry.

Finally, a fourth possible mechanism whereby β-actin mRNA can affect cell behaviour could be through the focal adhesion complex. We have shown that integin receptors can mediate RNA localization (Chicurel et al., 1998) and it is likely that one structure initiated by mRNA localization could be the focal adhesion. Like the lamellipod, the focal-adhesion complex requires the participation of many proteins to effect its function. The presence of RNA and RNA-binding proteins in actin-rich structures found

in the early stages of cell spreading would suggest that localized synthesis of these proteins occurs (de Hoog et al., 2004). Since focal adhesions are required for cell motility, it is possible that the localization of β-actin mRNA participates in the genesis of these structures, and in this way provides for the forward movement, in concert with its generation of actin monomers for protrusive extension of the lamellipod.

Since the discovery of β-actin mRNA localization in fibroblasts (Lawrence and Singer, 1986), localization seems to be a general feature of polarized cells. For instance, neurons localize β-actin mRNA to the growth cone of developing neurites (Zhang et al., 1999). The translation of β-actin protein in the growth cone is essential for neurite extension; delocalization of the mRNA results in growth cone retraction (Bassell and Singer, 2001). The field of RNA localization has developed rapidly since the discovery that mislocalization of the RNA can lead to a significantly altered phenotype or lethality in *Drosophila* development or yeast mating type switching (St Johnston, 1995; Long et al., 1997; Hazelrigg, 1998; Morris et al., 2000). Embryonic neural crest cells localize β-actin mRNA at their leading edge, in the direction of their migration. Disruption of the protein involved in this localization, the homologue of ZBP1 in *Xenopus*, results in severe embryological defects in forebrain development (Yaniv et al., 2003). In some of these cases mRNA localization is required for normal development and differentiation because the localized mRNA codes for nuclear determinants, and become segregated to create different cellular lineages. The specific localization we describe for β-actin is important for somatic cells: it determines their structure and function. For this aspect of RNA localization, proteins required for cell migration, response to environmental cues and consequent generation of cell polarity occur because of the spatial segregation of their cognate mRNAs, and are not, in the short term, related to transcription of genes. This provides a way by which the mechanism controlling the behaviour and structure of the cell can rapidly respond to environmental cues without any need for transcription; a process we characterize as 'gene expression in the cytoplasm'.

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