Regulation of local expression of cell adhesion and motility-related mRNAs in breast cancer cells by IMP1/ZBP1

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Summary

Metastasis involves tumor cell detachment from the primary tumor, and acquisition of migratory and invasive capabilities. These capabilities are mediated by multiple events, including loss of cell–cell contact, an increase in focal adhesion turnover and failure to maintain a normal cell polarity. We have previously reported that silencing of the expression of the zipcode-binding protein IMP1/ZBP1 in breast tumor patients is associated with metastasis. IMP1/ZBP1 selectively binds to a group of mRNAs that encode important mediators for cell adhesion and motility. Here, we show that in both T47D and MDA231 human breast carcinoma cells IMP1/ZBP1 functions to suppress cell invasion. Binding of ZBP1 to the mRNAs encoding E-cadherin, β-actin, α-actinin and the Arp2/3 complex facilitates localization of the mRNAs, which stabilizes cell–cell connections and focal adhesions. Our studies suggest a novel mechanism through which IMP1/ZBP1 simultaneously regulates the local expression of many cell-motility-related mRNAs to maintain cell adherence and polarity, decrease focal adhesion turnover and maintain a persistent and directional motility.

Key words: Cancer cell invasion, Post-transcriptional regulation, mRNA localization

Introduction

In the early stages of metastasis, cancer cells disseminate from the primary tumor and obtain invasive capabilities that are mediated by multiple events, including changes in cell–cell contact, loss of cell internal polarity, an increase in focal adhesion turnover and acquisition of autonomous motility. The body usually resists metastasis through the actions of a class of proteins known as metastasis suppressors, such as BRMS1 and KISS1 (Jackson, 2007). In this work, we identify another suppressor, the zipcode-binding protein 1 also known as human IMP1 (hereafter referred to as ZBP1/IMP1), which functions to suppress invasiveness.

ZBP1 belongs to a highly conserved family of RNA-binding proteins that has been implicated in many aspects of post-transcriptional regulation of mRNAs (Yisraeli, 2005). ZBP1 directs the localization of β-actin mRNA to the leading edge in a variety of cell types, which results in the asymmetric translation of the β-actin protein and establishes cell polarity (Condeelis and Singer, 2005). In primary neurons, ZBP1 regulates localized translation of β-actin mRNA in growth cones through a Src-mediated signaling pathway (Huttelmaier et al., 2005; Sasaki et al., 2010). The human ortholog of ZBP1 (IMP1) was originally identified as a translational regulator of mRNA encoding insulin-like growth factor 2 (IGF-2), but has since been found to promote the localization of H19 and tau mRNAs, as well as stabilize CD44, β-TrCP1, and mRNAs encoding β-catenin (Atlas et al., 2004; Elcheva et al., 2009; Gu et al., 2008; Nielsen et al., 1999; Runge et al., 2000; Vikesaa et al., 2006). In human breast cancer cells, IMP1/ZBP1 binds to a plethora of mRNA targets involved in cell–cell adhesion and motility, selectively regulating their expression (Gu et al., 2009). Loss of ZBP1 function affects many important cellular processes, such as actin dynamics, cell proliferation, migration and invasiveness. This is probably due to the deregulation of mRNAs normally associated with the protein.

ZBP1 is actively expressed during embryonic development but is silenced or repressed in most normal adult tissues. Re-activation or elevated expression of the *ZBP1* gene has been detected in mammalian tumors of different origins and has been considered a feature marker of clinical samples (Gu et al., 2004; Ioannidis et al., 2005; Yisraeli, 2005). A number of studies have revealed the positive or negative involvement of ZBP1 in tumorigenesis and tumor progression, including cancer cell proliferation, invasion and metastasis (Liao et al., 2004; Ross et al., 2001; Tessier et al., 2004; Wang et al., 2004). Substantial evidence implicates the role of ZBP1 in breast cancer invasiveness. ZBP1-regulated β-actin mRNA localization is required for directional cell motility (Farina et al., 2003; Kislauskis et al., 1997). In non-metastatic carcinoma cells (Shestakova et al., 1999), disruption of the interaction between ZBP1 and β-actin mRNA converts the behavior of cells with a polarized movement to a phenotype known as ‘random walk’ (Shestakova et al., 2001). Rat metastatic MTLn3 cells do not localize β-actin mRNA and lack an intrinsic polarity owing to the

Journal of Cell Science

repression of ZBP1 expression. By contrast, MTC (non-metastatic) cells derived from the same tumor express high levels of ZBP1 (Wang et al., 2004). Moreover, ZBP1 is broadly expressed in non-metastatic breast cell lines and human tumors, but is downregulated in metastatic cells (Gu et al., 2009). A recent study reported that human metastatic MDA231 cells, which do not express IMP1/ZBP1, display neither lamellipodia nor bleb extensions at the leading edge and invade 3D Matrigel with a characteristic rounded morphology using a uropod-like structure (Poincloix et al., 2011). It is suggested that cells that are able to localize β-actin mRNA retain a stable and persistent polarity, leading to reduced responsiveness to orient towards exogenous chemotactic gradients; such responsiveness is required for cellular invasiveness and hence reduced metastatic potential (Lapidus et al., 2007). Interestingly, some in vivo studies reveal contradictory results for the role of ZBP1 in metastasis – a transgenic study indicated that targeted expression of ZBP1 in mouse breast induced tumorigenesis, and the levels of ZBP1 expression positively correlated with metastasis (Tessier et al., 2004); however, xenograft studies found that re-expression of ZBP1 in ZBP1-negative metastatic MTLn3 line reduces the metastatic potential of cell-derived breast tumors (Lapidus et al., 2007; Wang et al., 2004). Recent studies demonstrate that expression of the *ZBP1* gene in mammalian cancer cells is a cellular response to Wnt/β-catenin signaling, which is frequently active in embryogenesis and tumorigenesis (Gu et al., 2009; Noubissi et al., 2006). The β-catenin protein specifically binds to the ZBP1 promoter, and this transactivates ZBP1 expression. However, in metastatic cells, the ZBP1 gene is repressed due to methylation of its promoter, which prevents β-catenin from binding and results in the transcriptional inactivation of the gene. Repression of ZBP1 expression not only increases cell migration, but also promotes the proliferation of metastatic cells (Gu et al., 2009). Microarray assays identified ZBP1-bound mRNAs in breast cancer cells. Many of these mRNAs are important for cell–cell adhesion and cell migration and display a different expression pattern in the absence of ZBP1 expression (Gu et al., 2009).

We hypothesized that the ability of IMP1/ZBP1 to suppress human breast cancer invasion and metastasis could result from a combined effect of regulating mRNAs associated with motility and adhesion. To address this hypothesis, we used two human cell lines: T47D cells – a well-differentiated, nonmetastatic human breast carcinoma cell line that normally expresses IMP1; and MDA231 cells – a highly invasive cell line in which the IMP1/ZBP1 gene is repressed. We demonstrate that IMP1 downregulation increased the invasive potential of T47D cells, which positively correlated with E-cadherin and β-actin mRNA delocalization at cell–cell junctions. We identify a functional role for IMP1/ZBP1 in the turnover of focal adhesions, probably through the regulation of mRNAs encoding α-actinin and Arp2/3. This suggests that IMP1/ZBP1 is a master regulator of many mRNAs encoding cell-motility-related factors, maintaining cell adherence and polarity, reducing focal adhesion turnover and promoting persistent and directional motility; all of which play a role in preventing the early steps in cancer invasion.

Results

IMP1/ZBP1 inhibits invasiveness of human breast cancer cells

We previously reported that knockdown of IMP1/ZBP1 expression in nonmetastatic T47D cells increases cell migration (Gu et al., 2009). To further examine whether IMP1/ZBP1 is involved in invasiveness of human breast cancer cells, we transfected a lentiviral-based expression vector encoding green fluorescent protein (GFP)-IMP1 into metastatic MDA231 cells, which do not express endogenous IMP1. Western blotting was used to analyze GFP–IMP1 expression in selected stable cell lines (Fig. 1A). Cells were then subjected to an in vitro invasion assay on Matrigel-coated transwells (BD Biosciences). The ability to invade through a Matrigel layer towards a chemo-attractant in serum is a surrogate indicator of metastasis. Control MDA231 cells expressing GFP were highly invasive, but this was reduced by 36% when the GFP-IMP1 fusion protein was expressed (Fig. 1B,C). Control cells exhibited no difference in invasive activity compared with that of the parental MDA231 cells (not shown), indicating that lentiviral infection had no effect on the behavior of these cells. Moreover, in benign non-metastatic T47D cells, which display normal endogenous IMP1 expression, knockdown of IMP1 expression markedly increased the invasive capability compared with that of control cells (Fig. 1D). These data demonstrate an important role for IMP1/ZBP1 in repressing invasiveness of human breast cancer cells in vitro.

IMP1/ZBP1 affects the accumulation of E-cadherin protein at cell adhesions

We hypothesized that one of the mechanisms by which IMP1/ZBP1 was able to repress cancer cell invasion is through the regulation of associated mRNAs. In breast cancer cells, IMP1/ZBP1 binds to a group of mRNAs important for cell–cell contact and migration. E-cadherin mRNA is one of the mRNAs that has been found to be selectively stabilized by IMP1/ZBP1 (Gu et al., 2009). E-cadherin mRNA encodes a membrane protein that plays an important role in maintaining cell–cell adhesions and is considered to be an invasion suppressor. To reveal whether IMP1/ZBP1 also regulated E-cadherin localization at cell–cell junctions, we initially analyzed the cellular distribution of E-cadherin in control and knockdown T47D cells. T47D cells grow as epithelial clusters with a typical ‘cobblestone’ morphology. In control cells, E-cadherin protein accumulated at cell–cell adhesions; by contrast, this accumulation was reduced in knockdown cells (Fig. 2A, arrows). Using custom software to measure the intensity of immunostained E-cadherin protein, we observed that the average fluorescent intensities of E-cadherin at the cell contacts of control cells were almost double compared with those of the knockdown cells (Fig. 2B), suggesting that IMP1/ZBP1 downregulation impaired E-cadherin distribution to the cell–cell contacts. Moreover, the pattern of E-cadherin distribution in shRNA antagonising IMP1 expression in the T47 cell line (hereafter referred to as T47D/IMP1-shRNA) could be restored by the expression of a chicken *ZBP1* gene (Fig. 2A, bottom row). Western blotting showed that the expression levels of E-cadherin protein in control cells were ~10% higher than those in knockdown cells (Fig. 2C). Therefore, the attenuated accumulation of E-cadherin at the cell contacts of IMP1/ZBP1 knockdown T47D cells was not due to the changes in protein expression but might have resulted from decreased localization of the mRNA in the absence of IMP1/ZBP1.

We then analyzed the distribution of E-cadherin in MDA231 cells, which do not form cell–cell adhesions in culture. Unlike T47D cells, E-cadherin did not localize at the cell membrane

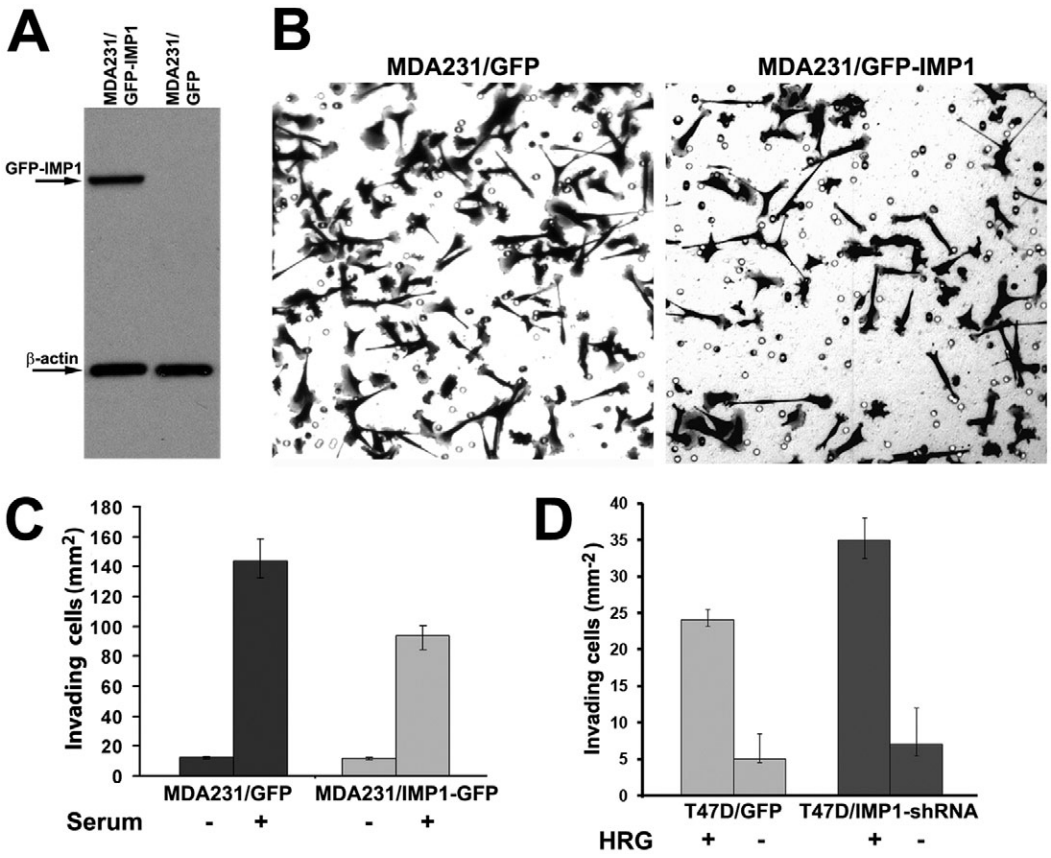

Fig. 1. ZBP1 represses invasiveness of human breast cancer cells. (**A**) Western blots showing GFP-IMP1 expression in MDA231 stable cell lines. (**B**) Invasion assay: MDA231/GFP and MDA231/GFP-IMP1 cells were plated in serum-free medium into the upper chamber of 8 μm pore Matrigel-coated transwell filters. The lower chamber contained medium with 10% serum. Cells that had invaded to the underside of the filter were stained and counted 12 hours later. (**C**) Ectopic expression of IMP1 inhibited invasion of MDA231 cells. Relative numbers shown in the figure represent the means ± s.e.m. of data from three independent experiments, $P<0.005$. (**D**) Loss of IMP1 increased the invasive capability of human T47D cells. Invasion assays were performed as described in B. T47D/GFP and T47D/IMP1-shRNA cells were plated in serum-free medium in the upper chamber of transwell filters. The lower chambers contained serum-free DMEM with or without 1 nM heregulin (Hrg). The relative numbers of invading cells shown in the figure represent the mean ± standard errors of the data from three independent experiments, $P<0.005$.

in MDA231 cells expressing either GFP or IMP1-GFP (supplementary material Fig. S1).

IMP1/ZBP1 facilitates the localization of E-cadherin and β-actin mRNAs at cell–cell contacts

To address the hypothesis that IMP1/ZBP1 regulates the accumulation of E-cadherin at cell adhesions through localization of E-cadherin mRNA, we performed fluorescent in situ hybridization (FISH) to detect cytoplasmic localization of E-cadherin mRNAs. Experiments showed that, in control cells, E-cadherin mRNA was mostly localized at the cell–cell contacts where the E-cadherin protein accumulated. However, localization of the mRNA was markedly reduced in knockdown cells (Fig. 3A, T47D cells expressing GFP – hereafter referred to as T47D/GFP – versus T47D/IMP1-shRNA). E-cadherin mRNA was localized at cell–cell contacts in 61% of control cells, whereas the localization was decreased to 28% when IMP1/ZBP1 was downregulated (Fig. 3B). These experiments indicate that the reduced accumulation of E-cadherin at cell–cell contacts and delocalization of mRNA encoding E-cadherin occur concurrently.

In motile cells, ZBP1-directed β-actin mRNA localization plays a vital role in affecting actin dynamics and establishing cellular polarity (Condeelis and Singer, 2005). Because β-actin mRNA is also one of the IMP1/ZBP1-bound mRNAs identified in breast cancer cells (Gu et al., 2009), we determined the localization of β-actin mRNA in control versus knockdown cells (Fig. 3C,D). In both clustered and single control cells, β-actin mRNA was localized near cell–cell adhesions and the cell leading edges. However, both clustered and individual cells showed a more diffuse localization pattern of β-actin mRNA in IMP1/ZBP1 knockdown cells, although some accumulation was seen (Fig. 3C, bottom). Nearly 70% of T47D cells localized β-actin mRNA near the leading edges of individual cells, whereas this localization decreased to 36% in knockdown cells (Fig. 3E). These data suggest that loss of IMP1/ZBP1 function could impair local translation of E-cadherin and β-actin mRNAs, resulting in attenuation of cell–cell adhesion and loss of cell polarity.

IMP1/ZBP1 stabilizes focal adhesions in breast cancer cells

The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration. To investigate whether the

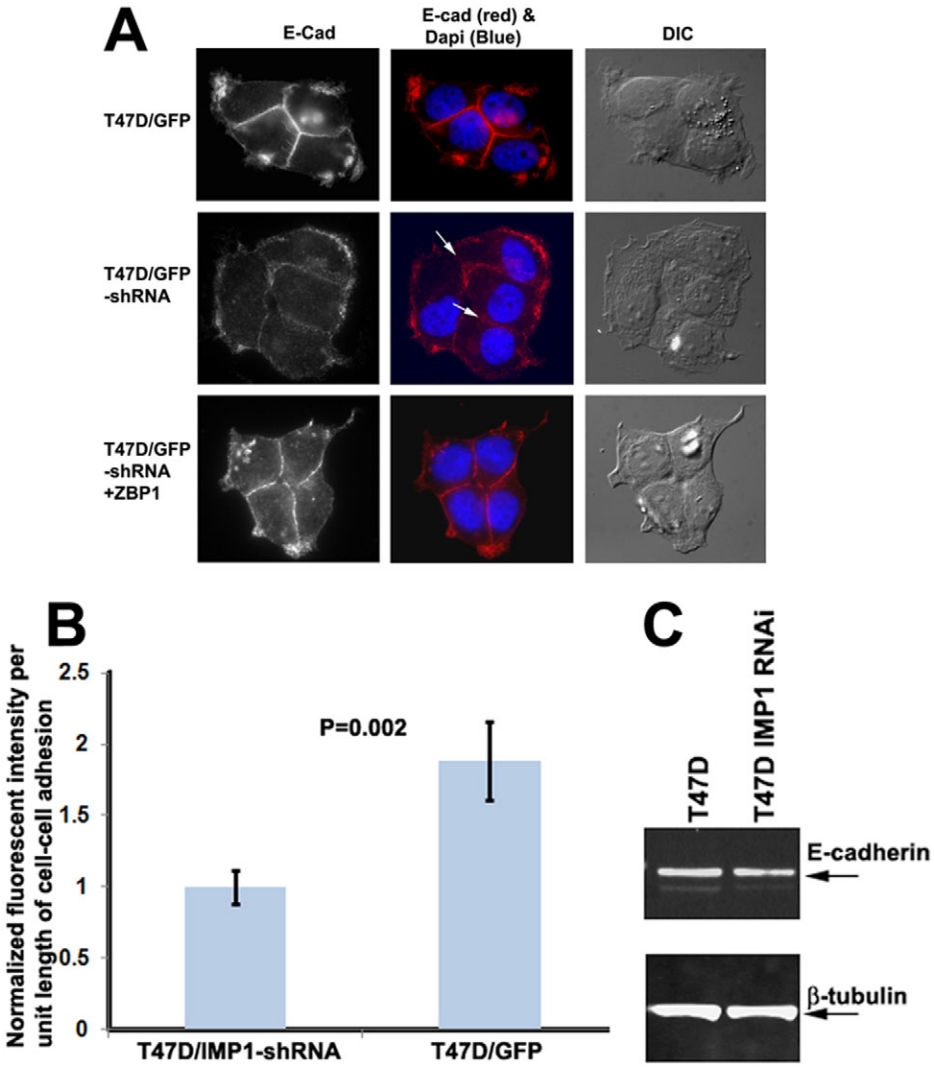

Fig. 2. IMP1/ZBP1 downregulation impairs accumulation of E-cadherin protein at the adhesion sites of human breast cancer cells. (**A**) Immunofluorescence showing the cellular expression and localization of E-cadherin in control (T47D/GFP) and knockdown (T47D/IMP1-shRNA) cells. The accumulation of E-cadherin at cell–cell adhesions was impaired when IMP1/ZBP1 was downregulated (arrows). However, the accumulation was restored after the chicken IMP1 ortholog was re-expressed in T47D/IMP1-shRNA cells. DIC, digital interference contrast. (**B**) The fluorescence intensities of immunostained E-cadherin protein at the cell contacts were quantified (see Materials and Methods) and normalized (T47D/IMP1-shRNA vs T47D/GFP). Quantified data were obtained from 38 cell–cell adhesion sites from 20 fields of T47D/IMP1-shRNA cells and 37 cell–cell adhesion sites from 20 fields of T47D/GFP cells in two independent experiments. (**C**) Western blots showing that the expression levels of E-cadherin protein in T47D/GFP cells were ~ 10% higher than those in T47D/IMP1-shRNA cells.

IMP1/ZBP1 effect on migration and invasion of carcinoma cells could be linked to the dynamics of focal adhesions, we examined the consequence of IMP1/ZBP1 depletion on focal adhesions. We analyzed focal adhesions by measuring their size and numbers in T47D and MDA231 cells in the presence or absence of IMP1/ZBP1 expression. Cells were stimulated with heregulin (T47D cells) or serum (MDA231 cells) for 20 minutes after culture in a medium lacking serum overnight. Immunofluorescence microscopy using antibodies against vinculin showed significant differences in the appearance and distribution of focal adhesions in IMP1-depleted cells when compared with cells expressing IMP1/ZBP1. Control cells formed large and elongated focal adhesions that were most prominent at the cell periphery (Fig. 4A, upper panels). By contrast, cells with downregulated IMP1 expression formed small and short punctate focal adhesions (Fig. 4A, lower panels). Similar observations of focal adhesion appearance were made in MDA231 cells (Fig. 4B). The average size of focal adhesions formed in T47D knockdown cells or MDA control cells was ~50% of that formed in control T47D ($P<0.001$) or MDA231 cells expressing ZBP1/IMP1 ($P<0.0001$), respectively (Fig. 4C). By contrast, the number of focal adhesions formed per cell in T47D knockdown or MDA control cells was approximately 2.4-fold or 2-fold more than the number of focal adhesions formed in T47D control ($P<0.0001$) or MDA231 knock-down cells ($P<0.066$), respectively (Fig. 4D). These results indicate that the role of IMP1/ZBP1 in affecting cell migration and metastasis correlated with impaired dynamics and stability of focal adhesions.

IMP1/ZBP1-expressing cells exhibit increased persistence and directional motility and decreased turnover of focal adhesions

We then used live-cell imaging to analyze the effects of IMP1/ZBP1 on cell motility and focal adhesion turnover. Mobility analysis was performed over a 12 hour period with parental metastatic MDA231 cells and MDA231 cells expressing IMP1/ZBP1. This provided an assessment of the differences in motility between genetically identical cell populations that differed only in their level of IMP1/ZBP1 expression. The experiments showed that, although the average velocity was not substantially reduced, IMP1/ZBP1 expression led to increased persistence and directionality in cell motility (Table 1). These results suggest that IMP1/ZBP1 was sufficient to provide these cancer cells with a greater ability to persist in their directional migration.

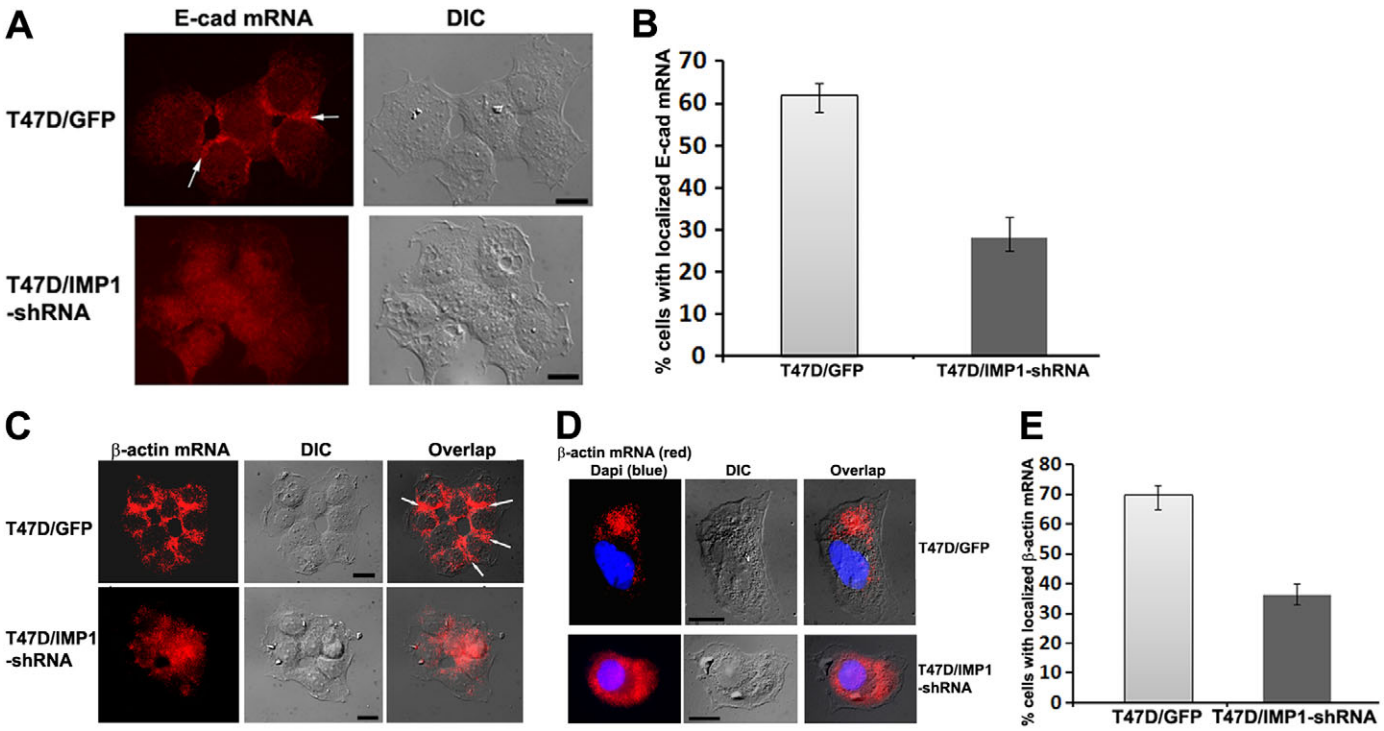

Fig. 3. Knockdown of IMP1/ZBP1 expression in T47D cells delocalizes E-cadherin mRNA and β-actin mRNA at cell–cell adhesions and leading edges. (**A**) Fluorescence in situ hybridization (FISH) of E-cadherin mRNA in T47D/GFP and T47D/IMP1-shRNA cells. Localization of E-cadherin mRNA at cell–cell contacts of T47D/GFP cells (upper panel, arrows). E-cadherin mRNA was more diffuse in T47D/IMP1-shRNA cells (lower panel). (**B**) The percentages of cells (n=100) with localized E-cadherin mRNA, which represent the means ± s.e.m. of the data from three experiments, P<0.001. FISH shows the localization of β-actin mRNA in clustered (**C**) and individual (**D**) T47D/GFP and T47D/IMP1-shRNA cells. Localization of β-actin mRNA at the cell–cell contacts is indicated with arrows (C, upper panel). Localization of β-actin mRNA at or near the leading edges of individual T47D cells was affected when IMP1 was knocked down (D, lower panel). DAPI stains the nuclei blue. (**E**) Graph showing the percentages of cells with localized β-actin mRNA in individual T47D cells, which represent the means ± s.e.m. of the data from three experiments (n=100), P<0.0005.

The cell–cell adhesions of T47D cells led to a lower intrinsic motility – hence, a direct comparison with MDA231 cells was not possible. Instead, we used analysis of cell shape, size and protrusion characteristics after heregulin stimulation, and these showed a substantial difference between IMP1/ZBP1 knockdown cells and wild type. Cells expressing ZBP1 exhibited a single broad, lamellipodial protrusion as early as 15 minutes after stimulation, whereas cells lacking ZBP1 produced filapodial projections only after at least 60 minutes and continued to extend them randomly for the entirety of the 6-hour experiment (supplementary material Movie 1). Thus, in less mobile epithelial cells, IMP1/ZBP1 might aid in regulating the response of the cells to an acute stimulation.

The turnover of focal adhesions in living cancer cells was evaluated using total internal reflection fluorescence (TIRF) microscopy. Transient transfection of MDA231 stable cell lines with plasmid constructs encoding paxillin-mCherry allowed us to track focal adhesion plaques as they formed and disappeared in motile cells on fibronectin-coated glass. A tracking algorithm was used to identify and follow the life span of paxillin fluorescence in TIRF over a 6-hour time period. We observed a substantial increase in the average lifetime of focal adhesions in cells expressing IMP1/ZBP1, as seen in Fig. 5 (supplementary material Movies 2, 3). This observation was consistent with the results in Fig. 4, suggesting that IMP1/ZBP1 might aid in creating a more stable adhesion complex to direct the motility of cancer cells.

IMP1/ZBP1-bound mRNAs involved in focal adhesion dynamics were delocalized in the absence of IMP1/ZBP1 expression

We reasoned that IMP1/ZBP1 expression altered the dynamics of focal adhesions through regulation of the IMP1 target mRNAs. To address this, we examined the localization of two IMP1/ZBP1-bound mRNAs encoding α-actinin and Arp-16 (a component of the Arp2/3 complex), in metastatic MDA231 cells. The protein products of both mRNAs perform crucial roles in regulating focal adhesion metabolism (DeMali et al., 2002; von Wichert et al., 2003). FISH analysis showed that, in control cells, the localizations of both mRNAs as well as β-actin mRNA were mostly perinuclear, with a few cells showing mRNA localization at the leading edges. However, in cells expressing IMP1/ZBP1, localization of α-actinin mRNA increased to 44% of the total cells, compared with the 21% observed in control MDA231/GFP cells (Fig. 6A). Similarly, Arp-16 mRNA increased from 25 to 51% of the total cells (Fig. 6B), and β-actin mRNA increased from 39 to 71% (Fig. 6C).

We then analyzed the pattern of cytoplasmic distribution of α-actinin, ARP-16 and β-actin mRNAs in MDA231/GFP and MDA231/GFP-IMP1 cells, which was determined as a polarization index using a custom MATLAB script. A higher

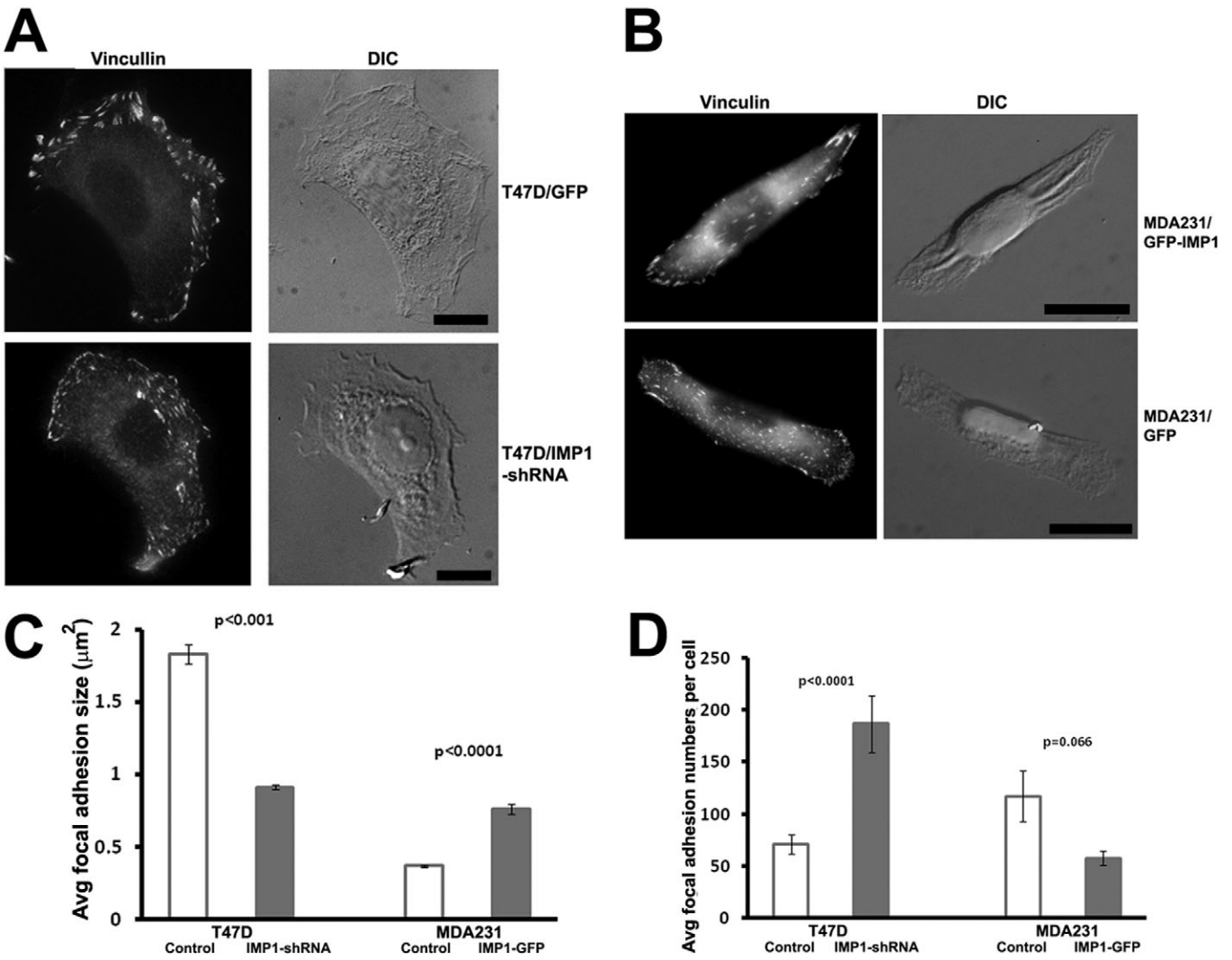

Fig. 4. IMP1/ZBP1 affects the stability of focal adhesions in breast cancer cells. (**A**) T47D/GFP, T47D/IMP1-shRNA and (**B**) MDA231/GFP and MDA21/GFP-IMP1 cells were serum-starved overnight in DMEM containing 0.5% BSA on collagen-coated coverslips and then treated with 1 nM heregulin or 10% serum-containing medium for 30 minutes, respectively (see Materials and Methods). Cells were then fixed and processed for immunofluorescence using antibodies against vinculin and Cy3-conjugated secondary antibodies. Scale bars: 10 μm (A,B). The average size (**C**) and number (**D**) of focal adhesions formed under each condition (T47D vs MDA231) was quantified (see Materials and Methods). Averages of 20 cells from each cell clone were examined from two independent experiments. Error bars indicate ± s.e.m. Controls comprised only of the medium.

polarization index indicates higher asymmetric distribution of the mRNAs. Using the method mentioned above, we analyzed averages of more than 50 cells from each cell clone for mRNA distribution. Bar graphs (Fig. 6D) show that polarization indices of α-actinin mRNA, ARP-16 mRNA and β-actin mRNA in MDA231/GFP-IMP1 cells are all higher than those in MDA231/GFP cells, indicating that expression of GFP-IMP1 in MDA-231 cells leads to more polarized distributions of the mRNAs. Owing to its high abundance, in the case of β-actin mRNA the difference between the polarization indices of MDA231/GFP-IMP1 and MDA231/GFP cells was less apparent because substantial amounts remain in the cell body and skew the index. In order to address the potential effects of cytoplasmic volume on the polarization index, we performed a control experiment using a cytoplasmic dye and observed no substantial difference between MDA231/GFP and MDA231/GFP-IMP1 cells in the distribution of the cytoplasm (Fig. 6D).

Consistent with the delocalization of α-actinin and Arp-16 mRNAs, their corresponding protein distribution was also altered in the absence of IMP1/ZBP1 expression. As with their mRNAs, both α-actinin and Arp-16 proteins were mostly perinuclear and evenly distributed within control MDA231 cells; however, when IMP1/ZBP1 was expressed, an accumulation of protein was observed at the cell protrusions or leading edges. Moreover, the proteins were colocalized to focal adhesions (supplementary material Fig. S2, arrows). Thus, localization of α-actinin and Arp-16 mRNAs to cell protrusions promotes localized expression of their proteins.

Discussion

The studies presented here demonstrate an important role for IMP1/ZBP1 in repressing invasiveness of human breast cancer cells. Repression of the expression of IMP1/ZBP1 substantially reduced accumulation of E-cadherin, a crucial cell adhesion

Table 1. IMP1/ZBP1-expressing cells exhibit increased persistent motility over 6 hours

	MDA231/GFP	MDA231/IMP1-GFP	*P*-value
Total path	350±31 μm	322±27 μm	0.480
Net path	100±17 μm	132±18 μm	0.186
Directionality	0.305±0.053	0.426±0.057	0.121
Average velocity	0.603±0.049 μm/minute	0.544±0.035 μm/minute	0.327
Average instantaneous speed	0.472±0.046 μm/minute	0.485±0.035 μm/minute	0.825
Average directional change	49.0±2.8°	37.7±3.4°	0.012
Average persistence	0.079±0.010	0.112±0.012	0.039

Motility analysis was performed by tracking MDA231/GFP and MDA231/GFP-IMP1 cells under regular growth conditions every 30 minutes. Cell motility was captured by phase-contrast microscopy and later processed by using ImageJ manual tracking and chemotaxis tool plugins available from the ImageJ website. The tracked positions were then used to compute motility statistics on the basis of previously reported parameters (Shestakova et al., 2001). A total of 20 cells were measured for each group.

protein, at cell–cell contacts and impaired the dynamics of focal adhesions – this resulted in a change from a polarized, adherent phenotype into one with unpolarized morphological and invasive behavior. The ability of IMP1/ZBP1 to localize bound mRNAs important for cell–cell adhesion and motility presumably results in a spatial and temporal regulation of specific protein synthesis – thus, a polarized, more adherent and less mobile cell phenotype can be maintained. Our data suggest a novel mechanism for repressing the invasion of breast cancer cells through the regulation of the localized expression of many adhesion- and motility-related mRNAs by IMP1/ZBP1.

Loss of IMP1/ZBP1 function resulted in delocalization of E-cadherin mRNA at the cell–cell contacts of T47D cells, which could subsequently impair the spatial organization of E-cadherin protein synthesis and consequently affect cell adhesion. E-cadherin has a widely recognized role in maintaining cell–cell adhesions and functions as an invasion/tumor suppressor. A transgenic mouse model has demonstrated that E-cadherin-mediated cell adhesion is crucial for preventing the progression from a well-differentiated adenoma to an invasive carcinoma (Perl et al., 1998). The adhesive function of E-cadherin prevents cell detachment and migration, thus inhibiting tumor cell invasion (Behrens et al., 1989; Berx et al., 1995; Birchmeier and Behrens, 1994; Frixen et al., 1991). In addition, re-expression of E-cadherin in epithelial cells where the protein is depleted leads to an inhibition of cell proliferation (Perl et al., 1998; Wong and Gumbiner, 2003) – furthermore, in some cases this inhibition is not dependent on cell adhesion but on the binding of E-cadherin to β-catenin and the subsequent inhibition of Wnt signaling and β-catenin or TCF transcriptional activity (Gottardi et al., 2001; Stockinger et al., 2001). Based on the fact that IMP1/ZBP1 is able to stabilize and regulate the localization of cell–cell contacts of E-cadherin mRNA, we conclude that IMP1/ZBP1 functions to repress breast cancer cell invasion and metastatic tumor growth in part through the regulation of E-cadherin mRNA expression.

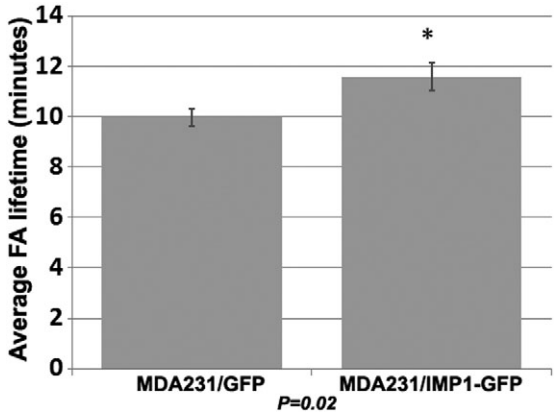

Fig. 5. IMP1/ZBP1 expression reduces turnover of focal adhesions. IMP1/ZBP1 expression decreased the turnover of focal adhesions in motile cancer cells. MDA231/GFP and MDA231/GFP-IMP1 cells were cultured on fibronectin-coated glass and were transiently transfected with plasmid constructs encoding paxillin-mCherry. Focal adhesion plaques were tracked as they were formed and degraded in motile cells by using total internal reflection fluorescence (TIRF) microscopy to monitor paxillin fluorescence over a 6-hour time period. Approximately 35 fields for each cell line were measured in three independent experiments with an average number of 220 adhesion paths per field (*$P<0.05$, error bars indicate ± s.e.m.).

The localization of cell surface transmembrane proteins is more complex than localization of cytosolic mRNAs and proteins. Cell surface transmembrane proteins are synthesized on and processed through the endoplasmic reticulum and Golgi complex prior to insertion at the cell surface. Once at the cell surface, proteins can begin a complex trafficking behavior, which includes exocytosis and endocytosis. A variety of membrane receptors and transporters, including AMPA and NMDA receptors, can exist as exocyst complexes beneath the plasma membrane (Washbourne et al., 2002). It is possible that IMP1/ZBP1 binds to partially translated E-cadherin mRNAs in a novel type of exocyst complex, linking localized translation with exocytosis. This would allow the complexes to be localized to cell–cell contacts where, in response to a cellular signal, they could be activated to complete translation and then be inserted into the plasma membrane. Several mechanisms have shown that regulation of E-cadherin expression can occur either genetically or epigenetically during tumor progression. The Snail-Slug family of transcription factors has been reported to play a major role in E-cadherin repression (Batlle et al., 2000; Bolos et al., 2003; Cano et al., 2000). E-cadherin gene expression is also downregulated by members of the two-handed zinc factor family, ZEB1 and ZEB2 (SIP-1) (Comijn et al., 2001; Perez-Moreno et al., 2001). Recently, hypermethylation and chromatin remodeling of the gene encoding E-cadherin have emerged as the main mechanisms for repression of E-cadherin in most carcinomas (Christofori and Semb, 1999; Liu et al., 2005). Our studies show that E-cadherin expression can be regulated

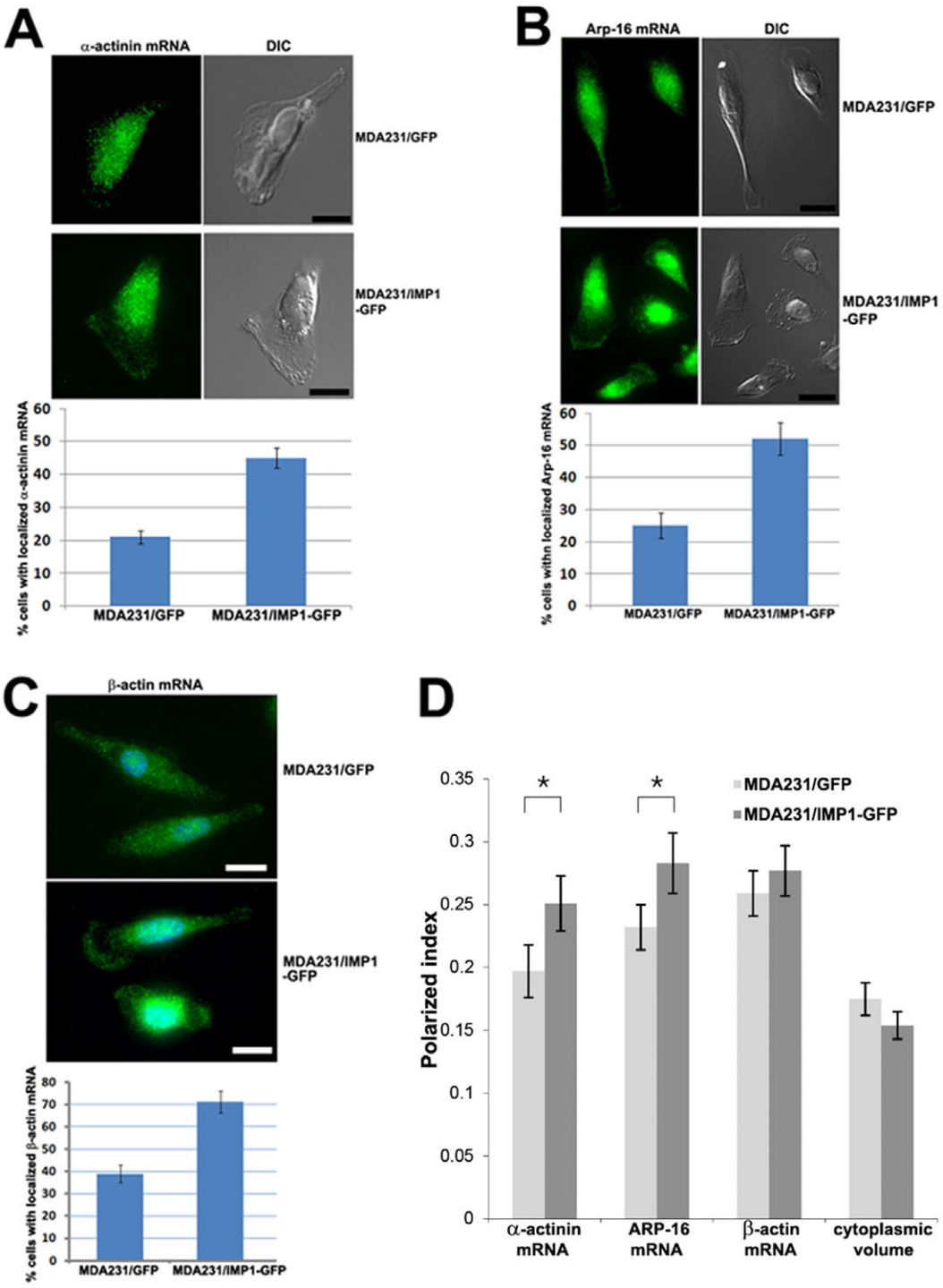

Fig. 6. Localization of α-actinin, ARP-16 and β-actin mRNAs in MDA231 cells results from IMP1/ZBP1 expression. In situ hybridization was performed on MDA231/GFP and MDA231/GFP-IMP1 cells to detect the localization of α-actinin mRNA (**A**), ARP-16 mRNA (**B**) and β-actin mRNA (**C**). Lower panels: percentage of cells localizing mRNA ($n=100$ cells from each cell clone, from two independent experiments). Error bars indicate ± s.e.m. (**D**) mRNA localization was quantified by an algorithm that assesses asymmetry (polarization index) using a custom MATLAB code based on the intensity-weighted centroid of the RNA and nucleus. Polarization index (max=1) was defined as the distance between the two centroids, normalized by the size of the cell ($n=50$ for each clone; error bars ± s.e.m.). A control experiment was performed to address the potential effect of cytoplasmic volume on RNA localization using CellTracker Orange dye (Invitrogen) – no substantial difference between MDA/IMP-GFP and MDA231/GFP cells was observed. $P<0.05$.

post-transcriptionally as well at both the stability (Gu et al., 2009) and localization levels, which effectively maintains cell adhesion and thus provides a brake on malignant cell invasiveness.

Cells expressing IMP1/ZBP1 exhibit larger and more solid focal adhesions, compared with the smaller, punctate shape of focal adhesions observed in cells lacking IMP1/ZBP1. Reduced

focal adhesion turnover is correlated with IMP1/ZBP1 expression, and this correlation is directly associated with persistent and directional cell motility. This suggests a direct connection between IMP1/ZBP1-mediated focal adhesion dynamics and cell invasive ability. Focal adhesions are specialized structures that mediate the cell protrusions required for cell attachment, cell migration and invasion. It has been reported that large focal adhesions are associated with the lateral and more rearward regions, where the cell spatially restricts membrane protrusion (Ballestrem et al., 2001; Wozniak et al., 2004). Signaling events that induce large focal adhesions inhibit membrane protrusion – an example is the activation of Rho (Arthur and Burridge, 2001; Worthylake and Burridge, 2003). By contrast, events such as platelet-derived growth factor (PDGF) treatment (Timpson et al., 2001), epidermal growth factor (EGF) treatment (Condeelis et al., 2001) or Src activation (Frame et al., 2002) lead to the conversion of large focal adhesions into smaller focal complexes and to an increase in membrane protrusion and cell migration. Our experimental data showed that IMP1/ZBP1 expression was associated with the formation of large focal adhesions and a reduction in the number of focal adhesions, indicating that IMP1/ZBP1 might change the motile behavior of cells. This pattern also resembled the appearance of focal adhesions in several cells whose motility was impaired, including cells deficient for focal adhesion kinase (FAK) or Src (Cary et al., 2002; Lahlou et al., 2007; Ren et al., 2000; Webb et al., 2004). Repression of IMP1/ZBP1 expression promoted focal adhesion turnover – presumably this step played an important role in generating cells with a more motile and invasive phenotype.

In order to investigate the molecular mechanism that induces the turnover of focal adhesions by IMP1/ZBP1, we examined the expression of α-actinin and Arp-16 mRNAs – both of these mRNAs have been identified as being IMP1/ZBP1-bound in breast cancer cells (Gu et al., 2009). α-Actinin is found in newly forming focal complexes and plays a crucial role in the maintenance of integrin–actin linkages and focal adhesions (Greenwood et al., 2000; Laukaitis et al., 2001; Rajfur et al., 2002). Focal adhesions devoid of α-actinin lack stability and display higher turnover rates (von Wichert et al., 2003). The Arp2/3 complex, which is a key component regulating the nucleation of actin polymerization and is important for forward protrusion, comprises seven subunits, including Arp-16 (Svitkina and Borisy, 1999). A transient interaction between vinculin, a major component of focal adhesions, and the Arp2/3 complex, has been determined in response to signals that trigger membrane protrusion (DeMali et al., 2002). In situ hybridization demonstrated that, in the absence of IMP1/ZBP1, not only was the localization of α-actinin and Arp-16 mRNAs at the leading edges impaired, but the asymmetric patterns of their cytoplasmic distribution were also altered. As a result of mRNA delocalization, their protein products were unable to accumulate at protrusions and focal adhesions. The changes in focal adhesion turnover observed when α-actinin and Arp-16 mRNA localization was impaired suggest a possible mechanism whereby IMP1/ZBP1 promotes focal adhesion assembly and stability by spatially regulating the translation of α-actinin and Arp-16 mRNAs, as well as other currently unidentified motility-relevant mRNAs.

In HeLa cells, the human homologs of ZBP1 (IMP1 and IMP3) have the ability to mediate the formation of invadopodia by post-transcriptional regulation of CD44 mRNA, resulting in an increased invasive capacity (Vikesaa et al., 2006). Our data provide contradictory evidence for the tendency of IMP1/ZBP1 to repress breast cancer cell invasiveness, by revealing the regulation of cell adhesion and motility-related mRNA targets. One way to reconcile the discrepancy is to consider that cancer cells arising from different origins behave differently in response to the complexity of ZBP1-mediated mRNA expression. For instance, ZBP1/IMP1 could repress invasion and chemo-attractant-induced movement in breast cancer cells by controlling localized expression of ZBP1/IMP1-associated mRNAs [this study and (Condeelis and Singer, 2005)] – furthermore, ZBP1/IMP1 could promote cancer progression by regulating the expression of proliferation-related molecules (Yisraeli, 2005). Cancer cells might be differentially affected depending on the level of ZBP1 expression, leading to changes in cell polarity, migration and invasiveness as a result of changes in the regulation of specific mRNAs (degradation and translation).

We conclude that IMP1/ZBP1 is a master mRNA regulator that plays a crucial role in maintaining cell contacts, stabilizing focal adhesions and establishing stable protrusions, all of which act to stabilize the nonmetastatic phenotype.

Materials and Methods

Stable cell lines and cell culture

The breast adenocarcinoma cell lines T47D/IMP1-shRNA and T47D/GFP cells have been described previously (Gu et al., 2009). Flag-tagged IMP1 cDNA was PCR amplified and subcloned into a lentiviral vector (kind gift of Xiuhua Meng) 3′ to the GFP gene. The GFP-Flag-IMP1 vector and control GFP vector were used to infect MDA231 cells. MDA231 cells infected with the above lentivirus were seeded in a six-well dish at 20% confluence, as previously described (Gu et al., 2009). Stably transfected cell clones were selected in a growth medium containing 1 μg/ml puromycin. Selected cell clones were then cultured at 37℃ in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and were further separated by FACS according to their green fluorescence intensities.

Cell invasion assays

Cell invasion experiments were performed using BD BioCoat growth factor reduced Matrigel invasion chambers according to the manufacturer's protocol (BD Biosciences). Briefly, transwell chambers were hydrated in serum-free DMEM medium for 2 hours in a cell incubator. Serum-starved cells were suspended in 400 μl DMEM medium supplemented with 0.5% bovine serum albumin (BSA) and placed into the upper chambers (2×10^4 cells for MDA231 cells and 4×10^4 cells for T47D cells). The chambers were inserted into 24-well culture dishes containing 500 μl DMEM medium with or without 10% FBS. Cells were allowed to invade through the Matrigel for 12 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.

Antibodies

Primary antibodies used in the experiments included lab-prepared rabbit polyclonal anti-ZBP1 (Gu et al., 2009), rabbit anti-E-cadherin (Covance), mouse monoclonal anti-vinculin (Sigma), rabbit anti-vinculin (Abcam), mouse anti-β-actin (Sigma), mouse anti-α-actinin (Covance) and mouse anti-Arp16 (Covance).

Western blotting

The primary antibodies used in western blotting were rabbit anti-ZBP1 and mouse anti-β-actin antibodies. Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat antibodies against mouse or rabbit. Western blots were developed using the enhanced chemiluminescence (ECL) method.

Focal adhesion assays

T47D and MDA231 cells were plated onto collagen-coated cover slips (Fisher catalogue number 12-545-84) for 12 hours. Cells on coverslips were starved in DMEM medium containing 0.5% BSA overnight. Cells were treated with DMEM containing 0.5% BSA and 1 nM heregulin (for T47D cells) or with DMEM containing 10% FBS (for MDA231 cells) for 30 minutes and were then fixed in 4%

formaldehyde for 15 minutes. Fixed cells were permeabilized in PBS containing 0.5% Triton X-100 for 10 minutes following incubation in PBS containing 0.1 M glycine for 20 minutes. Cells were incubated with primary antibodies against vinculin followed by Cy3- or Cy5-conjugated secondary antibody. Focal adhesions were visualized using an Olympus BX61 microscope, and images were captured using IP-Lab software.

The sizes and numbers of focal adhesions in each cell were determined using software written in IDL 6.2 (Interactive Data Language, ITT Visual Information Solution, Boulder, CO). The software first locates the intensity peaks and sets the focal adhesion threshold to 60% of the peak intensity – the boundary then defines a focal adhesion. Each individual focal adhesion was then visually inspected. In cases where two focal adhesions were touching or in close proximity to one another and the software was unable to distinguish between them, the boundary was determined visually. The software calculates the size of each adhesion and the total number of adhesions per cell. Two-sample *t*-tests and mixed linear models (Zeger and Liang, 1986) were used to test the difference in focal adhesion sizes between IMP1/ZBP1-expressing and nonexpressing cells.

Immunofluorescence analysis and imaging

Cells cultured on glass coverslips were permeabilized with 0.5% Triton X-100 and fixed with 4% paraformaldehyde (PFA) in PBS. The coverslips were incubated with primary antibodies followed by incubation with Cy3- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Fluorescent signals were detected by an Olympus BX61 microscope and analyzed using IPLab software version 3.61 (BD Biosciences). Software written in IDL 6.2 was used to determine the fluorescence intensities of E-cadherin at cell adhesions – briefly, the boundary between two adherent cells was segmented visually and the total fluorescence intensity was determined. The net fluorescence was obtained after the background fluorescence was subtracted. The fluorescence per unit length along the cell adhesion was determined by dividing the net fluorescence in the segmented area by half of the perimeter of the area (which approximates the length of the adhesion). A two-sample *t*-test was used to determine the difference of E-cadherin distribution at the cell adhesions between T47D/GFP and T47D/IMP1-shRNA cells.

Fluorescence in situ hybridization

T47D and MDA-231 cells were grown on coverslips and were fixed with 4% PFA in diethylpyrocarbonate (DEPC)-treated PBS. FISH was essentially performed as described previously (Huttelmaier et al., 2005). The probes used to detect β-actin mRNA have been described previously (Femino et al., 1998) and are listed here. For E-cadherin mRNA: 5′-CCTCAAAGACCTCCTGGATAAACTCTGGCCT-GTTGTCATTCTGATCTGTG-3′, 5′-GATCTTGGCTGAGGATGGTGTAGGC-GATGGCAGCATTGTAGGTGTTTATG-3′, 5′-TGGCAGTGTCCCTCCAAAT-CCGATACGTGATCTTCTGTTCCATGAATGTG-3′ and 5′-ACCTCCAACGT-GGTCACCTGGTCTTTATTCTGGTTATCCGAGAGCTTGAG-3′. For α-actinin: 5′-GATGGTGCGTGCGTGCTAGGGGCTAGATTTCTTTCTCCACCTTCTCTC-CA-3′, 5′-AGTGGATCATCCTTTCGAAGCTTTCCATAGTCAATCAACTCG-GGCCGGTG-3′, 5′-TTTGCCATTGATCTCCTGAGGTGTGATGGTTGTATAG-GGGTTGGTGCCT-3′ and 5′-ATAATTTTGTAAACTGTCACTTTGCGGGTA-GGGAGGCTCGGTGCCGCCTG-3′. For Arp P-16: 5′-TTCACTGCCTGACT-CTTGGTGTTGATAGGGGGGTTCTTCAGAGCTGCCTG-3′, 5′-CTCCTCCAG-CAGCAAGTGCCTTTTCATGCCATTGCAGTAACATAGCACTG-3′, 5′-TTT-GGTTGTTTTGGTCTTTGTACCAGCAATTCCCACTCCCGAGGCAGATA-3′ and 5′-CTAAGCACAAGCTCACTTCCCTCTTGGTCAGGTGGTTTGTTTTAG-AGCTA-3′. All probes used were designed, synthesized and labeled with Cy3 or Cy5 dye according to Singer lab protocols. The cytoplasmic volume was observed with the CellTracker Orange dye (Invitrogen).

Live-cell imaging of cancer cell motility

Cells were cultured on fibronectin-coated (Sigma) polystyrene wells in L-15 medium (Gibco) containing 10% FBS. Imaging was performed 24 hours after plating cells in a single well of a 12-well plate. Cell motility imaging of all experimental conditions was performed using transmitted light through a 10× or 20× Olympus phase objectives on an IX71 inverted Olympus microscope using multifield acquisition driven by MetaMorph software. Environmental conditions were controlled for the entirety of the 12 hour motility experiment through the use of a heated chamber. Motility analysis was performed on images at 30 minute intervals over the 12 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. Centroid coordinates were user-determined and later used to calculate the motility statistics presented in the results. Equations used to define the motility statistics can be found in the supplementary material.

Focal adhesion tracking in motile cancer cells

Cells were transfected with a plasmid encoding paxillin-mCherry by using the AMAXA nucleofector system (Amaxa, Cologne, Germany). After transfection, cells were plated on fibronectin-coated Mattek dishes for image acquisition 24 hours later. An Olympus TIRF IX71 system with heated chamber was utilized to image focal adhesions every minute for 3 hours. A 561 nm laser was used to acquire in TIRF mode with an Olympus 60×, 1.45 NA objective. An Andor iXon 512×512 Back Illuminated EMCCD Camera was used for acquisition, with the entire system driven through MetaMorph. Image analysis was performed using a custom code written in MATLAB. An automated particle-tracking algorithm (Crocker and Grier, 1996) was utilized to track individual adhesion complexes in motile cells. Adhesion complexes within the imaged field were tracked for a total of 3 hours. Each adhesion path was analyzed for its duration, length and complex size. A two-tailed Student's *t*-test was used to validate the significance of whether the experimental conditions regulate focal adhesions differently.

Analysis of cytoplasmic mRNA distribution

The intracellular polarization and localization of mRNA were quantified using a custom MATLAB code (H.Y.P., Tatjana Trcek and A.L.W. et al., unpublished). 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 mRNA within the cells.

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Journal of Cell Science

Supplementary Table-Motility Analysis Equations

Total path	$\sum_{i=1}^{n} \sqrt{(x_i - x_{i+1})^2 + (y_i - y_{i+1})^2}$
Net path	$\sqrt{(x_1 - x_n)^2 + (y_1 - y_n)^2}$
Directionality	(net path)/(total path)
Average Velocity	(total path)/(total time)
Average instantaneous speed	$\frac{\sum_{i=1}^{n} \sqrt{\left[\frac{(x_{i+1} - x_{i-1})}{60}\right]^2 + \left[\frac{(y_{i+1} - y_{i-1})}{60}\right]^2}}{n}$
Average directional change	$\frac{\sum_{i=1}^{n} \left\lvert \left[\tan^{-1}\frac{(y_{i+1} - y_{i-1})}{(x_{i+1} - x_{i-1})}\right] - \left[\tan^{-1}\frac{(y_{i+2} - y_i)}{(x_{i+2} - x_i)}\right] \right\rvert}{n}$
Average persistence	$\sum_{i=1}^{n} \frac{\Delta velocity}{\left(1 + \frac{\Delta direction_{(rad.)}}{36}\right)}$