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Original Paper

A role for VICKZ proteins in the progression of colorectal carcinomas: regulating lamellipodia formation

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Abstract

VICKZ proteins are a highly conserved family of RNA binding proteins, implicated in RNA regulatory processes such as intracellular RNA localization, RNA stability, and translational control. During embryogenesis, VICKZ proteins are required for neural crest migration and in adults, the proteins are overexpressed primarily in different cancers. We hypothesized that VICKZ proteins may play a role in cancer cell migration. In patients, VICKZ expression varies with tumour type, with over 60% of colon, lung, and ovarian tumours showing strong expression. In colorectal carcinomas (CRCs), expression is detected at early stages, and the frequency and intensity of staining increase with progression of the disease to lymph node metastases, of which 97% express the protein at high levels. Indeed, in stage II CRC, the level of VICKZ expression in the primary lesion correlates with the degree of lymph node metastasis. In culture, VICKZ proteins rapidly accumulate in processes at the leading edge of PMA-stimulated SW480 CRC cells, where they co-localize with β-actin mRNA. Two distinct cocktails of shRNAs, each targeting all three VICKZ paralogues, cause a dramatic drop in lamellipodia and ruffle formation in stimulated cells. Thus, VICKZ proteins help to facilitate the dynamic cell surface morphology required for cell motility. We propose that these proteins play an important role in CRC metastasis by shuttling requisite RNAs to the lamellipodia of migrating cells.

Keywords: actin cytoskeleton; Igf2 mRNA binding proteins; intracellular RNA localization; RNA binding proteins; colorectal carcinoma metastasis; immunohistochemistry; kymographs

Introduction

In both normal and neoplastic cells, the actin cytoskeleton drives cell motility, and many molecules regulating actin remodelling have been implicated in this process [1–3]. Polymerization of actin filaments near the cell membrane leads to the formation of filopodia, in which microfilaments are parallel and linearly arrayed, or lamellipodia, in which microfilaments are branched; actin polymerization-mediated protrusion of the leading edge drives the movement of the cell [4]. Shuttling RNAs to the leading edge of cells can ensure a ready supply of proteins that are needed when processes are quickly formed [5]. Thus, β-actin mRNA, as well as mRNAs coding for proteins in the Arp2/3 complex that generate actin branching, is localized in minutes to the leading edge of fibroblasts induced to migrate [6,7]. Disruption of β-actin mRNA localization was found to cause a loss of polarity coupled with a loss of migration in different cell types [8].

cVICKZ1, a β-actin mRNA binding protein (also termed ZBP-1 [9]), has been isolated from chick fibroblasts and is involved in intracellular localization of β-actin mRNA to the leading edge of migrating fibroblasts [10–12]. In the last few years, it has become clear that cVICKZ1 is a member of a family of highly related RNA binding proteins expressed in different organisms and cell types. Strikingly, screens for RNAs and antigens overexpressed in various tumours have 'fished out' members of this family. Overexpression of one or more of these proteins has been reported in pancreatic carcinomas [13,14], hepatocellular carcinomas [15], and CRCs [16] (reviewed in refs 9 and 17). Recently, hVICKZ3 was shown to be a prognostic indicator for metastases in renal cell carcinoma [18]. In contrast, the protein is either low or not detectable in

most normal adult tissues [13,19–21]. A possible function of these proteins in transformed cells has remained unclear.

In the process of analysing intracellular RNA localization in *Xenopus* oocytes, we identified and characterized a VICKZ protein homologue, xVICKZ3 (also termed Vg1 RBP) [22]. We found that in addition to its role in the oocyte, xVICKZ3 plays an essential role in cell migration during *Xenopus* embryogenesis: xVICKZ3 localizes to the leading edge of explanted migratory neural crest cells, and reduction of xVICKZ3 expression inhibits normal migration during development [23]. Not only neural crest, but also roof plate progenitor cells require xVICKZ3 in order to reach their proper destinations. Thus, xVICKZ3 is necessary for the migration of specific cell populations during embryogenesis.

We hypothesized that VICKZ proteins may also be playing a role in neoplastic cell migration. Using a pan-VICKZ antibody, we found that VICKZ proteins are highly expressed in certain types of cancer. An in-depth analysis of one of these types, CRC, revealed that VICKZ expression is tightly correlated with metastasis to lymph nodes, and VICKZ proteins appear to be useful prognostic indicators for CRC. To elucidate the biological function of these proteins in CRC, we examined their distribution and function in a human CRC cell line, SW480. VICKZ proteins localize to the leading edge of SW480 cells and are required for the dynamic cell surface morphology necessary for cell movement. These proteins appear to play an important role in CRC metastasis by trafficking RNAs required for migration to the leading edge of motile cells.

Materials and methods

Immunohistochemistry

Details of the techniques used for immunohistochemistry and to screen the tissue microarrays are described in detail in Supplementary File 1 (available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html).

Western blot

The concentration of extracts was determined using Bradford reagent (Bio-Rad). For western blot analysis, 20 μg of protein extract was separated on 10% SDS/PAGE and transferred to nitrocellulose membranes. After blocking in 5% dry milk, the blots were probed with either pan-xVICKZ (1 : 20 000) or tubulin (1 : 1000) (Sigma) antibodies, as previously described [23].

Cohort and statistical analyses

The CRC cohort #1 was collected randomly from the Department of Pathology archive (from the years 1999–2004) at the Hadassah Medical Center, Jerusalem, Israel. Cohort #2, collected randomly from the same archive, consisted of samples from patients (not included in cohort #1) with T3 CRC and either no lymph node metastases (N0) or four or more lymph node metastases (N2) according to the WHO TNM classification. [There were not enough patients available with invasive disease, T3, and one to three lymph node metastases (eg N1) to allow a statistically significant analysis of this group.] All patients in these cohorts had a single lesion and had not undergone neoadjuvant therapy. Both those who performed the staining and those who graded the samples were blinded to the clinical stage of the patient. Experiments using human tissue received IRB exemption by the IRB Chair.

TARP and colorectal carcinoma results were analysed by non-parametric one-way ANOVA (Kruskal–Wallis test) with post-test pair-wise comparisons. The correlation between VICKZ expression levels and frequency was analysed by calculating the Pearson correlation coefficient, r. Clinical stage and VICKZ expression correlation was tested using a Mann–Whitney comparison. All analyses were performed using GraphPad Prism 4 (GraphPad Software, Inc).

In situ hybridization and immunofluorescence

Sub-confluent SW480 cells were serum-starved for 6 h. To induce cells, PMA (Sigma) was added to a final concentration of 100 ng/ml. After 1 h, uninduced and induced cells were fixed and *in situ* hybridization was performed as described elsewhere (http://www.singerlab.org/protocols). Following *in situ* hybridization, cells were washed three times with 1× PBS, 5 mM $MgCl_2$, and blocked with CAS block supplemented with 1 mg/ml RNAse-free BSA (NEB) for 1 h. Affinity-purified xVICKZ3 antibody (1 : 100) was added to the blocking solution and incubated overnight at 4 °C. The next day, the coverslips were washed with 1× PBS, 5 mM $MgCl_2$, incubated with 1 : 100 anti-rabbit Cy-5-conjugated antibody (Jackson) for 1 h at room temperature, washed again three times, and mounted. Samples were viewed on an Olympus IX71 microscope equipped with a Quantix IQ-coolsnap CCD and run by Applied Precision software. Raw black and white images were processed by 2-D convolution using AutoDeBlur software (AutoQuant, Inc) and pseudo-coloured with Adobe Photoshop.

Time lapse microscopy

SW480 cells were grown in DMEM containing 10% FCS, l-glutamine (2 mm), penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO_2. SW480 cells seeded on laminin-coated coverslips were transiently transfected with a plasmid, termed pEGFP-hVICKZ1, containing the ORF of hVICKZ1 (hVICKZ1 cDNA was a kind gift of Dr Jan Christiansen) cloned into the expression vector pEGFP-C1

(Clontech), using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Cells were induced as described above. Images were collected at 5-min intervals at 37 °C by a cooled CCD camera (Sensicam; PCO imaging) that was mounted on an inverted Axiovert 200 microscope (Carl Zeiss MicroImaging, Inc) equipped with motorized stage and X63 air lens (Zeiss). Images were acquired using Image Pro (Media Cybernetics, Inc).

VICKZ knockdown

Details of the lentivirus-mediated shRNA knockdown of hVICKZ1, 2, and 3 are described in Supplementary File 1 (available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html).

Results

Variable VICKZ expression in different tumours

Given the evidence that VICKZ proteins are expressed in a number of different types of cancer, we decided to take a systematic approach to compare expression of the proteins in various neoplasias. Anti-VICKZ antibody raised against the *Xenopus* homologue xVICKZ3 recognizes all three human paralogues [24]. The use of this antibody on paraffin-embedded sections was calibrated using archived CRC samples, and specificity was demonstrated by the ability of purified VICKZ protein to titrate out staining (data not shown). The antibody was used to screen a tissue array containing 262 tumour samples. Figure 1 shows the percentage of samples, of each tumour type, expressing VICKZ proteins, at any level in the neoplastic cells, and the intensity of this expression per cancer. Among the seven different types of tumours examined, VICKZ expression is variable. Colon, lung, and ovarian carcinomas showed the most extensive expression, with greater than 60% of the samples expressing VICKZ proteins. A much smaller fraction (18% and 5%, respectively) of breast and prostate tumour samples were positive for VICKZ expression, while intermediate numbers of melanomas and brain tumours were positive for VICKZ. As reported previously [13,19–21], little or no expression is detected in non-neoplastic cells (eg Figure 2B and data not shown). We observed a strong correlation ($r = 0.992$) between the frequency of patients whose cancers are VICKZ-positive and the intensity at which the proteins are expressed in those cancers (see Figure 1).

VICKZ expression during the progression of CRC tumours and metastases

As CRC showed the most widespread and strongest expression of VICKZ proteins among the tumours analysed on the tissue array (Figure 1), we decided to examine the pattern of VICKZ expression as a function of CRC progression, from adenomas to CRC lymph node metastases. We analysed VICKZ expression in archived samples from 62 patients treated over the last 4 years (see Supplementary Figure 1, available online at http://www.interscience.wiley.com/jpages/0022-

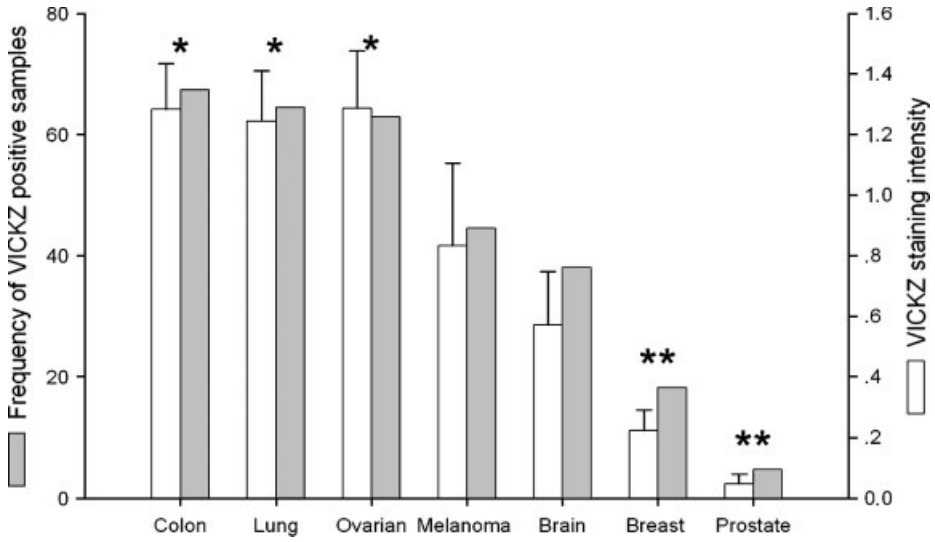

Figure 1. VICKZ expression in different types of tumours. Using the pan-VICKZ antibody, hVICKZ expression in 262 TMA samples from seven types of cancer was quantified, with each sample given a score from 0 (no staining) to 3 (extremely strong staining; see the Materials and methods section and Supplementary File 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html). The shaded bars represent the percentage of samples expressing hVICKZ (score 1–3) of each cancer type analysed. The bars marked with a single asterisk (colon, lung, and ovarian carcinomas) have a significantly higher percentage, in comparison with the bars marked with two asterisks (prostate and breast carcinomas) ($p < 0.001$). The open bars represent the hVICKZ mean staining score (with standard error of mean bars) for each tumour type studied. (The total number of samples analysed for each tumour type was as follows: colon, 46; lung, 45; ovarian, 35; melanoma, 16; brain, 21; breast, 54; prostrate, 43)

3417/suppmat/path.2376.html). An example of an adenocarcinoma that developed from a tubulovillous adenoma is shown in Figure 2A, which contains a section of a polyp protruding into the lumen of the colon, along with the underlying muscularis and serosa layers. This sample shows a typical progression for a CRC tumour with the different stages, all from the same patient, highlighted in Figures 2A and 2F. Generally, in normal colonic epithelia and surrounding tissues, VICKZ expression is essentially non-detectable (Figure 2B, B′, B″). In adenomas, VICKZ expression was clearly observed, particularly in areas of more

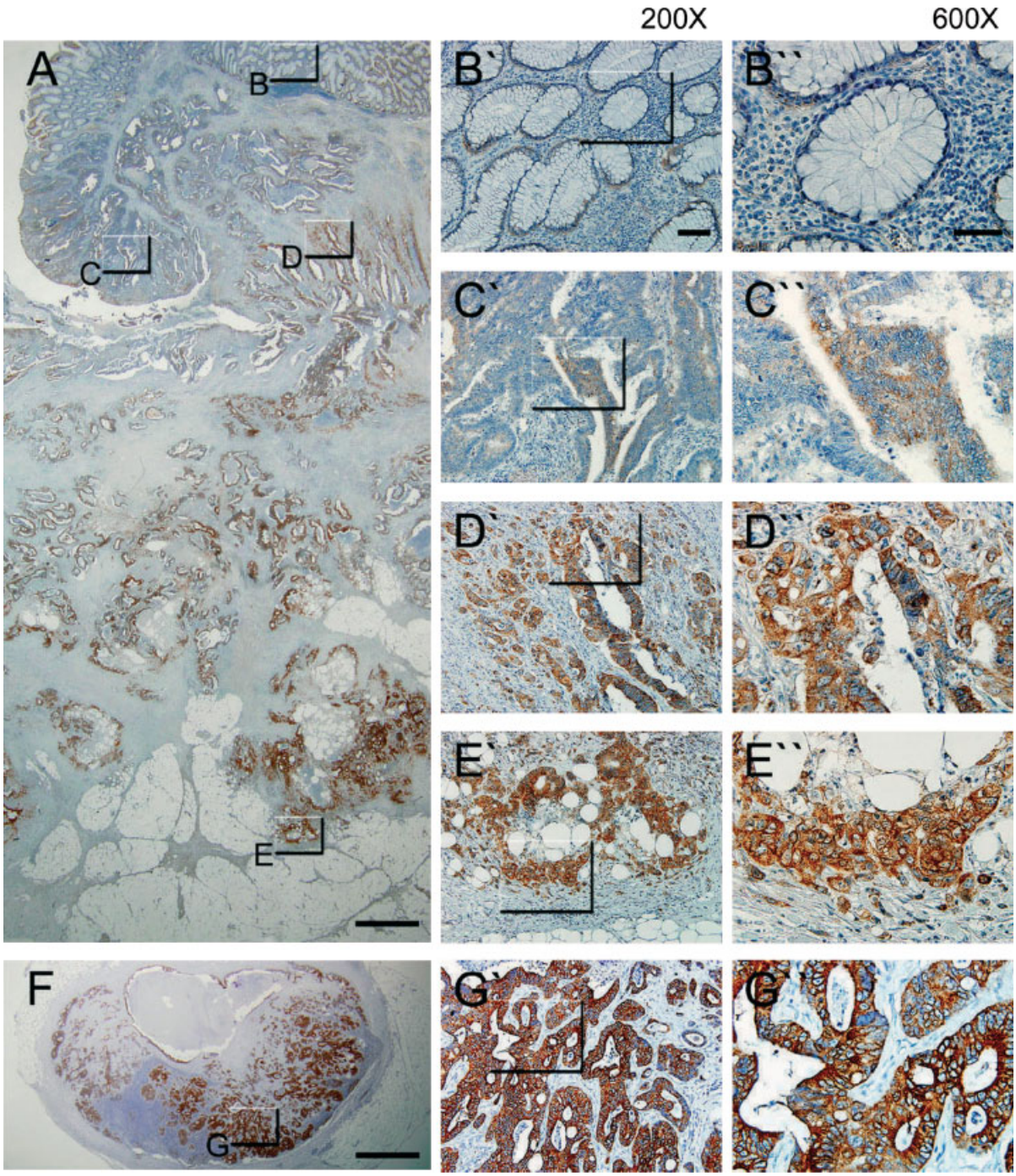

Figure 2. VICKZ protein expression increases through CRC tumourogenesis and invasion. Using the pan-VICKZ antibody, immunohistochemistry was performed on a section of a resected colon harbouring a lesion containing different stages of CRC progression. The brown colour represents VICKZ protein expression, and the blue, haematoxylin counterstain shows nuclei. (A) The entire section from one patient at low magnification (30×; scale bar = 1 mm). (B–E and B′–E′) Higher magnifications (200× and 600×, respectively) of the corresponding areas labelled in A (scale bars = 100 μm and 50 μm, respectively). A gradient of VICKZ protein expression is observed, with basal levels of expression in the normal region of the polyp (B), low levels in a region of dysplasia (C), moderate levels as the neoplastic cells migrate through the stem of the polyp (D), and higher levels in the cancer cells at the invasive front, where the cells are observed entering the mesenteric fat layer of the colon (E). Note that VICKZ expression can be seen in tumour cell clusters as well as in single tumour cells invading the adjacent stroma. The highest levels of VICKZ expression are observed in lymph node metastases (same patient) (F, G, G′, G″)

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severe dysplasia (Figure 2C, C′, C″). As neoplastic cells invade the tissue, a striking, graded pattern of expression is often observed (Figure 2D, D′, D″), with the invasive edge of the neoplasia, which is advancing through the subserosa, demonstrating strong staining (Figure 2E, E′, E″). Lymph node metastases showed extremely strong staining and the surrounding lymphocytes were completely negative for VICKZ expression (Figure 2F, G, G′, G″). Thus, CRC progression is characterized by increasing levels of VICKZ protein.

The samples were scored based on the average relative intensity of VICKZ expression in the neoplastic cells (see the Materials and methods section and Supplementary File 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html). VICKZ proteins were expressed in a high percentage of the tumours examined, with 87% (73/84) of the adenomas or adenocarcinomas, and 97% (58/60) of the metastases, positive for VICKZ expression. Significant differences, however, were observed when the intensity of expression of the proteins was compared. Thus, lymph node metastases showed much higher expression than the normal surrounding colonic epithelium ($p < 0.001$), adenomas ($p < 0.001$), or adenocarcinomas ($p < 0.001$), as judged by one-way ANOVA analysis using a Kruskal–Wallis test (Figure 3A). Given the gradient of VICKZ expression in invasive adenocarcinomas and the almost universal and high level of VICKZ expression in lymph node metastases, these results suggest that VICKZ proteins may play a role in facilitating metastasis in CRC. To test this hypothesis, we analysed, in a double-blind experiment, an additional 25 primary CRC samples from patients with a single lesion whose tumour had penetrated through the muscularis propria (T3), to determine whether their VICKZ levels correlated with the presence of lymph node metastasis (see Supplementary Figure 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html). Patients with no mesenteric lymph node metastases (N0) had significantly lower VICKZ expression in their primary tumours than those with four or more metastases (N2) ($p = 0.037$), a known marker of poor outcome (Figure 3B). Thus, VICKZ expression levels in the primary tumour correlate with the presence of lymph node metastases. Taken together with the fact that in *Xenopus*, VICKZ plays a role in cell migration, these results suggest that VICKZ proteins may be involved in facilitating CRC tumour cell migration.

Lamellipodia and ruffles of CRC SW480 cells are sites of VICKZ protein localization and mRNA targeting

VICKZ proteins have been proposed to facilitate motility via their ability to shuttle RNAs encoding proteins required for motion to the leading edge of migrating cells [17,25]. In neural tube explants, xVICKZ3 localizes to the leading edge of migrating neural crest cells [23]. In chick embryonic fibroblasts, cVICKZ1 shuttles β-actin mRNA from the nucleus to the leading edge of the cell [11], and overexpression

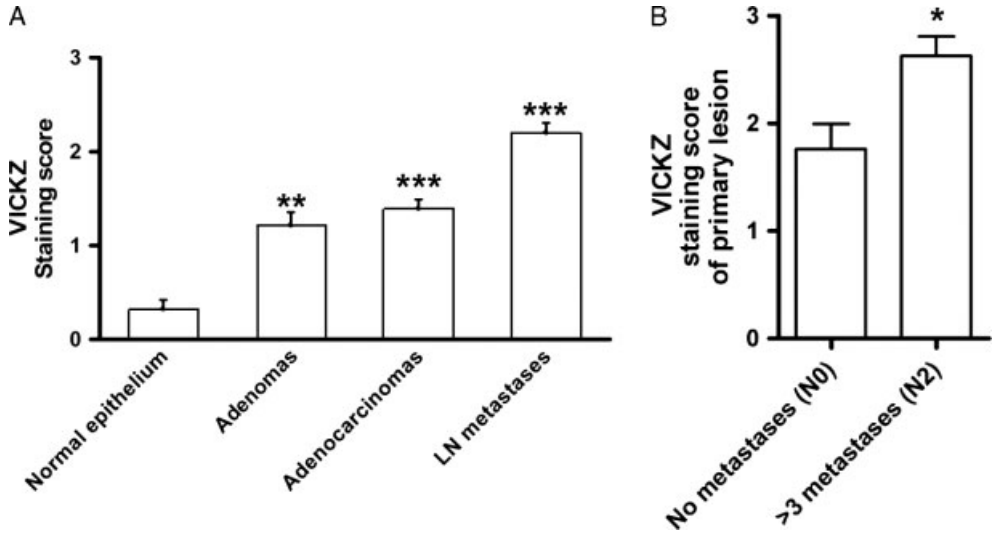

Figure 3. VICKZ protein expression increases during CRC invasion and predicts lymph node metastases. (A) One hundred and thirty-nine archival samples from CRC patients, representing normal epithelium, adenomas, adenocarcinomas, and lymph node (LN) metastases (cohort #1, see Supplementary Figure 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html), were stained with the pan-VICKZ antibody, scored, and graphed with standard error bars. The lymph node metastases show much higher VICKZ expression than any of the other groups ($p < 0.001$). Adenomas and adenocarcinomas are also significantly stronger in VICKZ expression than normal colonic epithelium ($p < 0.01$ and $p < 0.001$, respectively). (B) Pan VICKZ antibody was used to stain another 25 primary CRCs (cohort #2, see Supplementary Figure 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html) in order to test whether the intensity of VICKZ expression correlates with lymph node metastasis. The graph shows the mean score and standard error of mean for pan-VICKZ expression in CRC cohort #2. A significant correlation between metastasis and hVICKZ level of expression is observed ($p = 0.037$; two-tailed *t*-test)

of certain VICKZ deletion constructs inhibits β-actin mRNA localization and reduces cell motility [12,26]. In transformed cells, however, localization of VICKZ proteins with various mRNAs has been less clear [27,28].

To study whether VICKZ proteins are involved in CRC cell migration, we compared their intracellular distribution in unstimulated SW480 cells and SW480 cells stimulated to migrate by PMA. The SW480 cell line was isolated from a primary human adenocarcinoma of the colon and is metastasic when implanted in nude mice [29]. Western blot analysis showed that SW480 cells express VICKZ proteins and real-time RT-PCR analysis indicated that all three VICKZ paralogues are present in the cells (data not shown). Using the pan-VICKZ antibody, we examined the sub-cellular distribution of endogenous VICKZ proteins (Figure 4A). In both unstimulated and stimulated cells, VICKZ proteins were observed exclusively in the cytoplasm (Figure 4A). Protein staining was granular, as is often observed with RNP proteins [30]. In unstimulated cells, VICKZ proteins were fairly homogeneously distributed in the cytoplasm, with little VICKZ signal detectable at the cell surface. In stimulated cells, however, VICKZ proteins were clearly observed at the periphery of the lamellipodia as well (Figure 4A, arrow). To gain not only a spatial, but also a temporal view of the localization of VICKZ proteins to the leading edge of induced SW480 cells, we examined the distribution of a VICKZ protein in living cells. SW480 cells were transiently transfected with a GFP (green fluorescent protein)-hVICKZ1 fusion protein, stimulated by PMA, and filmed by time-lapse microscopy. The cells were seeded on coverslips coated with laminin, which inhibits their movement but not lamellipodia formation. In the series shown in Figure 4B, GFP-hVICKZ1 is clearly seen localizing to new lamellipodia of the stimulated cell, as identified by the phase contrast frames, taken in parallel. This localization activity is dynamic and not limited to any part of the cell; as protruding edges form, the GFP-hVICKZ1 protein localizes to the more developed lamellar areas, seen as darker, more pronounced ruffled areas in phase contrast. Cells transfected with GFP alone showed no localization of fluorescence in the lamellar areas (data not shown). Thus, GFP-hVICKZ1 localizes to protruding lamellipodia as they are being formed in stimulated CRC cells.

Lamellipodia, found at the leading edge of migrating cells, are filled with branched actin microfilaments and generate the driving force for cell migration [31]. In stimulated fibroblasts, mRNAs encoding β-actin and all seven components of the Arp2/3 complex (responsible for generating the branched microfilament network in lamellipodia) also localize to peripheral protrusions [6,7,32–34]. To determine whether RNA localization occurs in transformed CRC cells, we examined the distribution of β-actin, Arp2, and Arp3 mRNAs in stimulated SW480 cells. As can be seen in Figure 5A, all three of these mRNAs demonstrate a clear peripheral localization in the stimulated CRC cells, when compared with the distribution of

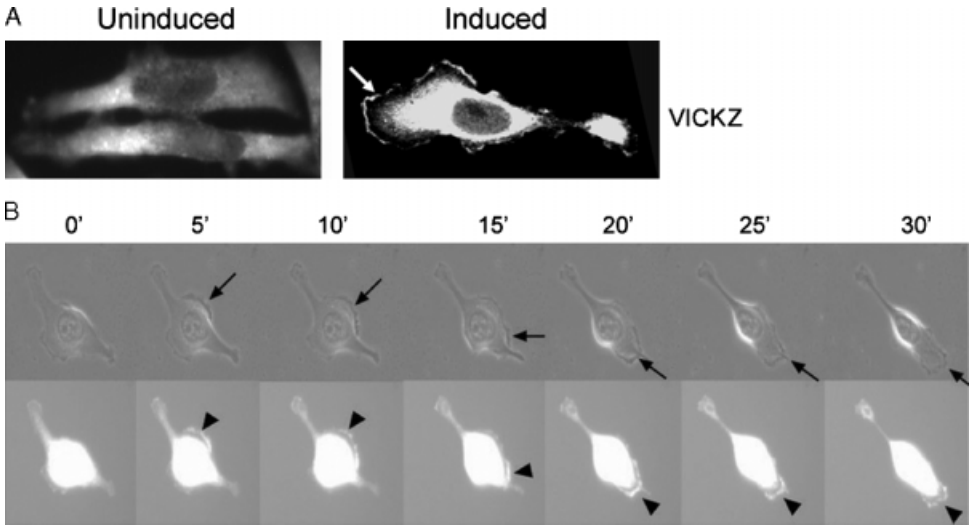

Figure 4. VICKZ proteins localize to the leading edge of induced SW480 cells and to protruding lamellipodia in live cells. (A) Starved SW480 cells were stimulated by PMA and fixed after 40 min. Indirect immunofluorescence was performed using the pan-VICKZ antibody as the primary antibody and an anti-rabbit, Cy-5-conjugated secondary antibody. Intracellular localization of VICKZ protein to the lamellipodial leading edge was observed in induced cells and is indicated by the white arrow. (B) SW480 cells were transfected with GFP-hVICKZ1 plasmid and seeded on laminin-coated coverslips. After 2 days, the cells were stimulated as described. Fluorescent time-lapse microscopy was used to study the cellular distribution of GFP-hVICKZ1 over time within living cells. Pictures were taken every 5 min. Upper row: phase contrast series of the cell shows the lamella as a dark structure at the edge of the cell. Black arrows point to the area where the lamella is most active. Lower row: corresponding fluorescent images show that GFP-hVICKZ1 clearly localizes to the protruding, well-developed lamella. The last three pictures show that as the lamella disappears, GFP-hVICKZ1 is delocalized from the cell edge, illustrating spatial as well as temporal localization. Because the fluorescent images were overexposed in order to detect the signal in the lamella, the nucleus in these pictures is masked by the overlying cytoplasmic signal

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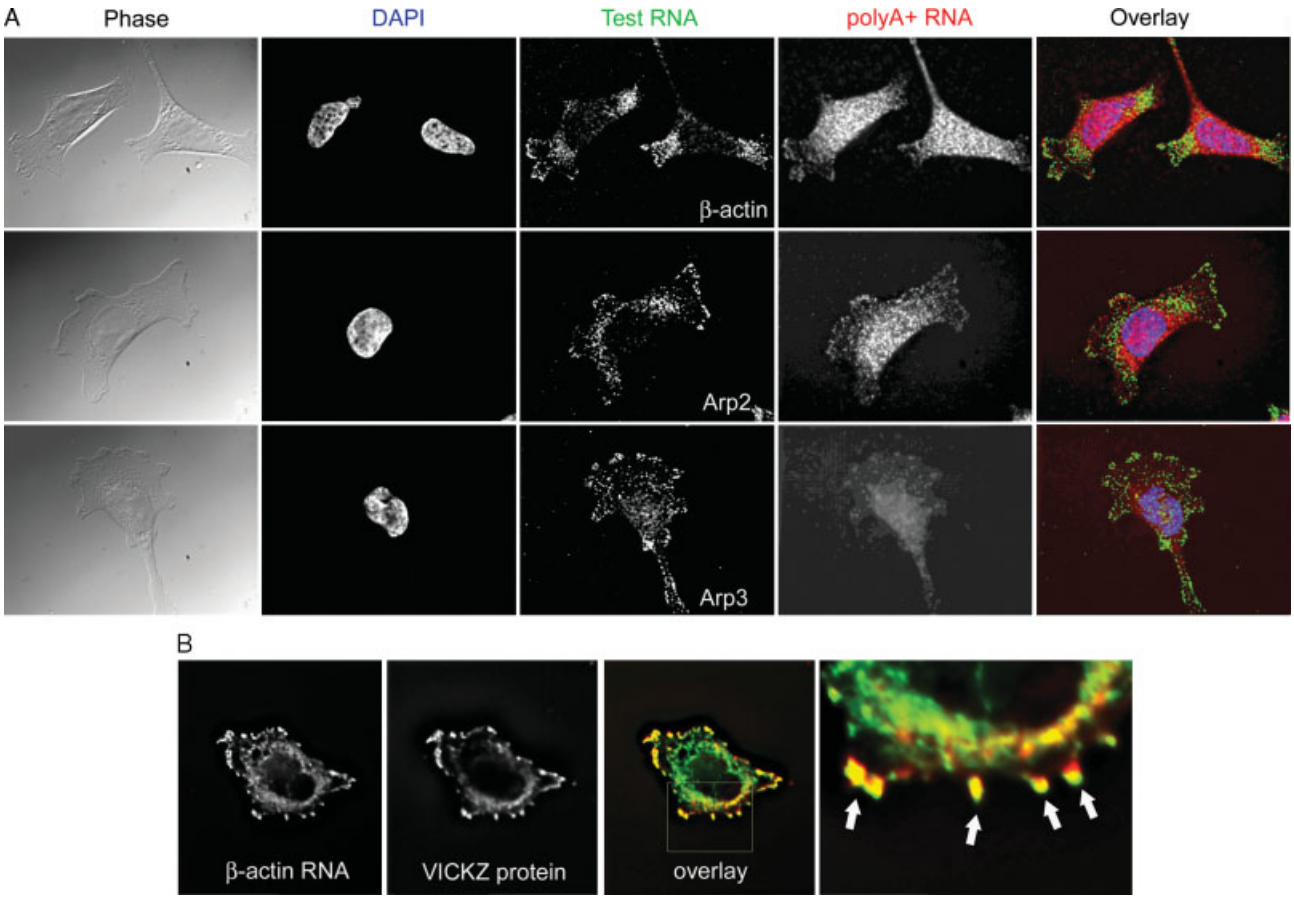

Figure 5. mRNAs localized to lamellipodia of induced cells. (A) SW480 cells were stimulated by PMA after overnight serum starvation and then fixed and hybridized with a mix of TRITC-labelled oligonucleotides recognizing polyA+ RNA as a control for non-localized RNA, and FITC-labelled oligonucleotides recognizing β-actin, Arp2, or Arp3 RNA. DAPI was used to stain nuclei. Black and white stacks of images were obtained at the different wavelengths, and deconvolution was performed on the Z-series stacks obtained for each cell. The three resulting images per cell were pseudo-coloured (poly A+ in red; test RNA in green; and DAPI in blue) and overlaid to facilitate comparison. (B) SW480 cells stimulated by PMA after overnight serum starvation were probed for co-localization of VICKZ proteins, using the pan-VICKZ antibody (in red), and β-actin mRNA (in green). The white arrows in the magnified region of the overlay (outlined by the white rectangle) indicate cell membrane protrusions with co-localized protein and mRNA (in yellow)

total polyA+ RNA in the same cells. Examination of the phase channel of these pictures indicates that the mRNAs are localized to the lamellipodial regions of the cells. β-actin mRNA is also a known target for at least some of the VICKZ proteins [10]. β-actin mRNA in fact co-localizes with VICKZ proteins to the periphery of the stimulated SW480 cells (Figure 5B). Thus, metastatic CRC cells traffic both VICKZ proteins and at least several mRNAs encoding proteins required for actin microfilament organization at the leading edge of the cell to peripheral lamellipodia.

VICKZ proteins are required for the formation of lamellipodia and ruffles in stimulated SW480 cells

To functionally test the role of VICKZ proteins in CRC cells, we developed shRNA vectors that induce VICKZ knockdown. shRNA constructs were cloned into a lentiviral vector that allowed for their induced expression upon addition of doxycycline (see Supplementary File 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html). When transcription of shRNAs directed against each of the three human VICKZ paralogues was induced in SW480 cells, an approximately two-thirds reduction of VICKZ expression was observed (Figure 6A). To analyse the effect of this knockdown, cells transduced with the shRNA cocktail were cultured in either the presence or the absence of doxycycline. Uninduced cells were labelled with MitoTracker; co-plated with unlabelled, doxycycline-induced cells (containing reduced VICKZ levels); and stimulated with PMA. Cells with normal levels of VICKZ proteins (MitoTracker-positive) have a smooth cell surface, with clearly detectable cortical actin microfilaments (Figure 6B, A′, B′). Cells with reduced VICKZ levels, however, show a subtle but discernible change in their cell periphery. Spike-like protrusions, also containing microfilaments, are present in these cells and give their surface a rough, irregular appearance (Figure 6B, C′, D′).

In order to quantify these differences, uninduced and induced cells were filmed following PMA stimulation.

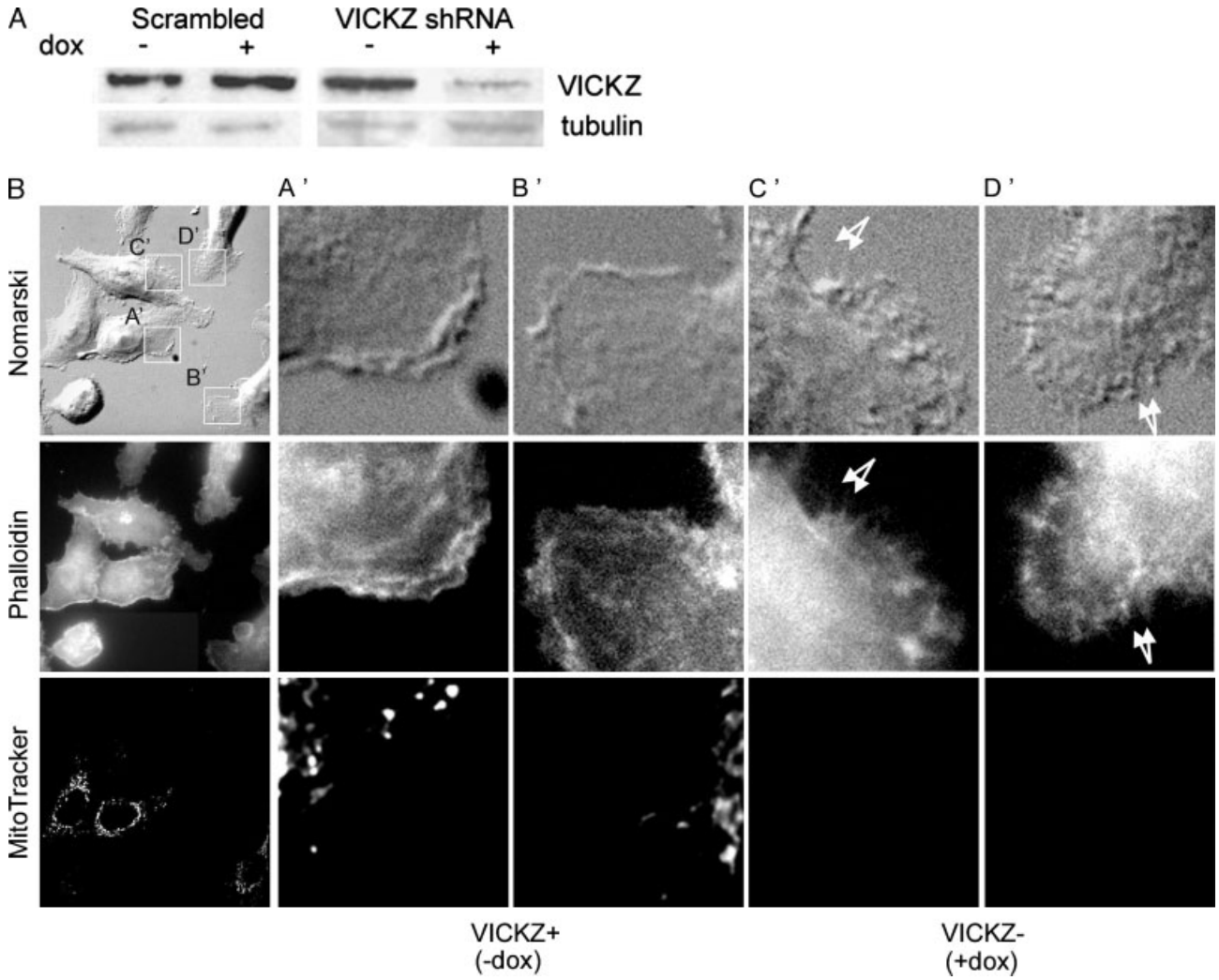

Figure 6. shRNA knockdown of VICKZ expression in SW480 cells. (A) SW480 cells transduced with either scrambled shRNA (Scrambled) or shRNAs against all three VICKZ paralogues (VICKZ shRNA) were grown for 6 days in the absence (−) or presence (+) of doxycycline. Protein extracts were prepared and subjected to western blot analysis, using the pan-VICKZ or tubulin antibody. Normalizing against the tubulin signal, a 66 ± 15% reduction in VICKZ expression was observed upon induction. (B) As detailed in Supplementary File 1 (available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html), uninduced SW480 cells transduced with shRNAs against all three VICKZ paralogues and labelled with TRITC-conjugated MitoTracker (bottom row) were co-plated with unlabelled, doxycycline-induced cells, serum-starved, stimulated with PMA, fixed, and stained with FITC-phalloidin in order to visualize actin microfilaments (middle row). Cells with normal levels of VICKZ (MitoTracker-positive; A′, B′) show smooth, normal membranes when viewed with Nomarski optics (top row), and contain cortical microfilaments (middle row). Cells with low levels of VICKZ (MitoTracker-negative; C′, D′) have a rough cell surface with multiple spike-like protrusions (white arrows) that stain positive for actin and are absent from uninduced cells

Cells transduced with the cocktail of vectors containing shRNAs against VICKZ proteins, but not incubated with doxycycline, showed robust membrane dynamics, similar to untreated cells (see Supplementary Movie 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html). Smooth lamellipodia formed rapidly, followed by the appearance of membrane folds, or ruffles, leading to the retraction of the lamellipodia. When the same pool of cells, however, was induced by incubation with doxycycline to reduce VICKZ expression, a very pronounced reduction in lamellipodia and ruffle formation was observed (see Supplementary Movie 2, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html). Quantification was performed by analysing kymographs generated at multiple sites along the membranes of recorded cells (Figure 7A). Expression of a scrambled shRNA (a negative control), or the presence of the lentiviral vectors containing VICKZ-specific shRNAs in the absence of doxycycline-induced shRNA transcription, had no significant effect on the rate of formation of either lamellipodia or ruffles (Figures 7A and 7B). Conversely, a striking inhibition in cell surface dynamics occurred when shRNAs were induced: an over two-fold reduction in lamellipodia formation and an over three-fold reduction in ruffle formation were observed following PMA stimulation. The differences (with and without doxycycline) were highly statistically significant ($p < 0.001$ and $p < 0.0001$, for lamellipodia and ruffle formation, respectively).

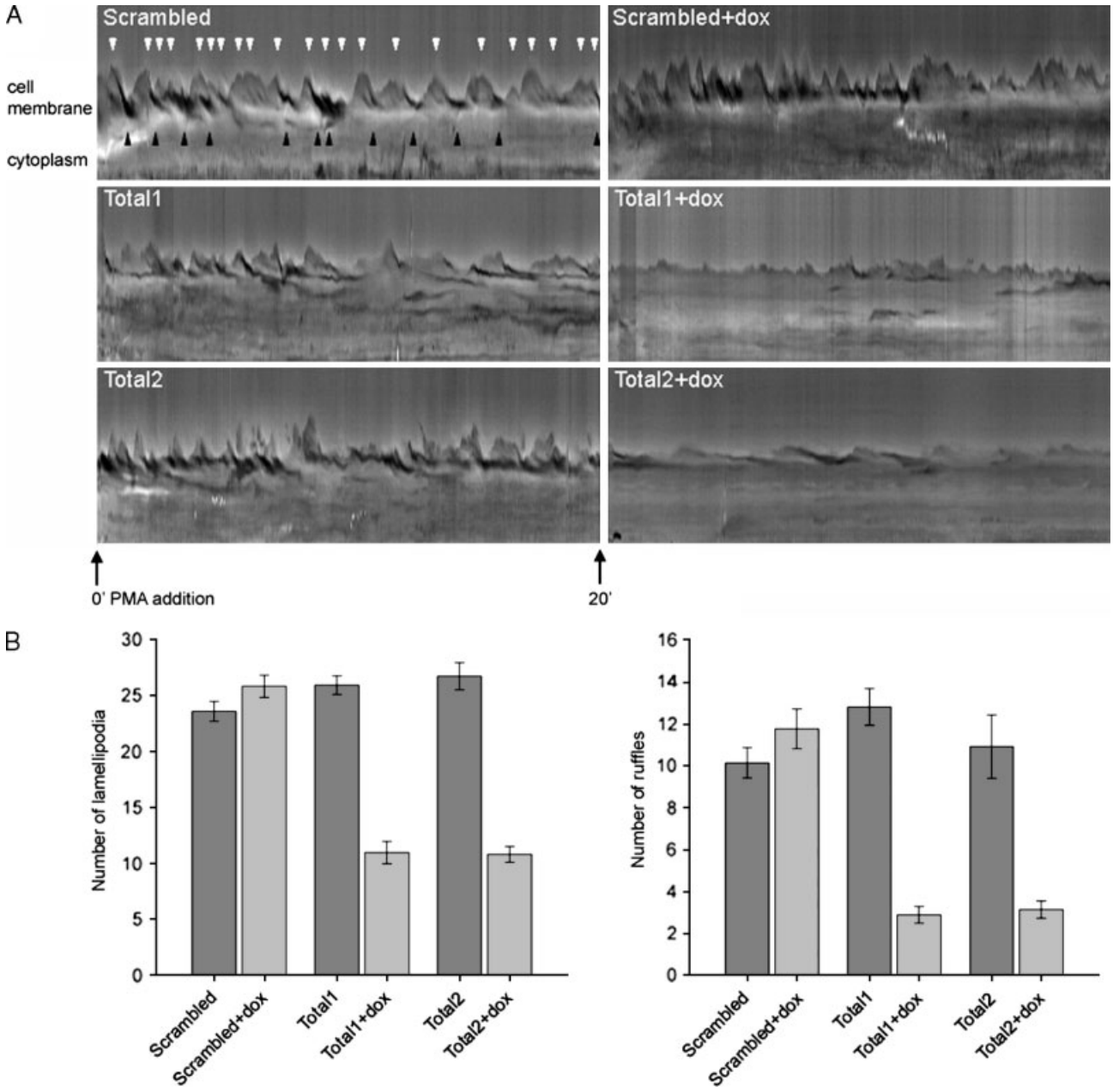

Figure 7. VICKZ KD in SW480 cells results in loss of lamellipodia and ruffles. SW480 cells were transduced with lentiviral vectors driving the expression, upon doxycycline (dox) induction, of either a scrambled shRNA (Scrambled) or one of two distinct pools of shRNAs directed against all three VICKZ homologues (Total1 or Total2). (A) Cells, induced or non-induced for shRNA production, were serum-starved and then stimulated by PMA. Pictures were taken every 3 s over the course of 20 min. Representative kymographs (assembled from 400 pictures each) are shown for each of the three types of transduced cells, without or with doxycycline induction. The white arrowheads show an example of how the peaks of lamellipodia extension (at the cell membrane) were determined for each kymograph; the black arrowheads indicate how the ruffles were counted. (B) The numbers of lamellipodia (left) and ruffles (right) were compared from a large set of kymographs obtained as described in A. From every cell examined, ten kymographs were constructed and analysed, with an average of 50 kymographs analysed per shRNA pool. For lamellipodia formation, a p value less than 0.001 was obtained when comparing plus and minus doxycycline in the 'Total' samples. For ruffle formation, a p value less than 0.0001 was obtained for the same samples

Similar reductions were observed for two distinct but partially overlapping cocktails of three shRNAs each (one against each VICKZ paralogue; see Supplementary File 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html), suggesting that the result was not an off-site effect specific for a particular set of shRNAs. Thus, VICKZ proteins are required for the formation of lamellipodia and ruffles in SW480 cells.

Discussion

Differential VICKZ expression in cancers

Using an antibody specific for all three hVICKZ paralogues, we have compared the expression of these proteins in different types of tumours displayed on a tissue microarray (TMA). Results similar to those obtained from the TMA were observed in the

completely independent screen of resected CRCs from an unrelated patient population. Given the potential difficulties in analysing TMAs (the small size of the samples and the relatively few neoplastic cells per sample), the data obtained reflect a minimal estimate of VICKZ expression in the assayed tumours. VICKZ expression has also been reported in diverse malignancies using a variety of methods (see ref 17). To the best of our knowledge, this study represents the first systematic approach to comparing VICKZ expression in different tumour types based on protein expression at the cellular level. Our results and much of the published data merge to form a general, consistent picture of high-level VICKZ protein expression in a large number of cancers, including colorectal, lung, ovarian, hepatocellular, pancreatic, renal cell, testicular, and B-cell lymphomas (Figure 1) [13–15,18,21,24]. Our data suggest that the frequency and level of expression of VICKZ proteins in breast cancer, however, are much lower than what has been cited in the literature [35]. One possible explanation lies in the fact that we have observed VICKZ proteins in normal duct cells in the breast (G Vainer and E Pikarsky, unpublished observations). This could explain why, based on non-quantitative RT-PCR, a larger proportion of the breast tumours were classified as positive.

VICKZ proteins in CRC tumour progression and metastasis

VICKZ proteins may play multiple roles during CRC development. VICKZ is first expressed in adenomas, well before metastasis begins. Given that at least one of the VICKZ proteins is reported to be positively regulated by Wnt signalling [36] and, in turn, can regulate Igf2 expression [37,38], early expression of VICKZ proteins could play a role in tumourigenesis through an Igf2-mediated pathway [39]. The graded VICKZ expression reported here, with the highest expression levels at the invasive front, suggests that VICKZ proteins help CRC reach the lymph nodes. In addition, we found that the level of VICKZ protein expression in the primary tumour predicts the extent of lymph node metastases. These facts argue that activation of VICKZ expression may help to promote the growth of epithelial tumours along with metastasis to the lymph nodes.

These results may be of particular clinical importance in post-operative stage II patients. In stage II colorectal cancer, the tumour has penetrated through the bowel wall, but without any detectable lymph node or distant metastases. Although the overall survival in these cases is approximately 70–80% 5 years after surgery, 40% of these patients will develop recurrent disease and would likely benefit from further treatment [40]. The search for predictive markers for metastatic CRC disease in these cases has not yielded clear-cut conclusions [41]. Our results here suggest that in cases of stage II CRC, further research comparing VICKZ expression levels and metastases may help in deciding on adjuvant therapy.

VICKZ proteins and cell migration

Reducing VICKZ protein expression by 66% causes striking inhibition of the highly dynamic cell surface morphology normally observed in PMA-stimulated SW480 cells (Figure 7). The presence of VICKZ protein, as well as β-actin, Arp2, and Arp3 mRNAs, at the leading edge of stimulated cells (Figures 4 and 5) suggests that the phenotype seen in the VICKZ knockdown may result from the inability to localize requisite mRNAs. The fact that actin microfilaments are present in the shRNA-expressing cells but organized into spike-like structures resembling filopodia (Figure 6 and Supplementary Movie 2, available at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html) raises the possibility that mRNAs encoding regulatory, rather than structural, elements of the peripheral actin network may be mediating the phenotype. Indeed, RNAi knockdown of WAVE or Arp3 causes the formation of filopodia without lamellipodia [42], a phenotype very similar to the VICKZ knockdown observed here. Cofilin RNA, known to be a target of VICKZ proteins [43], may also play a role in mediating the VICKZ knockdown phenotype; laser-activated caged cofilin protein generates a lamellipod at the site of activation [44]. We propose that VICKZ proteins help to shuttle not only β-actin mRNA, but also mRNAs encoding one or more of the components required for the dynamic actin cytoskeletal network present at the leading edge of migrating cells. Recent results in our lab (A Rubinstein *et al*, manuscript in preparation) and others [45,46] have shown that a number of mRNAs encoding receptors, kinases, cell adhesion molecules, and other membrane-associated proteins are VICKZ targets. The ability of VICKZ proteins to regulate RNAs such as these, as well as other RNAs such as CD44 in invadipodia [47], may explain why VICKZ protein levels are up-regulated in many metastatic tumours.

In breast cancer, VICKZ1 has been proposed to act as an anti-metastatic factor [25]. Our results indicate that in colorectal carcinomas, VICKZ proteins are in fact *pro-metastatic* factors. One possible explanation to reconcile these differences may stem from the way that the malignancies are regulated. Significantly, we found that the frequency and level of expression of VICKZ proteins in both primary breast carcinomas and metastases are low relative to those in colorectal carcinomas and metastases (see Figure 1). Overexpression of mVICKZ1 in mouse mammary glands, however, does lead to metastatic disease (at rates roughly dependent on the level of VICKZ expression), suggesting that abnormally high levels of VICKZ proteins can enhance metastases, even in breast cancer [48]. It remains to be seen how members of this protein family might interact with normal processes to give apparently opposite results.

The results presented here suggest that by regulating cell morphology dynamics, VICKZ proteins help to mediate CRC progression and lymph node metastasis. In addition to their ability to shuttle RNAs to

particular intracellular localizations, VICKZ proteins can function in a number of other ways in the post-transcriptional control of RNA [17,49,50]. These functions raise the possibility that VICKZ proteins may play additional roles in both normal and transformed cells. The identification of more VICKZ RNA targets, and the use of conditional and tissue-specific knockdowns of VICKZ paralogues, should lead to a fuller understanding of how these proteins function in both normal and neoplastic settings.

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Supplementary material

Supplementary material may be found at the web address http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2376.html

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