ERK2 drives tumour cell migration in three-dimensional microenvironments by suppressing expression of Rab17 and liprin-β2

Anne von Thun[1], Marc Birtwistle[2], Gabriela Kalna[1], Joan Grindlay[1], David Strachan[1], Walter Kolch[2], Alexander von Kriegsheim[2,*] and Jim C. Norman[1,*]

[1]Beatson Institute for Cancer Research, Glasgow, G61 1BD, UK
[2]Systems Biology Ireland, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland
*Authors for correspondence (j.norman@beatson.gla.ac.uk; alex.vonkriegsheim@ucd.ie)

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Summary

Upregulation of the extracellular signal-regulated kinase (ERK) pathway has been shown to contribute to tumour invasion and progression. Because the two predominant ERK isoforms (ERK1 and ERK2, also known as MAPK3 and MAPK1, respectively) are highly homologous and have indistinguishable kinase activities in vitro, both enzymes were believed to be redundant and interchangeable. To challenge this view, we show that ERK2 silencing inhibits invasive migration of MDA-MB-231 cells, and re-expression of ERK2 but not ERK1 restores the normal invasive phenotype. A detailed quantitative analysis of cell movement on 3D matrices indicates that ERK2 knockdown impairs cellular motility by decreasing the migration velocity as well as increasing the time that cells spend not moving. Using gene expression arrays we found that the expression of the genes for Rab17 and liprin-β2 was increased by knockdown of ERK2 and restored to normal levels following re-expression of ERK2, but not ERK1. Both play inhibitory roles in the invasive behaviour of three independent cancer cell lines. Importantly, knockdown of either Rab17 or liprin-β2 restores invasiveness of ERK2-depleted cells, indicating that ERK2 drives invasion of MDA-MB-231 cells by suppressing expression of these genes.

Key words: ERK2, cell migration, invasion, cancer, Rab17, Liprin-β2

Introduction

The extracellular signal-regulated kinase (ERK) pathway is one of the most intensively studied mammalian MAPK pathways, and it is deregulated in approximately one-third of all human cancers (Reddy et al., 2003). Aberrant ERK signalling is prevalent in most cancers as a consequence of increased expression and/or mutations of upstream components of the cascade. The canonical role of this abnormal signalling is its positive influence on cell survival and proliferation. It is becoming clear, however, that the ERK pathway also controls tumour cell migration, invasion and progression (Klemke et al., 1997; Ochieng et al., 1991; Reddy et al., 2003; Rodier et al., 1995; Ueoka et al., 2000). ERK1 and ERK2 (also known as MAPK3 and MAPK1, respectively) show 85% sequence identity and have indistinguishable kinase activities in vitro (Boulton et al., 1991; Lefloch et al., 2008; Pouysségur et al., 2002). Therefore, both isoforms were believed to be redundant and interchangeable. However, accumulating evidence, in particular marked discrepancies in the ERK homozygous mutant phenotypes, challenges this view. Whereas ERK1-null mice are viable, fertile and of normal size (Pagès et al., 1999), ERK2-null mice are embryonic lethal (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003), suggesting specific roles for these two isoforms during embryogenesis. However, as ERK2 is the predominant isoform in most tissues, this apparent functional specificity might simply reflect differences in ERK expression or activity levels (Lefloch et al., 2008). Indeed, a recent study has shown that loss of either ERK1 or ERK2 slows proliferation of fibroblasts to an extent that reflects the expression level of the individual kinase (Voisin et al., 2010). Moreover, proliferation is reduced following conditional inactivation of the (more abundant) ERK2 isoform in neuronal precursor cells, but cell growth resumes when ERK1 activity is upregulated to compensate for loss of ERK2 (Samuels et al., 2008). Thus, although disruption of ERK1 and ERK2 can clearly produce different phenotypes in a range of biological contexts, it is unclear to what extent this reflects true functional differences between the kinases or other factors, such as gene dosage.

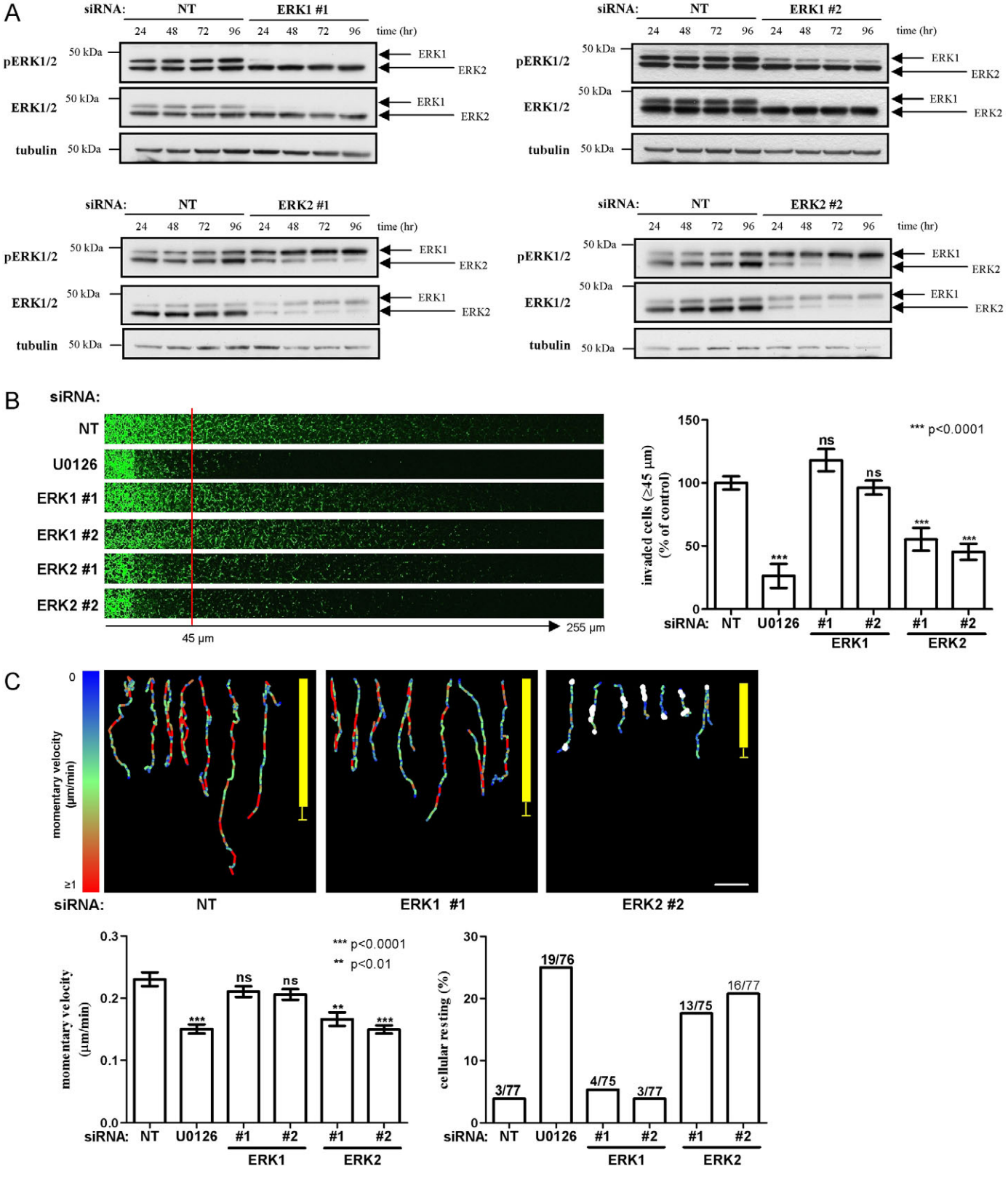
The aim of this study was to investigate the role of the two ERK isoforms in the invasiveness of tumour cell lines, and to determine whether ERK isoform differences with regards to cell migration are true functional disparities, or the result of different expression levels of the two kinases. We provide evidence that ERK2 (but not ERK1) is particularly important in the invasiveness of cancer cells. Furthermore, we report that ERK2 drives invasive migration in three-dimensional microenvironments by reducing the expression of two previously unidentified motility suppressor genes: *RAB17* and the gene encoding liprin-β2, *PPFIBP2*.

Results

Knockdown of ERK2 impairs invasion into Matrigel and migration on cell-derived matrix

We used the MDA-MB-231 breast cancer cell line to investigate the respective roles of ERK1 and ERK2 in invasive migration. We transiently knocked down either ERK1 or ERK2 (Fig. 1A)

with two independent short interfering RNAs (siRNAs) for each isoform, and plated the cells for inverted invasion assays. Although the knockdown of ERK1 was efficient and persisted for up to 96 hours following transfection (Fig. 1A), its suppression had no substantial effect on invasive migration into fibronectin-containing Matrigel (Fig. 1B). By contrast, invasion into Matrigel



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was clearly reduced when ERK2 was silenced (Fig. 1B). Addition of the MEK inhibitor, U0126, produced a slightly greater effect on migration than the ERK2 knockdown (Fig. 1B). Because impaired cell viability might adversely affect cell invasion and migration, we investigated whether ERK knockdown affected cell growth and apoptosis. We found that knocking down either ERK isoform had no effect on proliferation (supplementary material Fig. S1A) or apoptosis (supplementary material Fig. S1B) indicating that our invasion and motility results were not influenced by the effects of ERK signalling on cell viability.

Because the invasive process is difficult to see in Matrigel plugs, we monitored cell movement on plates coated with cell-derived matrix (CDM); a relatively thick, pliable matrix composed mainly of fibrillar collagen and fibronectin that recapitulates key aspects of the type of matrix found in connective tissues (Bass et al., 2007; Cukierman et al., 2001). We transfected MDA-MB-231 cells with ERK1 or ERK2 siRNAs and recorded cell movement on CDM over 16 hours. We noticed that ERK2 knockdown cells had a tendency to remain stationary for extended periods of time. To quantify this we defined a cell that moved less than 2 μm in 90 minutes as one that was engaged in 'cellular resting'. ERK2 knockdown or addition of U0126 markedly increased the proportion of cells that were resting, whereas ERK1 siRNA was ineffective in this regard (Fig. 1C). We also determined whether ERK2 siRNA influenced cell movement during the period in which cells were not resting. To do this we calculated frame-to-frame migration speeds, which we have called the 'momentary velocity' and compared values greater than zero. We found the momentary velocity to be significantly ($P \leq 0.01$) reduced following ERK2 knockdown or addition of U0126, but was it was unaffected by ERK1 siRNA (Fig. 1C). Taken together, these data indicate that knockdown of ERK2 decreases cell invasiveness, and that this is due to a combination of reduced momentary velocity and an increased tendency of ERK2 knockdown cells to remain immobile or rest for extended periods.

Fig. 1. Knockdown of ERK2 opposes invasion into Matrigel and migration of MDA-MB-231 cells on CDM. (**A**) MDA-MB-231 cells were transfected with non-targeting siRNAs (NT), or those targeting ERK1 or ERK2. The effectiveness of the ERK knockdown was assessed by western blotting over the course of 4 days following transfection. (**B**) MDA-MB-231 cells were transfected with non-targeting siRNAs (NT) or those targeting ERK1 or ERK2 and plated onto plugs of Matrigel supplemented with fibronectin (25 μg/ml). The MEK inhibitor, U0126 (10 μM) was included as indicated. 36 hours after this, invading cells were visualized by Calcein-AM staining. Serial optical sections were captured every 15 μm and are presented as a sequence in which the depth increases from left to right (left panel). Invasive migration was quantified by measuring the fluorescence intensity of cells penetrating the Matrigel plug to depths of ≥45 μm, and expressed relative to cells transfected with non-targeting (NT) siRNA. Values are means ± standard error of the mean (s.e.m.) of 18 replicates from three independent experiments (right panel). (**C**) MDA-MB-231 cells were transfected with non-targeting siRNAs (NT), or those targeting ERK1 or ERK2 and plated onto cell-derived matrix. Images were captured every 10 minutes over a 16-hour period and movies generated from these (supplementary material Movies 1 and 2). Cell movement was followed using cell-tracking software. Representative migration tracks are displayed in the upper panels and the migration speed is denoted by a colour code, the scale of which is indicated on the left side of the panels. The points at which cells moved less than 2 μm in 90 minutes (cellular resting) are indicated by white dots. Scale bar: 100 μm. The average vectorial (Euclidian) distance migrated by the cells under each experimental condition is depicted by the vertical yellow bar (means ± s.e.m.). Momentary migration velocities were calculated for each timeframe of the time-lapse experiment (lower left panel). Values are means ± s.e.m. of 15 fields from three independent experiments. Five cells were tracked for each field. Percentage of resting cells is given in the lower right panel with absolute numbers for each condition above the columns.

Ectopic expression of ERK2 (but not ERK1) restores the migratory characteristics of MDA-MB-231 cells after ERK2 knockdown

MDA-MB-231 cells predominantly express the ERK2 isoform. Therefore, observations that knockdown of ERK2 affects invasion (whereas ERK1 siRNA does not) do not necessarily reflect functional differences between the two kinases, but might be attributable to differences in their expression level (Lefloch et al., 2008; Voisin et al., 2010). To address this, we expressed recombinant ERK1 and siRNA-resistant ERK2 to equal levels after knocking down ERK2 (Fig. 2A, Fig. 3A). The expressed levels of SF-ERK1 and SF-ERK2 isoforms (see Materials and Methods) were similar to one another (Fig. 2A, Fig. 3A), their expression was maintained for up to 3 days following transfection (thus allowing sufficient time to carry out Matrigel invasion assays) (supplementary material Fig. S2A), and both recombinant kinases were equally phosphorylated by MEK in MDA-MB-231 cells (Fig. 2A, Fig. 3A). Moreover, expression of the recombinant ERKs compromised neither the extent, nor the persistence of ERK2 siRNA suppression (Fig. 2A, Fig. 3A; supplementary material Fig. S2A). Expression of recombinant ERK1 did not rescue the inhibitory effect of ERK2 knockdown on invasion, whereas siRNA-resistant ERK2 expression completely restored the invasive phenotype of MDA-MB-231 cells (Fig. 2B). Moreover, when cell migration within CDM was analysed, re-expression of siRNA-resistant ERK2 increased the momentary velocity and reduced the tendency of cells to pause (cellular resting) relative to that of control cells, whereas re-expression of ERK1 did not rescue the migratory defects of ERK2 knockdown cells (Fig. 3C).

The primary sequences of ERK1 and ERK2 diverge most in their N-terminal portions. A recent study found that ERK1 shuttles into the nucleus more slowly than ERK2, and domain swap experiments indicated that this property was conferred by a region in the longer N-terminal domain of ERK1 (Marchi et al., 2008). To address whether the information responsible for driving cell migration resides within the N-terminal portion of ERK2, we generated two chimeras; one containing the N-terminal region of ERK2 fused to the C-terminal portion of ERK1 (E2>E1) and another corresponding chimera of the N-terminal region of ERK1 fused to the C-terminal portion of ERK2 (E1>E2; Fig. 3B). Although expressed at lower levels than the native kinases, E2>E1 and E1>E2 were present at similar levels to one another (on an ERK2 knockdown background) and both chimeras were similarly phosphorylated at the TEY motif (Fig. 3B), suggesting an intact tertiary structure. Analysis of cell migration within CDM indicated that although expression of E1>E2 restored the migratory defects caused by ERK2 knockdown, E2>E1 was ineffective in this regard (Fig. 3D). These data indicate that the capacity of ERK2 to drive migration is not located within the divergent N-terminal portion of the kinase.

Identifying an ERK2-dependent gene expression signature

To determine whether isoform-specific regulation of gene expression was responsible for influence of ERK2 over cell migration and invasion, we expressed ERK1 or ERK2 in ERK2

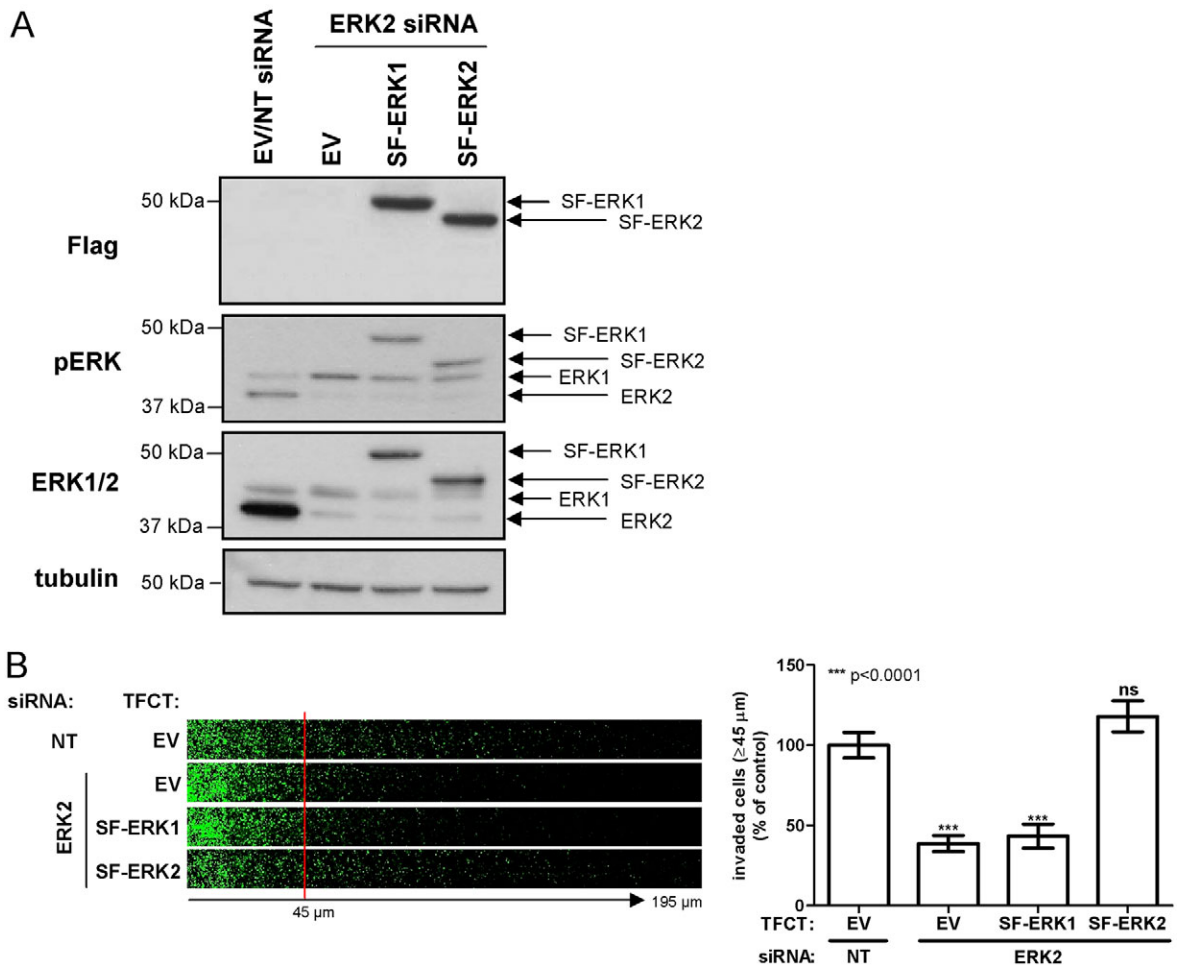
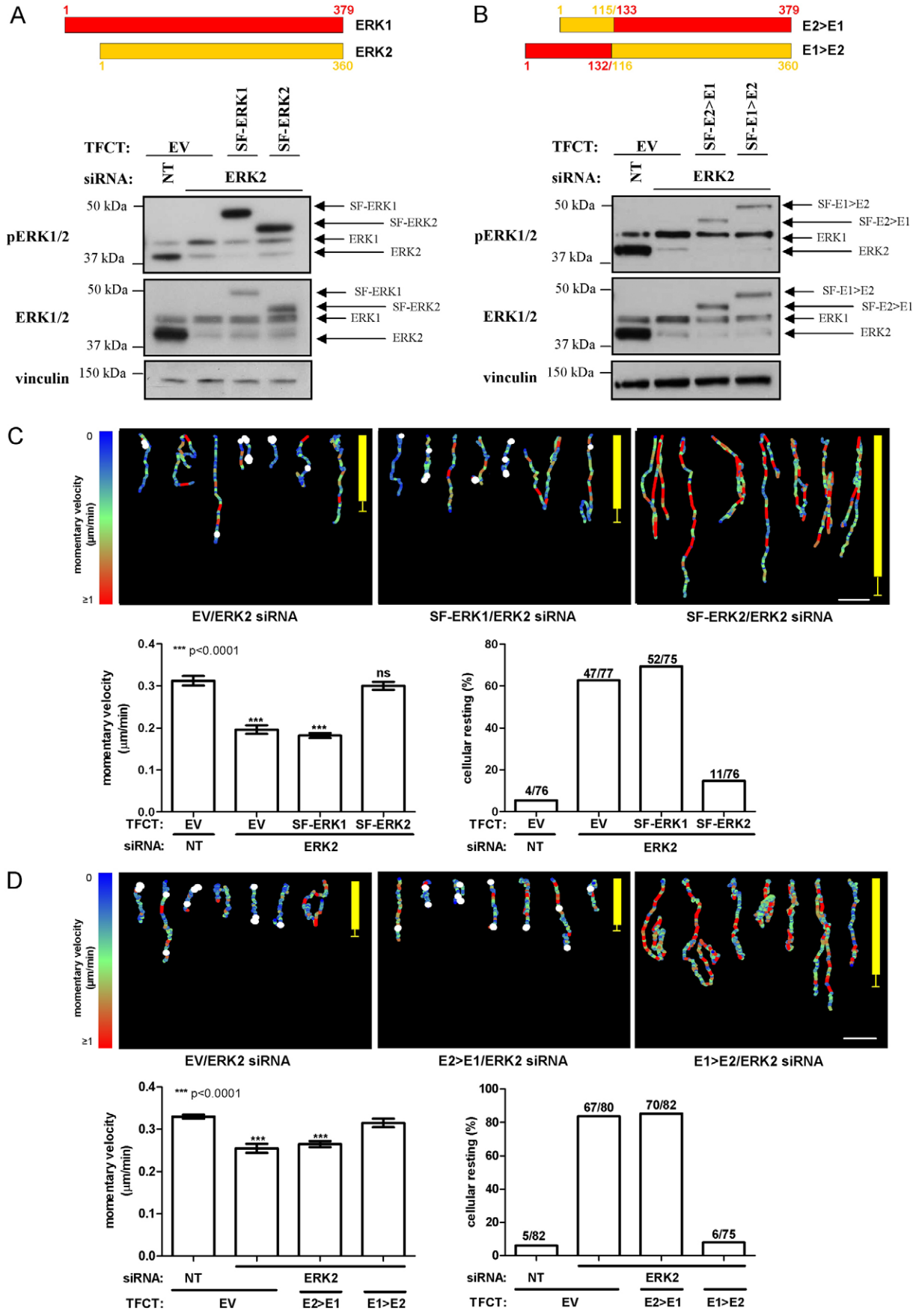


Fig. 2. Ectopic expression of ERK2 but not ERK1 restores invasion of ERK2 knockdown cells. (**A**) MDA-MB-231 cells were transfected with non-targeting siRNAs (NT), or siRNA targeting ERK2 in combination with expression plasmids for SF-ERK1, SF-ERK2 or an empty vector control (EV). Cells were harvested 2 days after transfection and ERK expression levels were determined by western blotting. (**B**) MDA-MB-231 cells were transfected with non-targeting siRNAs (NT), or siRNA targeting ERK2 in combination with expression plasmids for SF-ERK1, SF-ERK2 or an empty vector control (EV). Cells were plated onto plugs of fibronectin-supplemented Matrigel and invasion determined as for Fig. 1B.

knockdown cells (supplementary material Fig. S2B), plated them onto CDM for 16 hours and performed an Illumina gene expression array. These gene expression screens were performed with three independent biological replicates (i.e. separate experiments), and the reproducibility of the ERK knockdowns and re-expression of siRNA-resistant ERK2 and ERK1 across these three separate experiments is shown in supplementary material Fig. S2B. Knockdown of ERK2 altered the expression of a large number of genes and in most cases expression of these was restored to normal levels by ectopic expression of either ERK1 or ERK2. For instance, *EGR1* fell into this category, and regulation of *EGR1* expression by both ERKs has previously been demonstrated (Lefloch et al., 2008). However, we identified a subset of 27 genes whose expression was altered by knockdown of ERK2, but restored to normal levels by re-expression of siRNA-resistant ERK2 but not ERK1. Prominent amongst these were the genes for Ras-related protein 17 (*RAB17*) and liprin-β2 (*PPFIBP2*), whose mRNA levels were strongly increased by knockdown of ERK2, and normalised by re-expression of ERK2 (but not ERK1; Fig. 4A). Rab17 is a small GTPase, which has been shown to regulate intracellular transport of proteins and lipids (Zacchi et al., 1998). Rab17 expression is thought to be restricted to epithelial cells and there are indications that it is involved in the maintenance of epithelial polarity (McMurtrie et al., 1997). Liprin-β2 belongs to the family of LAR-interacting proteins (liprins) and has been shown predominantly to localise to membrane structures (Serra-Pagès et al., 1998).

We used quantitative real-time-PCR (qRT-PCR) to confirm that levels of *RAB17* and liprin-β2 (*PPFIBP2*) mRNAs were significantly ($P \leq 0.0001$) increased following knockdown of ERK2 (with two independent RNA duplexes), whereas ERK1 silencing did not elevate either *RAB17* or liprin-β2 (*PPFIBP2*) levels (Fig. 4B,C). Furthermore, re-expression of siRNA-resistant ERK2 or the E1>E2 chimera restored *RAB17* and liprin-β2 (*PPFIBP2*) to levels displayed by control cells, whereas expression of ERK1 or E2>E1 was ineffective in this regard (Fig. 4B,C).

We wished to evaluate the extent to which the ECM environment was responsible for these ERK2-specific changes in gene expression. We, therefore, knocked down ERK1 or ERK2, plated the cells onto plastic dishes (instead of CDM) and



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looked at the expression levels of *RAB17* and liprin-β2 (*PPFIBP2*). Clearly, ERK2 siRNA increased expression of *RAB17* and liprin-β2 (*PPFIBP2*) when cells were plated onto plastic surfaces, and knockdown of ERK1 also drove *RAB17* expression, but to a lesser extent (Fig. 4B,C). These data indicate that the presence of a 3D microenvironment is not a prerequisite for ERK2 to be able to suppress *RAB17* and liprin-β2 (*PPFIBP2*) expression, but that ERK1 might also acquire some capacity to control *RAB17* when cells are plated onto plastic. Unfortunately, we were unable to assess to what extent these changes in mRNA levels relate to changes in protein levels, as all the available antibodies that we have tested are not capable of recognising Rab17 or liprin-β2 protein in cell lysates.

To determine whether liprin-β2 and Rab17 influenced one another's expression, we knocked down either liprin-β2 or Rab17 and measured their respective mRNA levels by qRT-PCR. We found that liprin-β2 siRNA did not alter levels of *RAB17* mRNA, and neither did Rab17 knockdown affect liprin-β2 (*PPFIBP2*) expression (Fig. 4B,C). Next, we determined whether related members of the Rab or liprin family were regulated by ERK2. Expression of Rab20, a Rab GTPase that exhibits close homology to Rab17, was minimally affected by manipulation of ERK2 expression (Fig. 4D), and other members of the liprin family (*PPFIBP1*, *PPFIA1* and *PPFIA2*; encoding liprinβ1, -α2 and -α4) were also minimally affected by knockdown or re-expression of ERKs but not in the same fashion as was liprin-β2 (Fig. 4E).

ERK2 drives invasion by suppressing Rab17 and liprin-β2 expression

To determine whether Rab17 and liprin-β2 influence cell motility, we used siRNA to reduce their expression (supplementary material Fig. S3), and performed invasion assays. At this stage, we also looked at the behaviour of A2780 ovarian carcinoma cells stably transfected with the Rab11 GTPase Rab25 (A2780-Rab25 cells) and BE colon carcinoma cells because, similar to MDA-MB-231s, these cells invade fibronectin-containing Matrigel with high efficiency (Caswell et al., 2007; Moran-Jones et al., 2009). Interestingly, knockdown of either Rab17 or liprin-β2 promoted invasion of all three cell lines into Matrigel (Fig. 5A). Consistent with this, we found that overexpression of either GFP–Rab17 or GFP–liprin-β2 suppressed invasion of MDA-MB-231 and A2780-Rab25 cells (by comparison with expression of GFP alone; Fig. 5B). Moreover, overexpression of GFP–Rab17 or GFP–liprin-β2 reduced the momentary velocity and increased the tendency of MDA-MB-231 cells to pause (cellular resting) while migrating within CDM, in much the same way as did knockdown of ERK2, indicating that Rab17 and liprin-β2 act to restrict carcinoma cell migration in 3D microenvironments (Fig. 5C).

We wished to determine to what extent suppression of Rab17 and liprin-β2 by ERK2 was responsible for the ability of the kinase to drive invasion. To do this, we knocked down ERK2 in combination with knockdown of either Rab17 or liprin-β2. Interestingly, either Rab17 or liprin-β2 siRNA restored invasion of ERK2 knockdown cells to levels comparable with control cells (Fig. 6A). Moreover, Rab17 or liprin-β2 siRNA (with either a SMARTPool or two individual RNA duplexes) increased momentary velocity and reduced the tendency of ERK2 knockdown cells to pause (cellular resting) while migrating within CDM. Indeed, cells that had ERK2 knocked down in combination with either Rab17 or liprin-β2 migrated in a way that was indistinguishable from control cells (Fig. 6B). Taken together these data indicate that *RAB17* and liprin-β2 (*PPFIBP2*) are motility suppressor genes, and in order to drive invasion and migration of carcinoma cells in 3D microenvironments, ERK2 must reduce the expression of at least one of these genes.

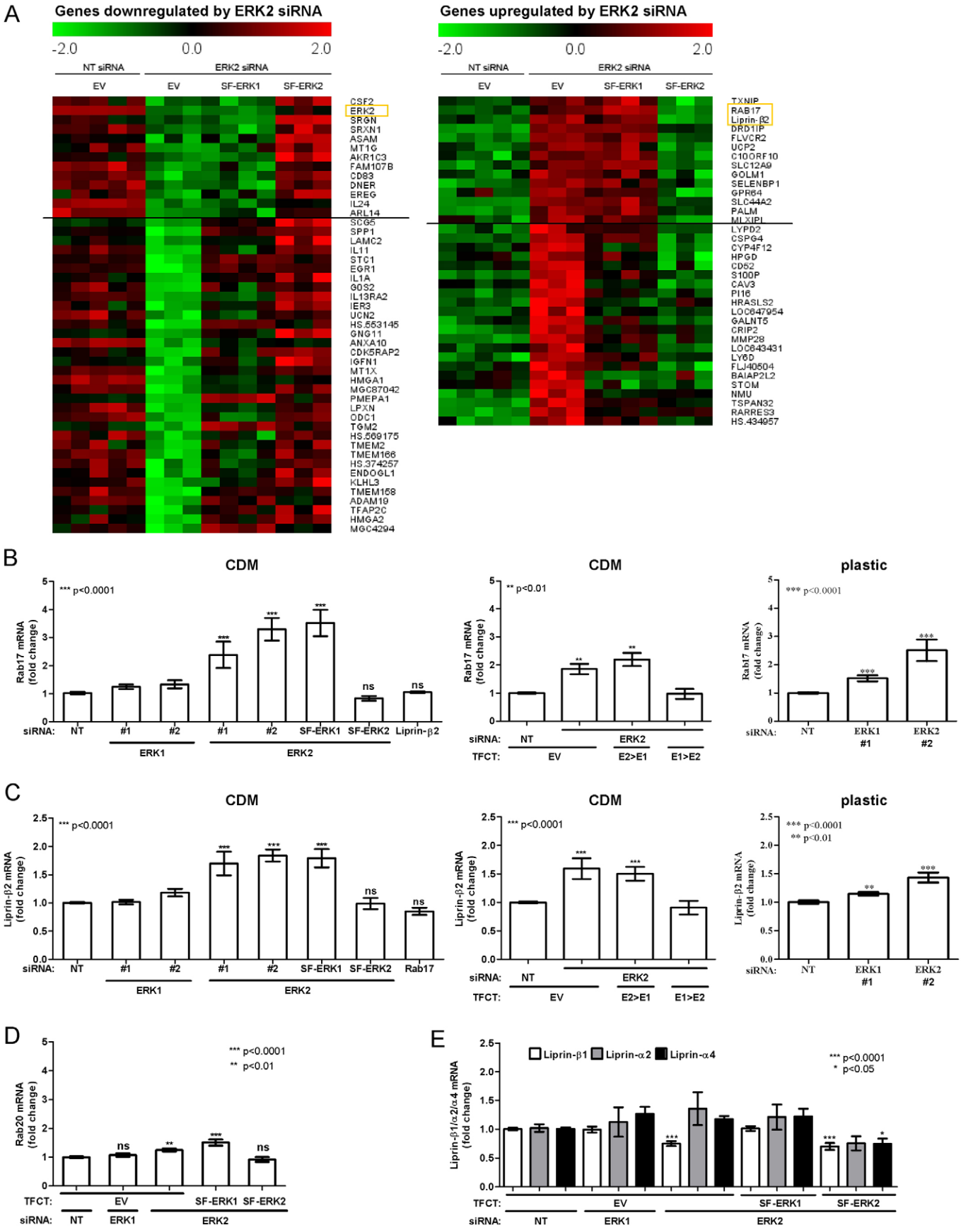
ERK2 drives migration on plastic surfaces, but not through Rab17 or liprin-β2

We wished to investigate the requirement for suppression of Rab17 and liprin-β2 in ERK2-dependent cell migration into scratch wounds. Knockdown of ERK2 (but not ERK1) delayed wound closure (Fig. 7A), and cell tracking analyses indicated that this was because of reduced migration speed, whilst the migratory persistence and the forward migration index (FMI; a quantitative measurement of persistent migration perpendicular to the wound edge) remained unaltered following ERK2 knockdown (Fig. 7B). Thus, ERK2 (but not ERK1) probably plays a role in cell migration in both 3D (Matrigel plugs and CDM) and 2D (scratch wound assays) microenvironments. However, in contrast to the situation in 3D microenvironments, Rab17 and liprin-β2 siRNAs were completely unable to restore the migration velocity of ERK2 knockdown cells (Fig. 7B), although the persistence of Rab17 and liprin-β2 knockdown cells was marginally increased (Fig. 7B). Taken together, these data indicate that although ERK2 controls cell movement in 3D microenvironments by suppressing Rab17 and liprin-β2, the pathways by which ERK2 controls cell migration on plastic do not involve these two previously unidentified ERK2 effectors.

Discussion

In recent years, much evidence supporting ERK isoform-specific functions has accumulated. Firstly, striking discrepancies between the ERK1 and ERK2 homozygous mutant phenotypes in mice argued for distinct roles of these kinases in embryogenesis (Bost et al., 2005; Hatano et al., 2003; Mazzucchelli et al., 2002; Pagès et al., 1999; Saba-El-Leil et al., 2003; Yao et al., 2003). Moreover, some studies have suggested specific roles for ERK2 in learning and memory, proliferation, and cell cycle progression (Frémin et al., 2007; Satoh et al., 2007; Vantaggiato et al., 2006). However, this view was challenged by Lefloch et al., who proposed ERK gene dosage as the reason for the differences in the ERK mutant phenotypes (Lefloch et al., 2008). More recently, genetic evidence indicates that ablation of either ERK1 or ERK2 had no effect on K-Ras-driven lung carcinogenesis because of the

Fig. 3. Expression of recombinant ERK2 or the E1>E2 chimera restores migration characteristics of ERK2-depleted cells. (**A**) Cells were transfected and as for Fig. 2A, harvested 2 days after transfection, and ERK expression levels were determined by western blotting. (**B**) MDA-MB-231 cells were transfected with non-targeting siRNAs (NT), or siRNA targeting ERK2 in combination with expression plasmids for SF-tagged E2>E1 or E1>E2 chimeras or an empty vector control (EV). In A and B the coordinates of the ERK chimeras are indicated with the portions of the primary sequence (with amino acid numbers in the appropriate colour) that derive from ERK1 and ERK2 being denoted in red and yellow, respectively. Cells were harvested 2 days after transfection and ERK expression levels were determined by western blotting. (**C**,**D**) Cell transfected as for A and B were plated onto CDM and their migration characteristics were analysed and presented as for Fig. 1C.



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ability of these kinases to compensate for one another (Blasco et al., 2011). However, this study looked primarily at primary tumour formation and did not consider potential isoform-specific roles for ERK1 and ERK2 in invasion and metastasis. By developing a system in which we can knockdown ERK2 and then ectopically express either ERK1 or ERK2 to similar levels, we have shown that ERK2 is the main driver of cell migration and invasion in 3D microenvironments in a way that is not influenced by gene dosage.

The role of the ERKs in cell migration has been studied extensively and it is clear that these kinases play a key role in tumour progression by regulating cell invasiveness (Klemke et al., 1997; Ochieng et al., 1991; Reddy et al., 2003; Rodier et al., 1995; Ueoka et al., 2000). However, little is known about the respective contributions of ERK1 and ERK2 to cell migration. Our finding that it is ERK2, and not ERK1, that contributes to cell migration in 3D microenvironments is certainly in agreement with observations in vivo, in that *Erk2*$^{+/-}$ mice show a delay in wound healing compared with Erk2 wild-type mice after partial-thickness burns (Satoh et al., 2009). Studies in zebrafish further support this view as ERK2 but not ERK1 morphants show defects in cytoskeletal reorganisation processes, which lead to anterior-to-posterior migration retardations (Krens et al., 2008). More recently, by using a retroviral system to express ERK 1 and 2 to similar levels, a study has demonstrated a specific role for ERK2 in cell migration in MCF-10A cells, which was due to the participation of this ERK isoform in Ras-induced epithelial-to-mesenchymal transformation (EMT) (Shin et al. 2010).

Both ERKs have indistinguishable kinase activities in vitro (Lefloch et al., 2008) and so far no isoform-specific protein interaction domains have been identified, thus leaving the question of how isoform specificity can be achieved unanswered. However, Marchi et al. have shown isoform-specific nuclear shuttling rates caused by differences in the N-terminal amino acid sequence (Marchi et al., 2008), suggesting that ERK1 and ERK2 perform different roles within the nucleus. We find that, although the expression of most ERK target genes is controlled equally by ERK1 and ERK2, a small subset of ERK targets (including Rab17 and liprin-β2) are specific to ERK2. At this stage we do not know how ERK2 suppresses Rab17 and liprin-β2, but the rate at which they are upregulated following ERK inhibition (it takes 24 hours before the mRNA for Rab17 is increased following addition of U0126; not shown), suggests that the Rab17 and liprin-β2 genes are targeted indirectly by ERK2. Here we report that the ability of ERK2 to control expression of Rab17 and liprin-β2 (and thereby cell migration and invasion) is not conferred by sequences within the divergent N-terminal portion of the kinase. This region of the ERKs is known to be responsible for the nuclear–cytoplasmic shuttling of ERK1 and ERK2, indicating that the capacity to selectively control Rab17 and liprin-β2 expression is unlikely to be linked to the nuclear import and/or export rates for these kinases.

However, despite having identified ERK2-specific events that play a clear role in its ability to drive invasion, at this stage we can only speculate on how specificity is accomplished. Isoform-specific scaffolds might localise the kinases differently and thereby provide specificity. although none have been identified to date. It is also possible that ERK1 and ERK2 possess distinct protein interactomes or varying affinities for the same substrate. Shin et al. demonstrated that ERK2 induced EMT through DEF motif-dependent signalling (Shin et al., 2010). Because both ERK isoforms bind to DEF motifs (Turjanski et al., 2007), this observation argues for distinct substrate affinities in vivo. Moreover, ERK2 has recently been identified as an unconventional DNA-binding protein, which specifically binds to a [G/C]AA [G/C] consensus motif (Hu et al., 2009). Although DNA-binding was shown to be independent of the kinase activity of ERK2 (Hu et al., 2009), it is possible that active ERK bound to the promoter region can influence transcription by phosphorylating neighbouring transcription factors. Interestingly, both ERK isoforms harbour the crucial residues involved in DNA-binding, but amino acids flanking these residues are only partially conserved between the two kinases. Thus, differing DNA binding grooves between the two kinases could exist and might account for differences in regulation of gene expression.

Rab17 has been shown to influence receptor-mediated transcytosis and recycling of receptors to the apical membrane in non-transformed epithelial cells (Hunziker and Peters, 1998; Zacchi et al., 1998). Recently, a study by Singh et al. found that Rab17 was one of many genes to be upregulated in cancer cell lines exhibiting a more epithelial morphology, whereas Rab17 was downregulated in cells displaying a more mesenchymal morphology (Singh et al., 2009). This indicates that Rab17 is associated with the maintenance of a polarised epithelial morphology and this might explain why Rab17 levels must be reduced in order for metastatic tumour cells to migrate with mesenchymal characteristics. Given that Rab17 would be expected to control membrane trafficking, it is interesting to speculate as to how it might suppress cell migration. Recycling of α5β1 integrin is known to be key to tumour cell migration and invasion (Caswell et al., 2008; Caswell et al., 2007; Muller et al., 2009) and we have preliminary data suggesting that overexpression of Rab17 leads to the accumulation of β1-integrin within large endosomes to which Rab17 itself is also localised (data not shown). Taken together with reports suggesting that Rab17 opposes the return of receptors to the plasma membrane (Hunziker and Peters, 1998; Zacchi et al., 1998), this moots that Rab17 could be an integrin recycling suppressor whose expression must be reduced for cells to migrate efficiently, and future work will explore this further.

The role of liprin family members, in particular the α-liprins, has been well established in synaptic transmission. It is clear from reverse genetic studies in vertebrates, *Drosophila* and *C. elegans* that liprin mutants have defects in synaptic vesicle

Fig. 4. Rab17 and liprin-β2 transcription is regulated in an ERK2-dependent manner. (**A**) MDA-MB-231 cells were transfected with non-targeting siRNAs (NT) or an siRNA targeting ERK2 in combination with expression plasmids for SF-ERK1, SF-ERK2 or an empty vector control (EV) and plated onto CDM. RNA was extracted, labelled and comparative whole-genome expression profiling was performed using Illumina HT-12 v4 expression chips. The heat maps display genes that are down- (left panel) or upregulated (right panel) in an ERK2-dependent manner and these are ranked as described in Materials and Methods. (**B**,**C**) MDA-MB-231 cells were transfected with the indicated siRNAs and ERK expression plasmids, and plated onto CDM or plastic dishes as indicated. qRT-PCR was performed to validate the differential regulation of *RAB17* (B) or liprin-β2 (*PPFIBP2*) (C). Data were normalised to GAPDH and values, relative to the control transfected samples, are expressed as means ± s.e.m. of 18 replicates from six independent experiments. (**D**,**E**) qRT-PCR was performed to assess ERK2-dependent expression of *RAB20* and *PPFIBP1, PPFIA1* and *PPFIA2* (liprin-β1, α2 and α4). Data were analysed as in B.

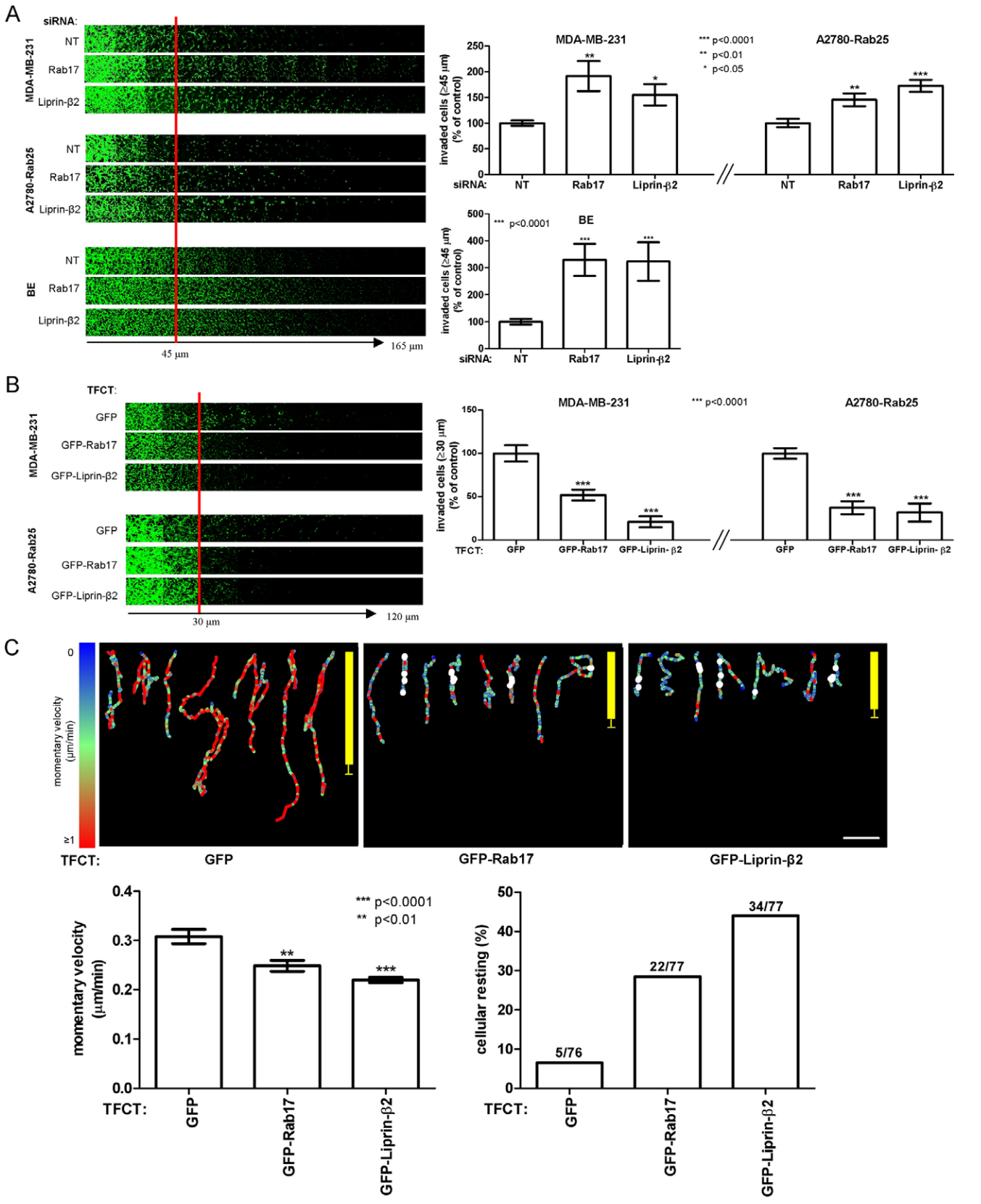

Fig. 5. Rab17 and liprin-β2 are inhibitors of cell migration and invasion. (**A**) MDA-MB-231, A2780-Rab25 and BE cells were transfected with non-targeting siRNAs (NT), siRNAs targeting Rab17 or liprin-β2. Cells were plated into plugs of fibronectin-supplemented Matrigel and invasion determined as for Fig. 1B. (**B**) MDA-MB-231 and A2780-Rab25 cells were transfected with expression plasmids for GFP–Rab17, GFP–liprin-β2 or GFP alone. Cells were plated into plugs of fibronectin-supplemented Matrigel and invasion determined as for Fig. 1B. (**C**) Transfected cells from B were seeded onto CDM and their migration characteristics were analysed and presented as for Fig. 1C.

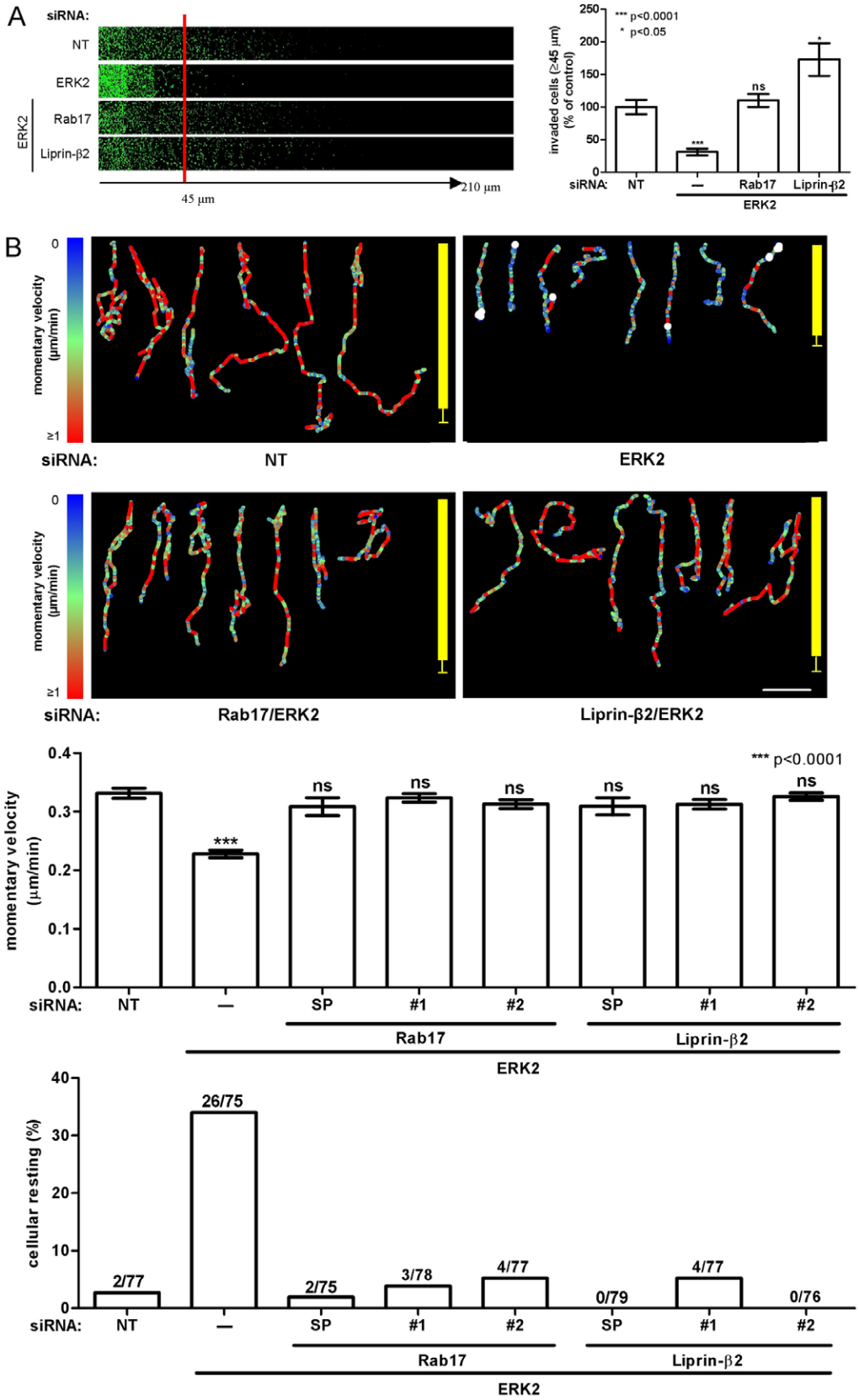

Fig. 6. Knockdown of Rab17 or liprin-β2 restores the invasiveness and migratory characteristics of ERK2 knockdown cells. MDA-MB-231 cells were transfected with non-targeting siRNAs (NT) or siRNAs targeting ERK2 in combination with individual or SMARTPool (SP) oligonucleotides targeting Rab17 or liprin-β2. Cells were plated onto (**A**) plugs of fibronectin-supplemented Matrigel and invasion determined as for Fig. 1B or (**B**) onto CDM and their migration characteristics were analysed and presented as for Fig. 1C.

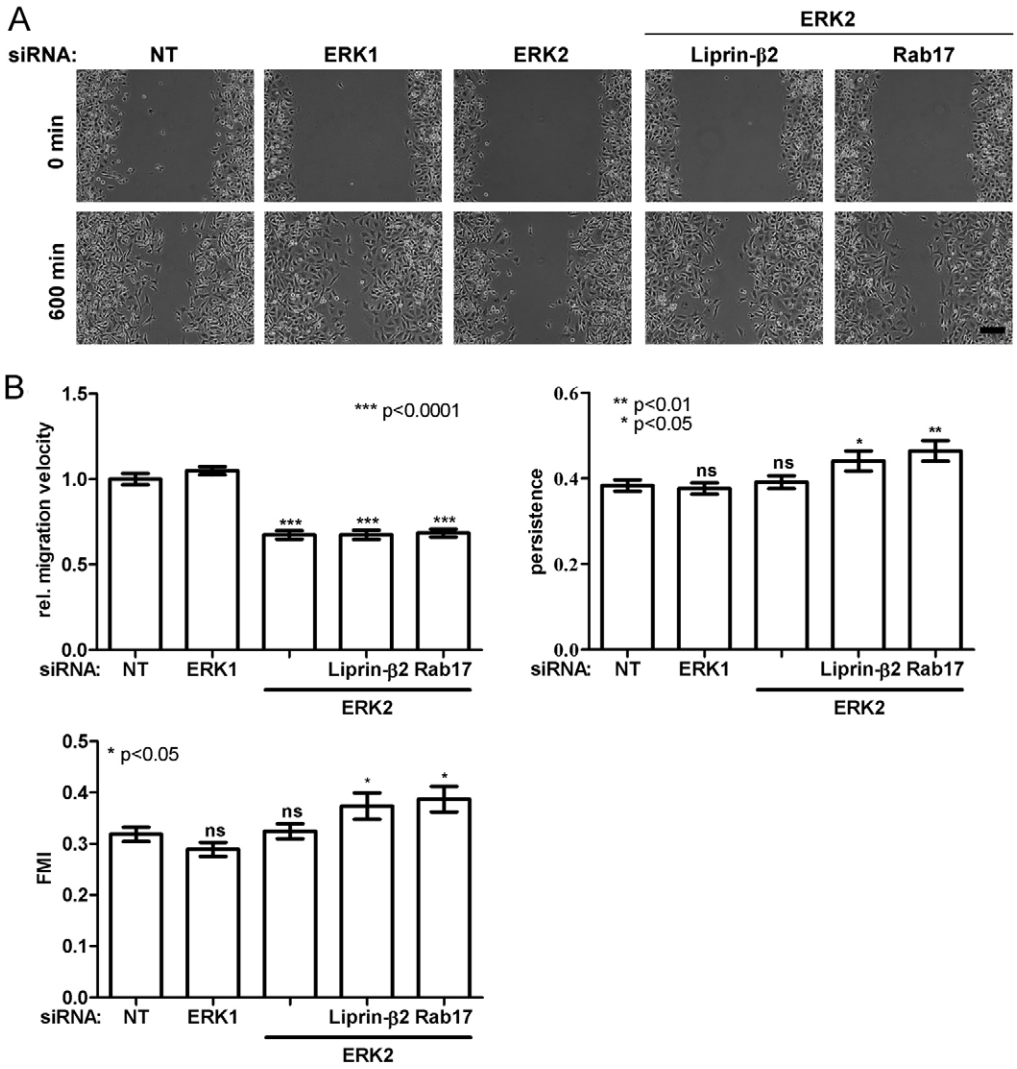

Fig. 7. ERK2 impairs cell migration on plastic surfaces through downstream effectors other than Rab17 and liprin-β2. MDA-MB-231 cells were transfected with non-targeting siRNAs (NT), or siRNAs targeting ERK1 or ERK2 in combination with those targeting Rab17 or liprin-β2, and plated onto six-well dishes so that they reached confluence 48 hours after transfection. A wound was introduced by scratching and wound closure monitored using time-lapse microscopy. (**A**) Representative images from two time points. (**B**) Cell movement was followed using cell-tracking software. Migration velocity, persistence and forward migration index (FMI) were extracted from the trackplots. Values are means ± s.e.m. of 15 fields from three independent experiments. Five cells or more were tracked for each field.

transport, in which synaptic vesicles accumulate in axons (Stryker and Johnson, 2007). Thus, liprins control vesicular transport, and this is due to their ability to act as scaffolds to recruit and stabilise a number of different proteins to the sites of exocytosis (Stryker and Johnson, 2007). There is a report that liprin-α1 might regulate integrin trafficking (Asperti et al., 2009), consistent with a role for these proteins in cell migration by controlling trafficking events. More recently liprin-α1 has been shown to contribute to the invasiveness of MDA-MB-231 cells and their ability to migrate persistently on 2D surfaces; both of which are integrin-regulated events (Astro et al., 2011). Although it is thought that they function by heterodimerising with α-liprins, there is little data available concerning the function of β-liprins. We found that liprin-β2 is a suppressor of cell migration and invasion downstream of ERK2 and so it is probable that it functions either by promoting the trafficking of anti-migratory receptors or by inhibiting the recycling of pro-invasive ones. Notably, liprins have been identified as inhibitors of cell invasion in head and neck squamous carcinoma cells (Tan et al., 2008); however, further work will be needed to establish how liprin-β2 acts to oppose tumour cell invasion and whether this involves recycling of integrins or other receptors involved in cell adhesion and migration. It is unclear from our studies how Rab17 and liprin-β2 interact functionally, but our observations that their silencing yields indistinguishable migratory phenotypes suggest that both proteins operate in series on the same pathway.

In conclusion, this study has identified Rab17 and liprin-β2 as previously unknown inhibitors of cell motility whose expression is regulated by ERK2 in MDA-MB-231 cells. Moreover, we have demonstrated that suppression of either Rab17 or liprin-β2 can completely compensate for loss of ERK2. Thus, we propose one way for ERK2 to drive invasiveness is by suppressing Rab17 and/or liprin-β2. The potential for inhibitors of the MAPK pathway to be used as anticancer agents is now being assessed clinically, and our finding that ERK2-mediated suppression of Rab17 and liprin-β2 drives cancer invasiveness will be important to the interpretation of data from these studies.

Materials and Methods

Cell culture

MDA-MB-231 and BE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (100 μg/ml) and 10% FCS. A2780-Rab25 cells were grown in RPMI-1640 medium containing glutamine (100 μg/ml) and 10% FCS. All cells were maintained at 37℃ under a humidified 5% CO_2 atmosphere.

Plasmid constructs

The ERK1 and ERK2 genes were amplified by PCR from a cDNA library using the following primers: *ERK1 att*B1 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCGGCGGCGGCGGCTCAGGG-3′, *ERK1 att*B2 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACTAGGGGGCCTCCAGCACTCC-3′, *ERK2 att*B1 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCGGCGGCGGCGGCGGCGG-3′, *ERK2 att*B2 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATTAAGATCTGTATCCTGGCTG-3′. ERK1 and ERK2 were recombined with the strep-tag II and FLAG-tag (SF)-TAP Gateway expression vector (Gloeckner et al., 2007). siRNA-resistant ERK2 expression vectors were generated by site-directed mutagenesis (QuikChange Multi Site-Directed Mutagenesis Kit from Stratagene) using the following primer: 5′-GCCTACGGCATGGTGTGTAGTGCTTATGATAATGTCAACAAAGTTCG-3′. ERK chimeras were generated as follows. For E2>E1 an internal *Hin*dIII restriction site was removed using the following primer 5′-GGGCCAAGCTCTTCCCCAAGTCAGACTCC-3′, and a new *Hin*dIII site was introduced by site-directed mutagenesis using the primer 5′-CTGACCTGTACAAGCTTCTGAAAAGCCAGCAGCTGAGCAATGACC-3′. For E1>E2 a new *Hin*dIII site was introduced using the following primer: 5′-CAGGACCTCATGGAAACAGATCTTTACAAGCTTTTGAAGACACAACACC-3′. Next, both expression plasmids were digested with *Hin*dIII, which cut within the SF-tag, and the ERK sequences (LYKLLK, restriction site is underlined), and the respective domains were swapped.

EGFP-C1-Rab17 was a gift from Jeremy Simpson (University College, Dublin, Ireland). pCMV6-GFP-Liprin-β2 expression vector was obtained from Origene.

Western blotting

The following antibodies were used for immunoblotting: tubulin (Insight), ERK1/2 (Sigma), pERK (Sigma), PARP (BD), vinculin (F9, a gift from Victor Koteliansky, Alnylam Pharmaceuticals, Cambridge, MA), FLAG (Sigma).

Transfection and RNA interference

Transfection of siRNA duplexes into MDA-MB-231 and BE cells was performed using HiPerFect (Qiagen) according to the manufacturer's instructions. Expression vectors were introduced using the Amaxa Nucleofector System according to the manufacturer's protocol. siRNA oligonucleotides and respective vectors were transfected into A2780-Rab25 cells using Amaxa. Assays were set up 48 hours (MDA-MB-231 and BE) and 24 hours (A2780-Rab25) after transfection. siRNA oligonucleotides were: *ERK1*, 5′-GACCGGAUGUUAACCUUUA-3′ (no. 1), 5′-CCUGCGACCUUAAGAUUUG-3′ (no. 2); *ERK2*, 5′-CACCAACCAUCGAGCAAAU-3′ (no. 1), 5′-GGUGUGCUCUGCUUAUGAU-3′ (no. 2); *RAB17* (SMARTpool, Dharmacon) and 5′-GAAGUGGCUCCGUGGGUAA-3′ (no. 1), 5′-ACGCUGCGCUUCUGGUGUA-3′ (no. 2); *PPFIBP2* (liprin-β2; SMARTpool, Dharmacon) and 5′-AGAUAAGGACCGUCGGAUA-3′ (no. 1), 5′-3′ (no. 2).

Inverted invasion assay

Inverted invasion assays (Hennigan et al., 1994) were performed as described previously (Caswell et al., 2007). Cells were allowed to invade towards a gradient of EGF (30 nM) and 10% serum for 2 days.

Migration studies

Cell-derived matrix (CDM) was generated as described previously (Bass et al., 2007; Cukierman et al., 2001). 70,000 cells were plated onto CDM 4 hours prior to time-lapse microscopy. For scratch wound assays, knockdown cells were plated in six-well dishes so that they reached confluence 48 hours post transfection, at which point they were wounded with a plastic pipette tip. Migration was monitored with a 10× objective using a Nikon time-lapse microscope and migration characteristics were analyzed using the manual tracking tool of ImageJ, a customised ImageJ macro and MATLAB script.

Gene expression array

Isolated RNA was labelled using a TotalPrep RNA Labeling Kit (Ambion). Comparative whole-genome expression profiling was performed using two Illumina HumanHT-12 v4 Expression BeadChips. Gene signal profiles of 24 samples were normalised and analysed in Partek® Genomics Suite Software, version 6.5. Quantile normalisation and log2 transformation of the data was followed by removal of batch effects between three groups of replicates. Outliers were removed and the remaining 19 samples re-normalised. Differentiated genes were identified by ANOVA and post-hoc linear contrasts performed between all pairs of experimental conditions. Multiple test corrections were performed for all calculated *P*-values. Genes that showed significant changes in expression level when comparing ERK2 knockdown with control (step-up P-value <0.05) and inverse changes when comparing ERK2 knockdown with re-expression of ERK2 (step-up P-value <0.05, fold change $>\pm 1.3$) were identified. ERK2-specific genes also had to meet the criterion of a step-up P-value >0.5 when comparing ERK2 knockdown cells with ERK1 re-expression.

qRT-PCR

The RNeasy kit (Qiagen) was used to isolate total RNA from relevant cell lines plated onto cell-derived matrix. cDNA was prepared using an Improm II Reverse Transcription kit (Promega). qRT-PCR reactions were prepared using the SYBR Green qRT-PCR kit (Finnzymes). Primers for respective genes were obtained from Qiagen. Amplified products were analysed by a Chromo4 Continuous Fluorescence Detector (BioRad) and Opticon Monitor3 software. $\Delta\Delta C(t)$ was determined as previously described (Livak and Schmittgen, 2001) using GAPDH as a reference. Control transfected transcript levels were assigned the arbitrary value of 1.

Proliferation assay

MDA-MB-231 cells were transfected with non-targeting siRNAs (NT), or those targeting ERK1 or ERK2. One day after transfection 40,000 cells were seeded into a six-well dish. Cells were counted on the following 5 days using a Casey Counter. As a negative control, cells were treated with the MEK inhibitor, U0126 (10 μM).

Statistical analysis

All experiments were performed in triplicate. Comparisons of invasion, momentary velocities and qRT-PCR were assessed using nonparametric Mann–Whitney *U*-tests. *P*-values of less than 0.05 were considered significant.

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