A proteomic approach to identify endosomal cargoes controlling cancer invasiveness

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Key words:

SILAC, Rab17, Vamp8, neuropilin-2, ductal carcinoma in situ, invasive ductal carcinoma, late endosome, cell migration, invasion, breast cancer.
ABSTRACT

We have previously shown that Rab17 - a small GTPase associated with epithelial polarity - is specifically suppressed by ERK2 signalling to promote an invasive phenotype. However, the mechanisms through which Rab17 loss permits invasiveness, and the endosomal cargoes that are responsible for mediating this are not known. Using quantitative mass spectrometry-based proteomics, we have found that knockdown of Rab17 leads to highly selective reduction in the cellular levels of a v-SNARE (Vamp8). Moreover, proteomics and immunofluorescence indicate that Vamp-8 is associated with Rab17 at late endosomes. Reduced levels of Vamp8 promote transition between ductal carcinoma in situ (DCIS) and a more invasive phenotype. We developed an unbiased proteomic approach to elucidate the complement of receptors that redistributes between endosomes and the plasma membrane, and have pinpointed neuropilin-2 (NRP2) as a key pro-invasive cargo of Rab17/Vamp8-regulated trafficking. Indeed, reduced Rab17 or Vamp8 levels lead to increased mobilisation of NRP2-containing late endosomes and upregulated cell surface expression of NRP2. Finally, we show that NRP2 is required for the basement membrane disruption which accompanies transition between DCIS and a more invasive phenotype.
INTRODUCTION

The membrane trafficking events controlled by Rab GTPases influence cellular processes that accompany cancer initiation and progression, including loss of cell polarity, and the drive to invasion and metastasis (Cox et al., 2014; De Franceschi et al., 2015; Goldenring, 2013). The contribution made by Rab GTPases to acquisition of invasive behaviour has been intensively studied, and the molecular machinery which is responsible for trafficking receptors controlling cell adhesion and cell migration is becoming well-understood (Miller et al., 2000; Rainero & Norman, 2013). There is good evidence that Rab11 GTPase control of integrin and receptor tyrosine kinase (RTK) recycling drives invasive migration in cancer, and expression of Rab11s and their effectors, such as Rab-coupling protein (RCP) is linked to metastasis (Caswell et al., 2008). Furthermore, Rab21 is closely associated with integrin trafficking and the contribution made by this Rab GTPase to cancer invasiveness and suppression of apoptosis is now well-documented (Alanko et al., 2015; Hognas et al., 2012).

Loss of epithelial cell polarity is a key event in cancer progression, and one that is generally accepted to precede acquisition of invasive and metastatic capabilities (McCaffrey & Macara, 2011). For example, in breast cancer, epithelial polarity is progressively lost as tumours progress from pre-malignant lesions to invasive carcinoma. Ductal carcinoma in situ (DCIS), which are a heterogeneous group of lesions characterised by intraductal proliferation of malignant epithelial cells, possess an intact basement membrane (which may be considered to be a remnant basolateral domain) but the cells display no functional apical surface (Wiechmann & Kuerer, 2008). The progression of DCIS to invasive ductal carcinoma (IDC) occurs when the basement membrane becomes disrupted and this last vestige of epithelial polarity is lost. Despite the fact that transition between DCIS and IDC is an accepted watershed in disease aggressiveness, the key molecular and cellular drivers of this process are not well understood (Clark et al., 2011; Hannemann et al., 2006; Vincent-Salomon et al., 2008). Since the DCIS to IDC transition involves loss of aspects of epithelial polarity, this is likely to be driven by alterations to Rab GTPase levels and/or function. It is
already known that Rab25, a Rab11 GTPase whose expression is largely restricted to epithelia, is often lost in breast cancer (Cheng et al, 2010), and deletion of Rab25 accelerates tumourigenesis in a mouse model of colon cancer (Nam et al, 2010). One interpretation of this is that loss of Rab25 leads to disruption of trafficking pathways that maintain aspects of normal epithelial polarity. Rab17 was the first epithelial cell specific small GTPases to be identified, and its expression is induced as epithelial cells polarise (Hunziker & Peters, 1998; Lutcke et al, 1993; Zacchi et al, 1998). There is some debate as to the trafficking pathways controlled by Rab17, but a consensus is emerging that this Rab GTPase is associated with transcytosis of receptors, such as the polymeric immune globulin receptor (dlgA) and the transferrin receptor (TfnR) from the basolateral to the apical surface of epithelial cells (Hansen et al, 1999; Zacchi et al, 1998). We have recently identified Rab17 as a gene which is suppressed during the acquisition of invasive migration that accompanies upregulation of ERK2 kinase signalling (von Thun et al, 2012). Furthermore, we have shown that Rab17 levels must be suppressed for ERK2 to drive breast cancer invasiveness. Consistently, reduced Rab17 has been shown to be associated with increased aggressiveness in hepatocellular carcinoma (Wang et al, 2015). Taken together these studies suggest that Rab17 may oppose events that accompany the DCIS to IDC transition – such as the loss of cell polarity and the acquisition of invasiveness – and this would be likely to be mediated by controlling endosomal trafficking of pro- and/or anti-invasive receptor cargoes.

As there is no reason to propose that the known receptor cargoes of Rab17-regulated transport – such as dlgA and TfnR – would be drivers of the DCIS to IDC transition or cancer invasiveness, we developed an unbiased mass spectrometry (MS)-based proteomic approach to investigate Rab17’s interactome and the protein cargoes whose trafficking it influences. To do this we combined quantitative SILAC-based proteomics with a biotinylation-based method to separate plasma membrane from endosomal proteins. From this approach we found that Rab17 mediates its anti-invasive effects via interaction with the
SNARE protein Vamp8, and that Rab17-Vamp8 influences the endosomal-plasma membrane distribution of a number of cargoes that might be expected to influence invasiveness. Prominent amongst these is neuropilin-2 (NRP2) which moves from endosomes to the plasma membrane following Rab17 depletion. We conclude by showing that NRP2 is required for the DCIS to IDC transition and the acquisition of invasiveness that is driven by depletion of Rab17-Vamp8.
RESULTS

Low levels of Rab17 mRNA correlate with poor breast cancer survival

We have previously shown that reduced Rab17 mRNA levels are associated with invasive migration of breast cancer cells (von Thun et al, 2012). To determine whether this observation is pertinent to aggressiveness of the human disease we interrogated the caBIG, GEO and TCGA repositories via the ‘kmplot’ analysis tool (Gyorffy et al, 2013). Consistent with our previous results, we found that low levels of Rab17 expression in breast tumours was strongly associated with poor overall patient survival (p<0.0001) (Fig. 1A), and this was statistically significant in all breast cancer intrinsic subtypes (Fig. S1 A-C), with the exception of HER-enriched subtype (Fig. S1D). Survival of ovarian cancer patients was also associated with low Rab17 expression (p<0.0001) (Fig. S1E), but we were unable to demonstrate any correlation between Rab17 levels in lung or gastric cancers (not shown).

Quantitative proteomics indicate that Rab17 controls Vamp8 levels

We hypothesised that Rab17 may restrict invasiveness by controlling the expression or trafficking of proteins that are invasion suppressors. To search for these we knocked-down Rab17 (Fig. S1F) and performed a SILAC-based global proteomic mass spectrometry analysis. We labelled control and Rab17 knockdown MDA-MB-231 breast cancer cells with light and heavy SILAC amino acids respectively (forward (Fw) experiment), and with heavy and light amino acids respectively (reverse (Rev) experiment). We then prepared cells extracts, and quantified them on an LTQ-Orbitrap. MS data were analysed with the MaxQuant computational platform. Accurate protein quantification highlighted high reproducibility between Fw and Rev experiments. Despite the large number of proteins that were unambiguously identified and quantified, we found that only a single protein was significantly downregulated (in both Fw and Rev experiments) in Rab17 knockdown cells.
and this was the v-SNARE, Vamp8 (Fig. 1B; supplementary spreadsheet 1). We confirmed this result by knocking-down Rab17 in MDA-MB-231 cells using either a single siRNA oligonucleotide or pooled siRNAs followed by Western blotting. (Fig. 1C, D). Moreover, this analysis indicated that levels of Vamp3 and Vamp7 (the most closely related Vamp to Vamp8) were unaffected by knockdown of Rab17 (Fig.1C). Furthermore, since Rab17 knockdown suppressed Vamp8 protein but not levels of its mRNA, we conclude that Rab17 controls Vamp8 expression post-transcriptionally (Fig, S2A, B).

Our previous work has identified Rab17 to be a key effector of MAP kinase signalling. Following suppression of ERK2 (but not ERK1), Rab17 levels are increased and this leads to increased invasiveness (von Thun et al, 2012). To determine whether the ability of MAP kinase signalling to influence Rab17 was, by turns, capable of controlling Vamp8 levels, we knocked down ERK2 and measured Vamp8 levels by Western blotting. Knockdown of ERK2 led to increased levels of the mRNA for Rab17 and Vamp8 protein (Fig. 1E), further supporting the view that Rab17 controls Vamp8 levels and suggesting that suppression of this v-SNARE may underlie ERK2’s ability to drive invasiveness.

The Rab17-Vamp8 complex is localised to late endosomes

We used MS-based proteomics to investigate Rab17’s interactome. We expressed GFP-tagged Rab17 (GFP-Rab17) or GFP in MDA-MB-231 cells and immunoprecipitated these using GFP-trap beads. A label-free quantification approach was then used to identify a set of 95 proteins that constitute the Rab17 interactome, and Vamp8 was a prominent component of this (Fig. 1F; supplementary spreadsheet 2). We used Western blotting to confirm the association between Rab17 and Vamp8 (but not Vamp7), and also found that Rab24 (a Rab GTPase which is closely related to Rab17) did not coimmunoprecipitate with Vamp8 indicating that there was a degree of specificity to the Rab17-Vamp8 interaction (Fig. 1G). To determine whether Vamp8 and Rab17 were able to interact directly with each other,
we produced the cytoplasmic region of Vamp8 (Vamp8cyto) and GST-tagged-Rab17s (both the wild-type and a constitutively active GTPase-deficient mutant of the protein) in E.Coli and purified these. However, we were unable to demonstrate co-precipitation of purified soluble Vamp8cyto with glutathione bead-conjugated Rab17s (Fig. S2C) indicating that these proteins either do not interact directly or interact transiently. Next, we used STRING software (http://string-db.org) (Szklarczyk et al, 2015) to examine known functional interactions between proteins of the Rab17 interactome. In addition to the anticipated Rab protein/signal transduction and SNARE complex groups, we found that Rab17 associated with proteins related to lysosomes (Fig. S2D). Indeed, when we annotated our Rab17 interactome to denote the intracellular compartmentalisation of its components, we found that 43% of Rab17’s interactors may be classified as late endosomal and/or lysosomal proteins (Fig. 1H).

GFP-Rab17 displayed significant co-localisation (Pearson’s coefficient=0.82 ± 0.089) with endogenous Vamp8 at vesicular structures in the perinuclear region of MDA-MB-231 cells, consistent with late endosomal localisation of Rab17/Vamp8 (Fig. 2A). Indeed, endogenous Vamp8 displayed significant colocalisation with both endogenous (Fig. 2B, C; Pearson’s coefficient ~ 0.6) and GFP-tagged (Fig. S3) markers of late endosomes, such as CD63 and Lamp1/2. As described in Fig. 1, we observed a (50-60%) reduction in Vamp8 levels following Rab17 knockdown. Correspondingly, the late endosomal distribution of Vamp8 was disrupted by following siRNA of Rab17. Thus the residual Vamp8 in Rab17 knockdown cells was distributed more generally in the central region of the cell, and its colocalisation with CD63-positive structures was significantly reduced (Fig. 2C, D).

**Rab17 and Vamp8 oppose breast cancer cell invasiveness in 3D models**

Our identification of Vamp8 as an interactor of Rab17, and the fact that Vamp8 was downregulated in Rab17-depleted cells, prompted us to determine whether Vamp8 contributed to Rab17’s ability to function as a suppressor of breast cancer cell invasiveness.
Initially, we investigated the consequences of knocking down Rab17 or Vamp8 on the ability of MDA-MB-231 cells to invade into plugs of Matrigel supplemented with fibronectin. siRNA of Rab17 increased invasiveness of MDA-MB-231 cells. Interestingly, siRNA of Vamp8 also promoted invasiveness into Matrigel plugs, consistent with the view that Vamp8 is functionally linked to Rab17 (Fig. 3A). These data led us to hypothesise that Rab17 and Vamp8-dependent trafficking might drive the transition between DCIS and IDC in the mammary gland, thus contributing to breast cancer progression. Experimentally, this transition may be modelled using the MCF10DCIS.com breast cancer cell line. MCF10DCIS.com cells are estrogen receptor (ER)-negative premalignant mammary carcinoma cells which are derived from the 'normal' MCF10A cell line and are known to form well-defined comedo-like DCIS structures when injected as subcutaneous or intraductal xenografts (Behbod et al, 2009; Miller et al, 2000). However, with time these lesions spontaneously progress to invasive carcinoma characterised by disruption of their surrounding basement membrane, and the development of invasive outgrowth. Elements of this progression may be recapitulated in 3D culture (Jedeszko et al, 2009). Since the levels of Vamp8 in MCF10DCIS.com cells were dependent on Rab17 expression (Fig. S4A, B), we used this model to study the role of Rab17 and Vamp8 in the DCIS to IDC transition. When MCF10DCIS.com cells were cultured for 3 days in Matrigel they formed well-organised comedo-like structures which were surrounded by basement membranes evidenced by immunofluorescence staining for the basolateral marker β4 integrin and the basement membrane component laminin-V (Fig. 3B). We quantitatively assessed the shape of these organoids and found that MCF10DCIS.com cells formed structures that were roughly spherical – as reflected by a low deviation from circularity of a cross sectional focal plane (Fig. 3C). However, when either Rab17 or Vamp8 were knocked-down in MCF10DCIS.com cells (using either a SMARTPool or individual siRNA sequences), structures with a high degree of sphericity were able to initially form (not shown), but their symmetry became significantly disrupted after 3 days of culture in Matrigel (Fig. 3C). Consistently, immunofluorescence staining for β4 integrin and laminin-V indicated that siRNA of Rab17 or
Vamp8 drove substantial disruption of the basement membrane surrounding the comedo-like structure allowing MCF10DCIS.com cells to migrate out of the organoid (Fig. 3B). Furthermore, the ability of siRNAs targeting Rab17 or Vamp8 to disrupt the sphericity of MCF10DCIS.com organoids was completely reversed by expression of siRNA resistant (rescue) versions of GFP-Rab17 and GFP-Vamp8 respectively (Fig. 3C; Fig. S4C, D). Finally, combined knockdown of Rab17 and Vamp8 did not increase the disruption of sphericity evoked by individual siRNA of these proteins, consistent with Rab17 and Vamp8 operating on the same signalling axis (Fig. S4E). Taken together these data are consistent with a role for Vamp8 in mediating Rab17’s anti-invasive functions, and that this is evident in a 3D model of the DCIS to IDC transition.

**Vamp8 levels are associated with tumour grade in breast cancer**

Our data indicating the likelihood that Rab17 exerts its anti-invasive effects by maintaining Vamp8 levels, prompted us to investigate the relationship between Vamp8 and invasive pathology in breast cancer. In the normal mouse mammary gland, Vamp8 appeared to be associated with the luminal face of the ductal epithelium (Fig. 4A). Expression of the polyoma middle-T antigen (PyMT) under control of the MMTV mammary-specific promoter (MMTV-PyMT), results in formation of tumours in the mouse breast with high penetrance. MMTV-PyMT tumours have a gene expression profile similar to human luminal B cancers (Herschkowitz et al, 2007), and most of these progress from DCIS-like structures to IDC in a similar fashion to the human disease. We, therefore, looked at the distribution of Vamp8 in a number of tumours from MMTV-PyMT mice and compared this with their histopathology. We found that Vamp8 levels were high in the less advanced MMTV-PyMT tumours that displayed DCIS-like pathology, whereas in more invasive tumours with IDC-like characteristics Vamp8 levels were low (Fig. 4A). We then deployed a large tissue microarray (TMA) containing 542 human breast tumours to test whether this inverse
relationship between Vamp8 levels and features of tumour aggressiveness may be observed in human breast cancer. Furthermore, because some Vamp8 immunoreactivity observed in breast tumours appeared to be diffusely distributed within the cells, we scored our TMA only for Vamp8 that was localised membranous structures that were either in the cell periphery or within the cell. There was a highly significant inverse correlation between membranous Vamp8 staining and tumour grade. Indeed, the majority of low grade (grade I) tumours had high levels of Vamp8, whereas most high grade (grade III) tumours displayed less intense membranous Vamp8 staining (Table 1; Fig. 4B). Moreover, Vamp8 levels were significantly correlated with breast cancer molecular subtype in a way that was consistent with tumour grade. For example, Vamp8 levels were low in the majority of the highly aggressive ER-negative subtype, whereas a minority of ER-positive tumours had high Vamp8 levels (Table 1). These data indicate that loss of Vamp8 occurs as murine mammary tumours progress from DCIS to IDC and that Vamp8 levels are maintained in less aggressive luminal A and low grade human tumours in comparison to more aggressive cancers, which is consistent with an anti-invasive role for Rab17/Vamp8.

**A SILAC-based mass spectrometry screen reveals NRP2 to be a Rab17 cargo**

We hypothesised that Rab17/Vamp8-regulated trafficking pathways might suppress invasion by either reducing delivery of pro-invasive receptors to the plasma membrane, or by increasing recycling of anti-invasive cargoes. We designed a strategy (schematically summarised in Fig. 5A) which allowed us to identify alterations to receptor distribution between endosomes and the plasma membrane in an unbiased way. To do this we combined SILAC-based MS proteomics with compartment-specific protein biotinylation approaches. As before, we labelled control and Rab17 knockdown MDA-MB-231 cells with light and heavy SILAC amino acids (Fw experiment), and with heavy and light amino acids respectively (Rev experiment). We then surface-labelled these cells at 4ºC with membrane
impermeant sulpho-NHS-SS-Biotin and isolated the fraction of plasma membrane proteins using streptavidin affinity chromatography – this complement of proteins we termed the ‘surface proteome’. In a separate set of culture dishes, we warmed SILAC-labelled surface-biotinylated cells to 37°C for 20 min to allow surface-labelled receptors to distribute between endosomes and the plasma membrane. As the sulpho-NHS-SS-Biotin reagent contains a disulphide linker that may be cleaved by reducing agents, we were able to remove biotin from receptors remaining at the cell surface by treatment with the membrane impermeant reducing agent, MesNa. The remaining endosomally-localised biotinylated receptors were then isolated using streptavidin beads, and this fraction of proteins we termed the ‘internalised proteome’. The surface and internalised proteome from the Fw and Rev experiments were then analysed by high resolution mass spectrometry. This provided unambiguous identification of 1640 surface proteome and 2272 internalised proteome components and, as expected, the majority of protein isolated using this surface biotinylation approach were membrane and/or membrane-associated proteins (not shown). We then looked for proteins whose relative abundance between surface and endosomal compartments was reproducibly (in the Fw and Rev experiments) influenced by Rab17 knockdown. We categorised these according to whether their abundance was co-ordinately or differentially regulated at the cell surface and the endosome by Rab17 knockdown. The largest category constituted those proteins whose abundance was co-ordinately increased in the surface proteome and the internalised proteome by Rab17 knockdown (upper right quadrant in Fig. 5B; supplementary spreadsheet 3). Prominent amongst these were the pro invasive transmembrane matrix metalloprotease MT1-MMP and the receptor tyrosine kinase, EGFR1, indicating that degradation of these receptors may be suppressed following Rab17 knockdown.

The purpose of this study was to identify novel cargoes of Rab17 - i.e. proteins that were distributed differently between the cell surface and endosomes following siRNA of Rab17. We, therefore, focussed our attention on those hits whose abundance moved in opposite
directions in the surface proteome and internalised proteome following Rab17 knockdown (upper left quadrant in Fig. 5B; supplementary spreadsheet 3). Interestingly, this analysis revealed neuropilin-2 (NRP2), which is a co-receptor for semaphorins and vascular endothelial growth factor (VEGF), to be the protein whose surface expression was most significantly increased (1.5 fold) at the cell surface and reduced (1.2 fold) in the internalised proteome when Rab17 was silenced (Fig. 5B). Western blotting confirmed that siRNA of Rab17 increased the quantity of NRP2 at the cell surface and decreased the endosomal pool of NRP2, whilst its total cellular content remained unchanged (Fig. 5C). Furthermore, we found that knockdown of Vamp8 evoked similar alterations to NRP2’s plasma membrane/endosomal distribution to that elicited by siRNA of Rab17 (Fig. 5C).

** Trafficking of NRP2-positive vesicles is altered by suppression of Rab17/Vamp8 **

Our proteomic analysis indicated that NRP2 redistributes from endosomes to the cell surface following Rab17 knockdown, suggesting that the endosomal trafficking of this receptor is regulated by Rab17/Vamp8. To visualise the trafficking of NRP2, we transfected MDA-MB-231 cells with GFP-tagged NRP2 in combination with the late endosomal marker mCherry-Lamp1 and performed fluorescence live cell imaging followed by quantitative vesicle tracking. GFP-NRP2 was localised to large Lamp1-positive late endosomes which were located primarily in the perinuclear region, and quantitative analysis indicated that less than 40% of NRP2-positive structures occupied an area of less than 2 μm² (Fig. 6A, B). Moreover, NRP2-positive vesicles had low motility and many of the larger vesicles were largely stationary during the course of the movies. By contrast, following siRNA of either Rab17 or Vamp8, the number of smaller NRP2-positive vesicles increased dramatically (>70% of structures being less than 2μm²), and quantitative tracking analysis indicated that the speed of their movement significantly increased (Fig. 6A, B). Moreover, the population of smaller vesicles that was evoked by knockdown of Rab17 or Vamp8 still overlapped
substantially with Lamp1-containing structures. Taken together, these data indicate that Rab17 and Vamp8 act to restrict the movement of a late endosomal/lysosomal compartment in which NRP2 is concentrated and, following suppression of either of these two trafficking regulators, NRP2 is localised to a population of smaller and more motile late endosomes. This observation is consistent with increased mobilisation of NRP2-containing late endosomes from the perinuclear region to more peripheral cellular locations and to the plasma membrane.

**NRP2 is required for invasiveness evoked by suppression of Rab17/Vamp8**

Our MS analysis indicated that NRP2 redistributes from endosomes to the cell surface following Rab17 knockdown suggesting that this receptor is a pro-invasive cargo whose recycling is opposed by Rab17/Vamp8. We, therefore, provoked breast cancer cell invasiveness by knocking-down Rab17 or Vamp8 and tested the requirement for NRP2 in this. Knockdown of NRP2 had no effect on the ability of MCF10DCIS.com cells to assemble basement membrane-like structures and to form spherical acini when grown in 3D Matrigel cultures (Fig. 7A, B). However, siRNA of NRP2 (using either a SMARTPool siRNA or an individual siRNA sequence) opposed the ability of Rab17 and Vamp8 knockdown to drive basement membrane degradation and to disrupt acinar sphericity (Fig. 7A, B). Consistently, we found that siRNA of NRP2 completely opposed the ability of both Rab17 and Vamp8 knockdown to drive invasion of MDA-MB-231 cells into Matrigel plugs (Fig. 7C).
DISCUSSION

The available database information concerning Rab17 and its interactors is insufficient to infer potential effectors and pathways through which this GTPase acts to suppress cancer invasiveness and progression. We, therefore, determined Rab17’s interactome and its influence on the cellular proteome in an unbiased way. The use of high accuracy MS-based approaches has enabled us to do this, and to show that Rab17 has a close physical and functional relationship with the SNARE protein, Vamp8. Rab GTPases are known to recruit specific effector proteins that bind to SNAREs giving specificity to vesicle fusion (Angers & Merz, 2011). Nevertheless, to our knowledge, our study is the first to provide an indication that the stability and cellular levels of a SNARE protein may be controlled by a Rab GTPase. Moreover, because Vamp8 is the only component of the proteome that is significantly altered following Rab17 knockdown, the relationship between this GTPase and Vamp8 stability is likely to be highly selective. Our mass spectrometric and immunofluorescence data indicate that Vamp8 is localised primarily to late endosomes, and that its recruitment to this compartment is strongly dependent on Rab17. Thus, these data are consistent with a view that Rab17 acts primarily to recruit Vamp8 to late endosomes and, when Rab17 levels are low, Vamp8 becomes mislocalised and is subsequently degraded. We have found that neither proteosomal nor lysosomal mechanisms are responsible for degradation of mislocalised Vamp8 (not shown). However, a report that caspases regulate Vamp8 expression and function in dendritic cells indicates the possibility that Rab17 could protect Vamp8 from caspase cleavage by localising it to late endosomes (Ho et al, 2009). Increased Vamp8 is primarily thought to promote homotypic fusion of either early or late endosomes (Antonin et al, 2000; Pryor et al, 2004). Endosomal compartments are constantly engaged in homotypic fusion and fission, and the relative rates at which these two processes occur dictate the size and identity these compartments. Our observation that NRP2-containing late endosomes are large when Vamp8 levels are high is consistent with a role for this v-SNARE in homotypic fusion of these compartments. Thus, activation of ERK2 which leads to
downregulation of Rab17 and, in turn, reduced Vamp8 levels might be expected to shift the homotypic fusion/fission balance of late endosomes in favour of fission to promote mobilisation of NRP2-containing late endosomes to the plasma membrane to drive invasion.

Rab17 expression has been known for a number of years to be associated with establishment of a polarised epithelial phenotype (Hunziker & Peters, 1998; Lutcke et al, 1993; Zacchi et al, 1998), and its downregulation is linked to loss of epithelial polarity (Lutcke et al, 1993). Our observations that downregulation of Rab17 accompanies events such as the DCIS to IDC transition and the disruption of acinar morphology (including breaching of the basement membrane) are consistent with a role for Rab17 in maintaining epithelial morphology and opposing epithelial to mesenchymal transition or EMT. Thus it is probable that cargoes trafficked by Rab17 are involved in maintenance of epithelial polarity and, consistently, Rab17 has been shown to control trafficking events, such as transcytosis, that are peculiar to polarised epithelia (Hunziker & Peters, 1998; Zacchi et al, 1998). Vamp8 has been functionally linked to transcytosis (Pocard et al, 2007). Indeed, most evidence indicates that this SNARE, like Rab17, plays an important role in controlling trafficking events that support maintenance of epithelial polarity. In epithelial cells, Vamp8 is responsible for sorting apical cargoes, such as dipeptidyl peptidase IV (DPPIV), to the apical plasma membrane (Pocard et al, 2007). Many tumour cells retain some, but not all, of the characteristics of a polarised epithelium. For instance, comedo-forming tumour cells, such as MCF10DCIS.com, have a basement membrane which corresponds to a basolateral region, but do not have a well-defined apical domain. Thus, it is interesting to speculate about the role played by the transcytotic machinery in partially polarised comedo-like organoids and other less polarised cells. One possibility is that in the absence of an apical domain (such as is encountered in MCF10DCIS.com cells), proteins such as Rab17 and Vamp8 might still function to move receptors that could potentially disturb basement membrane integrity away from the plasma membrane and into other compartments.
Therefore, we developed an unbiased approach to identify the receptors whose distribution between the plasma membrane and endosomes was altered following loss of Rab17.

Characterisation of the endomembrane proteome is complicated by the fact that it is difficult, if not impossible, to obtain purified endosomal fractions that are uncontaminated by components from other cellular subcompartments. To overcome this, we have used a biotin-labelling approach which allows enrichment of internalised protein cargo, and combined this with a quantitative SILAC method designed to highlight consistent changes in plasma membrane/endosomal distribution evoked in response to a defined pro-invasive stimulus. By focusing on these changes, interference from proteins that are non-specifically associated with plasma membrane and/or endosomal isolates is minimised. The receptor whose distribution is most significantly altered by Rab17 knockdown is NRP2. The neuropilins are well-established to contribute to axonal guidance and angiogenesis (Raimondi & Ruhrberg, 2013), however the roles played by these receptors in epithelial cells has received less attention. Neuropilins and the receptors with which they collaborate, such as VEGF receptors, are generally thought to be restricted to the apical/luminal domains of epithelial ducts to reduce their exposure to growth factor ligands that are abundant in the interstitium (Wild et al, 2012). Consistently, upregulation and/or mislocalisation of NRP1 and NRP2 to the basolateral region correlates with invasiveness and poor prognosis in a number of tumour types (Wild et al, 2012). Our data indicate that Rab17/Vamp8 promotes removal of NRP2, and other potentially proinvasive receptors (such as MT1-MMP), from the basolateral membrane so that they can be routed to cellular locales where they will not disrupt basement membrane integrity. Thus, in normal epithelia the role of Rab17/Vamp8 might be to transcytose these receptors to the apical domain, but in partially transformed comedo-like tumours Rab17/Vamp8 strives to oppose the transition to invasive carcinoma by actively retaining NRP2 safely within the late endosomal system. Thus, Rab17/Vamp8-dependent sequestration of NRP2 within the endosomes of tumour cells likely represents a remnant of the transcytotic system which still acts to suppress basement membrane disruption and
DCIS to IDC transition. Suppression of Rab17 levels by the activation of ERK2 signalling, likely represents a key event in loss of the last vestiges of epithelial polarity which allows tumours to invade and metastasise. Our use of novel MS proteomics to identify NRP2 as a cargo of the Rab17/Vamp8 pathway indicates that loss of control over trafficking of this receptor is a key event in the DCIS to IDC transition.
**MATERIALS AND METHODS**

*Cell culture and transfection*

MDA-MB-231 cells were cultured as described previously (von Thun et al, 2012). MCF10ADCIS.com cells were kindly provided by Prof. Philippe Chavrier (Institut Curie, Paris, France) and were cultured and transfected as described previously (Macpherson et al, 2014). Rab17 siRNA oligos were SMARTpool, #1 5'-GAAGUGGCUCCGUGGUAA-3’ or #2 5’-ACGCUGCUCUUCUGUGUA-3’ (Dharmacon). Vamp8 and NRP2 siRNA oligos were SMARTpool (Dharmacon, CA, USA). EGFP-C1-Rab17 was a gift from Jeremy Simpson (University College, Ireland). CD63- and Rab11-GFP constructs were as described previously (Macpherson et al, 2014). GFP-NRP2 in pCMV6-AC-GFP was from Amsbio (MG223943), and GFP-Vamp8 was a gift from Thierry Galli (Addgene plasmid #42311 (Paumet et al, 2000)). Cells were transfected using the Nucleofector system as described previously (Macpherson et al, 2014). SILAC-labeled MDA-MB-231 cells were obtained by culturing in SILAC-DMEM lacking arginine and lysine (Life Technologies, CA, USA) supplemented with L-arginine and L-lysine (SILAC light) (Sigma-Aldrich, St. Louis, MO, USA) or $^{13}$C$_6^{15}$N$_4$ L-arginine and $^{13}$C$_6^{15}$N$_2$ L-lysine (SILAC heavy; Cambridge Isotope Laboratories, Tewksbury, MA, USA). 3D culture of MCF10DCIS.com cells and inverted invasion assays were performed as described previously (Macpherson et al, 2014).

*qRT-PCR and Western blotting*

Primers for GAPDH, Rab17 and Vamp8 were obtained from Qiagen (cat# QT01192646, QT00009590 and QT00086639 respectively) NRP2 primers were ordered from Thermo Fisher Scientific (Wittmann et al, 2015) and qRT-PCR experiments were performed conducted as described previously (von Thun et al, 2012). Antibodies recognising Vamp8 (Synaptic systems, Göttingen, Germany), Vamp7 and Vamp3 were a gift from Andrew
Peden (University of Sheffield, UK). Antibodies against β tubulin (Sigma-Aldrich, Dorset, England), GFP (Abcam, Cambridge, UK), laminin V (Millipore, Darmstadt, Germany), β4 integrin (BD Biosciences, Becton, Dickinson and Company), NRP2 (R&D, Abingdon, UK), and ERK1/2 (Santa Cruz K-23), were used for Western blotting.

Recombinant protein expression, pulldown and site directed mutagenesis

Vamp8 amino acids 1-74 was sub-cloned into pGEX-6P-1 from GFP-Vamp8 plasmid using EcoR1 and Xho1 restriction sites. GST-Vamp8cyto and GST-Rab17 were transformed into OneShot® BL21(DE3) E.coli (Invitrogen) grown at 37 ºC for 6 hours and then induced with IPTG for 16 hours. Bacteria were lysed by sonication in PBS and proteins purified using glutathione-sepharose beads (GE Healthcare). Vamp8 was subsequently cut from the beads with PreScission protease (GE Healthcare) 1.6 units per 10 µl cleavage buffer (50 mM Tris-HCL pH 6.8, 150 mM NaCl, 1 mM EDTA, 0.01% Triton X-100, 1 mM DTT). The resulting protein was quantified via NuPAGE Tris-acetate gel and equal quantities of Vamp8 and Rab17 were incubated for 16 hours at 4ºC. The beads were then washed twice with PBS and the input and beads run on NuPAGE Tris-acetate gel and whole protein levels analysed with InstantBlue™ Coomassie stain (Expedeon).

Site directed mutagenesis was carried out using QuikChange Lightning kit (Agilent) to create GST-Rab17Q77L (forward 5’-TGTGGTACTTCTTCCAGGCCAGCTGTGTCC-3’ and reverse 5’-GGACACAGCTGGCCTGGAGAAGTACCACA-3’) and the two rescue vectors to si-#1 GFP-Rab17 T81C_C84G_C87A_G90T forward 5’-GCCAAGCTGGACTTACCAACTGACCCGCTTCCCAGGAGAACCAG-3’

Reverse 5’-CTGGTTCTCTGGGAAGCGGCTCAGTTGGTAAGTCCAGCTTGGCCTGCACGC-3’, and GFP-Vamp8 to si-#2 c234t_t237g_c240a_c243t forward 5’-CTGGTTGGGAGAAGTCTTGTTTCTTGATATGTGTGATTGTTTTATTATCATCATCTCT

CTTC-3’ reverse 5’-
Surface and internalised proteome analysis

SILAC-labelled MDA-MB-231 cells were transfected with siRNAs targeting Rab17 or a non-targeting control and plated into 10cm plastic dishes. 48hr later cells were surface-biotinylated by incubation with 0.2 mM sulpho-NHS-SS-Biotin (Thermo Fisher Scientific, Paisley, UK) in PBS at 4 °C for 1 hr. To study the internalised proteome, surface-biotinylated cells were transferred to SILAC-DMEM (Gibco, Life technologies) at 37 °C for 20 min to allow internalisation of labelled protein, and then biotin remaining at the cell surface was removed by incubation with a cell impermeant reducing agent (MesNa) 20mM (Fluka Sigma-Aldrich, Dorset, England) in Tris-buffered saline (pH8.6) for 50 min at 4 °C. Remaining MesNa was quenched by incubation with iodoacetamide (IAA; 20mM Sigma-Aldrich). Cells were lysed in 200 mM NaCl, 75 mM Tris, 15 mM NaF, 1.5 mM Na3VO4, 7.5 mM EGTA, 1.5% Triton X-100, 0.75% Igepal CA-630, 0.5 mg/ml leupeptin (Melford, Suffolk, UK), 0.25 mg/ml aprotinin (Sigma), and 5 mM 4-(2-Aminoethyl)benzynesulphonyl fluoride (AEBSF) (Melford) and lysates cleared by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatants from the heavy and light SILAC labelled cells were then mixed and biotinylated proteins were captured by incubation with streptavidin-agarose beads (UPSTATE, Millipore) for 1 h at 4 °C with constant rotation. Following extensive washing, specifically-associated proteins were eluted by treatment with cleavage with 0.1 M DTT (0.1M) in a Tris buffer (pH 7.5).
**MS analysis**

Proteins were separated on 4-12% gradient NuPAGE Novex Bis-Tris gel (Life Technologies), visualised by staining of the gels with Comassie blue and digested in-gel (8 gel slices were cut for the Surface proteome/Internalised proteome; 5 gel slices for Rab17 IP) using trypsin (Shevchenko et al, 2006) and desalted as described previously (Rappsilber et al, 2007). For the proteome of cells silenced for Rab17, proteins were trypsin digested using the filter-aid sample preparation (FASP) method and 50 µg of peptides separated by strong anion exchange chromatography on StageTip as previously described (Wisniewski, J. R. and Mann M. J Proteome Res 8:5674; 2009). Digested peptides were loaded onto an EASY-nLC connected online to an LTQ-Orbitrap Elite mass spectrometer (Thermo-Fisher Scientific). Peptides were separated using a 20 cm fused silica emitter (New Objective) packed in house with reversed-phase Reprosil Pur Basic 1.9 µm (Dr. Maisch GmbH) and eluted with a flow of 200 nl/min from 5% to 20-30% of buffer containing 80% ACN in 0.5% acetic acid, in a 90 min (190 min gradient for the proteome of si-Rab17 cells) linear gradient. The full scan MS spectra were acquired at a resolution of 120,000 at m/z 400. The top 10 most intense ions were sequentially isolated for fragmentation using high-energy collision dissociation, and recorded at resolution of 15,000. Data were acquired with Xcalibur software (Thermo-Fisher Scientific).

**MS data analysis**

The MS files were processed with the MaxQuant software (Cox & Mann, 2008) version 1.3.7.4 (proteome) and 1.3.8.2 (internalised proteome and interactome) and searched with the Andromeda search engine (Cox et al, 2011) against the human UniProt database (release-2012 01, 88,847 entries). The common reverse and contaminant hits (as defined in MaxQuant output) were removed. Only protein groups identified with at least one uniquely assigned peptide were used for the analysis. For the SILAC experiments, the SILAC ratios
between light and heavy peptides were calculated by MaxQuant. Protein groups were considered reproducibly quantified if identified and quantified in forward and reverse experiment. For label-free quantification proteins were quantified according to the label-free quantification algorithm available in MaxQuant (Cox et al, 2014).

*Surface proteome and internalised proteome (SILAC):* From the filtered list of quantified proteins, hits with opposite regulation (= opposite sign when SILAC ratio expressed as log₂) in the two experiments were excluded. To identify differentially trafficked proteins upon Rab17 knockdown, proteins belonging to the 1ˢᵗ or 3ʳᵈ of the SILAC ratio distribution of the forward and reverse experiment were considered. This statistical approach allowed us to select proteins with small but reproducible (in both forward and reverse SILAC experiments) changes in localization.

*Rab17 knockdown proteome (SILAC):* For forward and reverse experiment, protein groups with significant SILAC ratio were determined according to the Significance B (Cox et al, 2011), using 5% as FDR. The Significance B identifies outliers based on the standard deviation of the protein SILAC ratio of the main distribution and the protein abundance (Intensity measured at the MS). The significance B was chosen because, as typically shown for SILAC datasets (Cox et al, 2011), the distribution of the logarithmic SILAC ratios was dependent on the protein intensity, where the spread of nonregulated proteins was higher for low compared to high abundant proteins. For this reason, for low abundant proteins to be significant a higher deviation from the SILAC ratio main distribution is required. Conversely, for high abundant proteins a higher deviation is required.

*Interactome (label-free):* Three independent replicates were generated per condition (Ctl and Rab17 IP) and significantly enriched proteins were selected using a Welsh test-based analysis (5% FDR). The Welsh test is particularly suitable for this kind of samples, IP where Ctl and Rab17 samples could recover different number and amount of interacting proteins,
because this test is designed for studies where the two samples have unequal variances and unequal sample sizes.

**Immunofluorescence and immunohistochemistry**

Antibodies against CD63 (PeliCluster M1544), Lamp2 (BD Pharmingen 555803), FITC (Southern Biotech 1031-02) and phalloidin-Alexa 546 (Thermo Fisher Scientific, Paisley, UK) were used for immunofluorescence. Cells were stained and imaged using either 64x or 60x objective with an inverted confocal microscope (Fluoview FV1000; Olympus). To quantify localisation, the confocal images were analysed using colocalisation2 plugin in ImageJ and plotting the Pearson’s coefficient which indicates positively correlated pixels. Immunohistochemical staining was performed as described previously (Dozynkiewicz et al, 2012; Macpherson et al, 2014) – the antibody recognising Vamp8 was from Synapticsystems (Göttingen, Germany).

**Live cell imaging**

Cells were imaged using a 60x objective and an inverted confocal microscope (Fluoview FV1000; Olympus). Movies were captured with 0.85 sec frame intervals over a period of 2.85 min. Tracking of GFP-NRP2 vesicles was performed using Trackmate plugin (Tinevez et al, 2016) and size data was collected manually both using ImageJ software.
ACKNOWLEDGEMENTS

Supported by Cancer Research UK (grant numbers; JCN, C596/A18277 and C596/A17196; SZ, C596/A12935), and Breast Cancer Now (grant to JCN, IM and ED). We would like to thank Andrew Peden, Jez Simpson and Thierry Galli for their generous gifts of reagents. JDV was initially funded by “Fundacion Canaria Dr. Manuel Morales” and the Spanish Ministry of Education. ED was supported by a grant from Breast Cancer Now. We would like to thank Colin Nixon and his team for performing the immunohistochemistry. We thank the PRIDE team: the .raw MS files and search/identification files obtained with MaxQuant have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/cgi/GetDataset) via the PRIDE partner repository (Vizcaíno et al. Nucleic Acids Res 41:D1063 2013) with the dataset identifier PXD003745 (Username: reviewer29949@ebi.ac.uk ; Password: zfXLaNPq).
REFERENCES


Rainero E, Norman JC (2013) Late endosomal and lysosomal trafficking during integrin-mediated cell migration and invasion: cell matrix receptors are trafficked through the late endosomal pathway in a way that dictates how cells migrate. *BioEssays : news and reviews in molecular, cellular and developmental biology* **35**: 523-532


**Figures**

**A**

High Rab17 mRNA

Low Rab17 mRNA

**B**

Log2 (si-Rab17/si-Con) Fw

Significantly upregulated proteins

Significantly downregulated proteins

**C**

siRNA

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Vamp8
Vamp7
Vamp3
β Tubulin

**D**

siRNA

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Vamp8
β-Tub

**E**

siRNA

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Vamp8
ERK1
ERK2
Tubulin

**F**

Log Welch in NaN

Welch Difference

Specificity of the interactor

**G**

IP

GFP-GFP

Rab17-Rab24

anti-Vamp8
anti-Vamp7
anti-GFP

**H**

Log Welch protein

Welch Difference
Figure 1. Rab17 is a key regulator and interactor of the endosomal v-SNARE, Vamp8

(A) Kaplan-Meier plot showing influence of Rab17 expression on overall survival in breast cancer. The red and black lines represent patients with Rab17 expression above and below the median respectively. n=1778 patients (low Rab17); n=1776 patients (high Rab17). Log Rank test; p=1.2 X10^{-11}

(B) MDA-MB-231 cells which were SILAC-labelled with heavy and light amino acids were transfected with non-targeting si-RNA (si-Con) or an si-RNA targeting Rab17 (si-Rab17) respectively (forward (Fw) experiment). For the reverse (Rev) experiment these labelling conditions were swapped. Scatter plot indicates the SILAC ratio si-Rab17/si-Control (Log2 scale) of each protein obtained for Fw versus Rev experiment for the whole cell proteome. Blue dotted lines indicate the regions encompassing significantly regulated proteins (Significance B statistic test, False discovery rate of 5%, Perseus software)

(C-E) MDA-MB-231 cells were transfected with siRNAs targeting Rab17 (SMARTPool or individual oligo Rab17#1), Vamp8 (SMARTPool (SP)), ERK2 or non-targeting controls (si-Con#1 and si-Con#2). Vamp8, Vamp7, Vamp3 and ERK expression levels were then determined by Western blotting. In (E) Rab17 expression was determined using qPCR.

(F) MDA-MB-231 cells were transfected with GFP or GFP-Rab17. GFP-tagged proteins were isolated using GFP-Trap_A agarose beads (Chromotek, Planegg, Alemania). Proteins eluted from the beads were resolved by SDS-PAGE, stained with Comassie blue (Expeidon, Cambrige, UK) and in-gel digested for MS analysis. Statistical test of differences between means (Welch t-test) was performed to show the most differentially expressed proteins among three independent experimental replicates. The red dotted line indicates the significance threshold. Blue dots denote the 95 significant interactors identified.
MDA-MB-231 cells were transfected with GFP, GFP-Rab17 or GFP-Rab24. GFP-tagged proteins were isolated as for (A) and immunoprecipitates were analysed by Western blotting with antibodies recognising GFP, Vamp8 or Vamp7.

Zoom of plot in Fig. 1F showing the identity (Gene name) of late endosomal-lysosomal associated proteins (red dots) in the Rab17 interactome. 43% of the members of Rab17’s interactome belong to lysosomal/late endosomal categories and are annotated according to (Chapel et al, 2013).
Figure 2. Rab17 and Vamp8 colocalise at late endosomal membranes

(A) MDA-MB-231 cells were transfected with GFP or GFP-Rab17 (green signal), fixed and endogenous Vamp8 was then visualised using immunofluorescence (red signal). Pearson's
coefficients were determined using the Colocalization Colormap Image J plugin and are depicted both as a pseudocolour scale and graphically. Mean ± SEM, number of cells=18 (GFP), 22 (GFP-Rab17). *** p<0.001; U-Mann Whitney. Bar: 10 µm.

(B-D ) MDA-MB-231 cells were transfected with siRNAs targeting Rab17 (si-Rab17) or a non-targeting control (si-Con). Following this, cells were fixed and stained for endogenous Vamp8 (red) and either CD63 (green) or Lamp2 (green). The nuclei were counterstained with DAPI (blue). Dotted squares indicate the zoomed regions. Bar: 10 µm. Pearson’s coefficients were determined as for (A), ** p<0.01; U-Mann Whitney.
Figure 3. Rab17 and Vamp8 oppose breast cancer cell invasiveness

(A) MDA-MB-231 cells were transfected with siRNAs targeting Rab17 (si-Rab17), Vamp8 (si-Vamp8) or a non-targeting control (si-Con). Invasiveness into fibronectin-supplemented (25μg/ml) Matrigel (Thermo-Fisher) was determined using an inverted invasion assay. Invading cells were stained with Calcein-AM (Molecular Probes, Thermo-Fisher), and visualised by confocal microscopy. Serial optical sections were captured at 15μm intervals, and are presented as a sequence in which the individual optical sections are placed alongside one another with increasing depth from left to right as indicated. Migration was quantitated by measuring the fluorescence intensity of cells penetrating the Matrigel to depths of 45μm and greater, and expressing this as a percentage of the total fluorescence intensity of all cells within the plug. Values are mean ± SEM. n=12 measurements from three independent experiments. Kruskal Wallis (p<0.0001) and Dunn’s test for the multiple comparisons were performed (*p<0.05; ***p<0.001).

(B, C) MCF10DCIS.com cells were transfected with siRNAs targeting Rab17 (si-Rab17 (SMARTPool) or individual siRNA sequences Rab17#1 or Rab17#2), Vamp8 (si-Vamp8 (SMARTPool) or individual siRNA sequences Vamp8#1, Vamp8#2, or Vamp8#3) or a non-targeting control (si-Con), in combination with plasmids encoding siRNA-resistant forms of GFP-Rab17 (Rab17-rescue) or GFP-Vamp8 (Vamp8-rescue) as indicated. Cells were plated into Matrigel and cultured for 3 days, following which cells were fixed and laminin V and β4 integrin visualised by immunofluorescence (B; green signal). The actin cytoskeleton (red signal) and nuclei (blue signal) were visualized with phalloidin–Alexa-Fluor-546 and DAPI staining respectively (B). Bar in (B), 10 μm. In (C), acini were visualised using phase contrast microscopy. Circularity was determined by measuring the ratio between the width and length of each acini. Values are mean ± SEM, n values in (C) from left to right are: n=88 (Con), n=160 (Rab17#1), n=130 (Rab17#2), n=243 (NT), n=293 (Vamp8#1), n=222
(Vamp8#2), n=220 (Vamp8#3), n=168 (Con), n=174 (Rab17), n=201 (Vamp8), n=63 (Con),
n=51 (Rab17), n=39 (Vamp8), n=177 (Con), n=253 (Rab17#1), n=270 (Rab17#1+ Rab17-
rescue), n=173 (Con), n=174 (Vamp8#2), and n=176 (Vamp8#2+ Vamp8-rescue). Bar in
(C), 100 µm. Kruskal Wallis and Dunn’s test for the multiple comparisons were performed
(**p<0.001, **p<0.01, *p<0.05).
Figure 4. Low Vamp8 levels are associated with high tumour grade in breast cancer

Vamp8 (brown staining) was visualised in paraffin-embedded sections of normal mammary ducts and mammary gland tumours from MMTV-PyMT mice (A) and in a human breast cancer TMA (B) using immunohistochemistry. Squares indicate zoomed regions. Scale bars, 25 µm (A); 100µm (B). In (A) the tumours depicted are from the intra epithelial neoplasm or DCIS (centre panels), and the invasive ductal carcinoma (right panels) stages of the disease. In (B) representative examples of a low grade (Grade I) tumour with intense Vamp8 staining (left panels) and a high grade (Grade III) tumour with less-intense Vamp8 staining (right panels) are displayed.
Figure 5. SILAC-based mass spectrometry screen reveals NRP2 to be Rab17 cargo

(A) Workflow of the SILAC based Mass Spectrometric approaches used for the comparative analysis of biotin label surface (surface proteome) and internalised (internalised proteome) proteins in SILAC-labelled MDA-MB-231 cells. This experiment was performed in a Forward (as indicated in this diagram) and Reverse configuration in parallel.

(B) Scatter plot showing the SILAC ratio si-Rab17/si-Control (logarithmic scale) for each significantly regulated protein (grey dots). Upper-right and lower-left quadrant show proteins which are respectively up- or down-regulated in both the cell surface and internalised.
proteome fractions. The upper-left and lower-right quadrants display proteins that are regulated in opposite directions in the cell surface and internalised proteome fractions as indicated. n=2 SILAC experiments. Significance A statistic test, False Discovery Rate of 5%, Perseus software.

(C) MDA-MB-231 cells were transfected with siRNAs targeting Rab17, Vamp8 or a non-targeting control (Con). Cell surface and internalised proteome fractions were prepared as indicated in (A) and the presence of NRP2 in these was determined using Western blotting. The cellular NRP2, Vamp8 and β-tubulin levels were also determined (input).
A

Movie 1

NRP2
Lamp1
si-Con

Movie 2

NRP2
Lamp1
si-Rab17

Movie 3

NRP2
Lamp1
si-Vamp8

B

NRP2 vesicles detected per cell

% NRP2 vesicles under 2 µm²

Velocity of NRP2 vesicles (µm/sec)

Con  | Rab17  | Vamp8  
siRNA (SMARTPool)  | siRNA (SMARTPool)  | siRNA (SMARTPool)  

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***
**Figure 6. NRP2 is relocated to a population of small motile transport vesicles following Rab17 or Vamp8 knockdown**

MDA-MB-231 cells were transfected with GFP-tagged NRP2 and mCherry-tagged Lamp1 in combination with siRNAs targeting Rab17 (si-Rab17), Vamp8 (si-Vamp8) or a non-targeting control (si-Con). Transfected cells were imaged by time-lapse fluorescence confocal microscopy. Movies were captured with 0.85 sec frame intervals over a period of 2.85 min. Stills were extracted from supplementary movies 1 – 3, and GFP-NRP2 (green) and mCherry-Lamp1 (red) are displayed in the merged images (A; left panels). Bars, 10 µm. Movies were analysed using the TrackMate plug-in for ImageJ (Tinevez et al, 2016). The trackplots displayed in the centre panels (A) indicate the movement of GFP-NRP2 vesicles with the speed of vesicle movement being denoted by a pseudocolour scale and the positions of stationary vesicles are indicated using purple circles. The areas encompassed by the orange boxes in the centre panels are enlarged in right panels (A; zoom). The number, size and velocity of GFP-NRP2 vesicles was calculated and these values are plotted in (B). Values are mean ± SEM, number of cells is 26 (si-Con), 23 (si-Rab17) and 20 (si-Vamp8), ***p<0.0001 Mann-Whitney test.
Figure 7. NRP2 is required for invasiveness evoked by suppression of Rab17/Vamp8

(A, B) MCF10ADCIS.com cells were transfected with siRNAs targeting Rab17 (si-Rab17), Vamp8 (si-Vamp8), NRP2 (si-NRP2 (SMARTPool)) or an individual siRNA sequence (NRP2#1)) or a non-targeting control (si-Con) in the indicated combinations and plated on a
thin layer of Matrigel. After 3 days of culture, cells were visualized using phase contrast microscopy and the circularity of individual acini was determined. Bar: 50 μm (A). Circularity was measured in two independent experiments. Values in (A) are mean ± SEM. n=21 (si-Con), n=15 (si-Rab17), n=12 (si-Vamp8), n=15 (si-NRP2), n=15 (si-Rab17 plus si-NRP2), n=12 (si-Vamp8 plus si-NRP2), n=71 (Con), n=143 (NRP2#1), n=140 (NRP2#1+Rab17), and n=121 (NRP2#1+Vamp8). Kruskal Wallis test was performed (p<0.0001) and Dunn’s test for the multiple comparisons (***p<0.001). Then cells were fixed and laminin V and β4 integrin were visualized by immunofluorescence (green signal and counterstained for F-actin (red signal) and nuclei (blue signal). Bar: 25 μm (B). The Western blot in (A) indicates the efficiency of the NRP2 knockdown with tubulin used as a loading control.

(C) MDA-MB-231 cells were transfected with siRNAs targeting Rab17 (si-Rab17), Vamp8 (si-Vamp8), NRP2 (si-NRP2) or a non-targeting control (si-Con) in the indicated combinations and invasiveness into fibronectin-supplemented (25μg/ml) (Sigma-Aldrich) Matrigel was determined using an inverted invasion assay as for Fig. 4A. Values are mean ± SEM, n=6. Dunn’s test for multiple comparisons was performed (***p<0.001).
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* Molecular subtype were defined as follows: Estrogen receptor (ER) or progesterone negative (PR) positive, HER2 negative and low Ki67 <14% for luminal A; ER or PR positive and Ki67 >14% or HER2 positive for luminal B; ER negative PR negative and HER2 positive for HER2 like; and ER negative PR negative and HER2 negative for triple negative.
Figure S1 Relationship between Rab17 levels and breast and ovarian cancer survival

(A-D) Kaplan-Meier plots showing the relationship between Rab17 expression and overall survival in the indicated molecular subtypes of breast cancer. The red and black lines represent patients with Rab17 expression above and below the median respectively. Log rank test was performed in all cases. (A) Basal; n=1778 patients (low Rab17); n=1776 patients (high Rab17). p=0.059. (B) Luminal A; n=884 patients (low Rab17); n=880 patients (high Rab17). p=0.00013. (C) Luminal B; n=500 patients (low Rab17); n=501 patients (high Rab17). p=0.00022. (D) HER2 positive; n=104 patients (low Rab17); n=104 patients (high Rab17). p=0.78.

(E) Kaplan-Meier plot showing the relationship between Rab17 expression and overall survival in ovarian cancer; n=653 patients (low Rab17); n=652 patients (high Rab17). p=3 x10^-5; Log rank test.

(F) Use of qPCR to confirm the siRNA knockdown of Rab17 for the transfections pertaining to the forward and reverse SILAC experiments.
Figure S2  Rab17 and Vamp8 knockdown, and STRING analysis of Rab17 interactome

(A, B) MDA-MB-231 cells were transfected with siRNAs targeting Rab17 (si-Rab17), Vamp8 (si-Vamp8) or a non-targeting control (si-Con). Levels of mRNA for Vamp8 (A) and Rab17 (B) were determined using qRT-PCR. Data were normalised to GAPDH and values are mean ± SEM n>5. Kruskal-Wallis test was performed (p=0.0078) and Dunn’s test as “post-hoc” test for the multiple comparisons (**p<0.01).

(C) A mutant of Vamp8 lacking the transmembrane domain (Vamp8cyto) and GST-tagged-Rab17s (both the wild-type and a constitutively active GTPase-deficient mutant of the protein) were produced in E.Coli and purified. Purified proteins were incubated together, following which GST-tagged Rab17s were captured using glutathione beads (pull-down). The beads were washed and captured proteins were separated by SDS-PAGE and visualised using colloidal coomassie blue.

(D) Most highly connected network of physical and functional protein-protein interactions within proteins of the Rab17 interactome (95 proteins) identified by STRING analysis. The network shows the significant links between components of the Rab17 interactome. The nodes representing the lysosome, SNARE complexes and Rab-protein signal transduction are highlighted. Required confidence (score) = 0.4.
Figure S3  Colocalisation of Vamp8 with GFP-tagged late endosomal markers

MDA-MB-231 cells were transfected with GFP-tagged CD63 (CD63-GFP; green) or GFP-tagged Lamp1 (Lamp1-GFP; green). 24hr following transfection, cells were fixed and stained with antibodies recognising endogenous Vamp8 (red). Nuclei were counterstained using DAPI (blue). Bar, 10 μm. The area encompassed by the white squares are shown on the panels on the right (zoom)
Figure S4  Knockdown and rescue of Rab17 and Vamp8 expression in MCF10ADCIS.com cells

(A) MCF10ADCIS.com cells were transfected with an siRNA targeting Rab17 (si-Rab17) or a non-targeting control (si-Con) and the levels of mRNA for Rab17 determined using qRT-PCR. Cells were plated for 48, 72 and 96h after transfection. Data were normalised to GAPDH and values are expressed as means ± SEM.

(B) MCF10ADCIS.com cells were transfected with a siRNAs targeting Rab17 (si-Rab17), Vamp8 (si-Vamp8) or a non-targeting control (si-Con), and levels of Vamp8 were determined using Western blot 48, 72 and 96 h after transfection.

(C) MCF10ADCIS.com cells were transfected with an siRNA targeting Rab17 (si-Rab17#1) or a non-targeting control (si-Con) in combination with a plasmid encoding an siRNA resistant form of GFP-Rab17 (GFP-Rab17esc). The levels of mRNA for Rab17 were then determined using qRT-PCR.

(D) MCF10ADCIS.com cells were transfected with an siRNA targeting Vamp8 (si-Rab17#2) or a non-targeting control (si-Con) in combination with a plasmid encoding an siRNA resistant form of GFP-Vamp8 (GFP-Vamp8esc). Levels of Vamp8 were then determined using Western blot with actin used as a loading control.

(E) MCF10DCIS.com cells were transfected with siRNAs targeting Rab17 (Rab17), Vamp8 (Vamp8), both alone or in combination (Rab17 + Vamp8), or a non-targeting control (Con) Cells were plated into Matrigel and cultured for 3 days, following which cells were fixed and acini were visualised using phase contrast microscopy. Circularity was determined by measuring the ratio between the width and length of each acini. Values are mean ± SEM, n=198 (Con), n=266 (Rab17), n=291 (Vamp8) and n=192 (Rab17 and Vamp8). ** p<0.01, ***p<0.001 Mann-Whitney test.
SUPPLEMENTARY SPREADSHEETS

Supplementary spreadsheet 1  SILAC analysis of the influence of Rab17 knockdown on the cellular proteome

This spreadsheet supports the data presented in Fig. 1B. MDA-MB-231 cells which were SILAC-labelled with heavy and light amino acids were transfected with non-targeting si-RNA (si-Con) or an si-RNA targeting Rab17 (si-Rab17) respectively (forward (Fw) experiment). For the reverse (Rev) experiment these labelling conditions were swapped. Columns E and F indicate the SILAC ratio si-Rab17/si-Con (Log2 scale) of each protein obtained for Fw (si-Rab17/si-Con) versus Rev experiment for the whole cell proteome. (Significance B statistic test, False discovery rate of 5%, Perseus software).

Click here to download spreadsheet 1
Supplementary spreadsheet 2  Label-free MS analysis of the Rab17 interactome

This spreadsheet supports the data presented in Fig. 2A. MDA-MB-231 cells were transfected with GFP or Rab17-GFP. GFP-tagged proteins were isolated using GFP-Trap_A agarose beads (Chromotek, Planegg, Alemania). Proteins eluted from the beads were resolved by SDS-PAGE, stained with Comassie blue (Expeleon, Cambridge, UK) and in-gel digested for MS analysis. Three independent replicates were generated per condition (column E-G; Con IP; column H-J, Rab17 IP). Significantly enriched proteins were selected using a Welsh test-based analysis (5% FDR).

Click here to download spreadsheet 2
**Supplementary spreadsheet 3  SILAC analysis of the influence of Rab17 knockdown on the surface and internalised proteomes of MDA-MB-231 cells**

This spreadsheet supports the data presented in Fig. 6A & B. SILAC ratios (si-Rab17/si-Con) for the surface and internalised proteomes were determined for the forward a (Fw) and reverse (Rev) experiments and these are presented in columns E-H. From the filtered list of quantified proteins, hits with opposite regulation (= opposite sign when SILAC ratio expressed as log₂) in the two experiments were excluded. To identify differentially trafficked proteins upon Rab17 knockdown, proteins belonging to the 1st or 3rd of the SILAC ratio distribution of the forward and reverse experiment were considered. This statistical approach allowed us to select proteins with small but reproducible (in both forward and reverse SILAC experiments) changes in localization.

Click here to download spreadsheet 3
SUPPLEMENTARY MOVIES
Supplementary movies 1 – 3 Fluorescence time-lapse imaging of GFP-NRP2 dynamics in living cells.

MDA-MB-231 cells were transfected with GFP-tagged NRP2 and mCherry-tagged Lamp1 in combination with siRNAs targeting Rab17 (si-Rab17) (movie 2), Vamp8 (si-Vamp8) (movie 3) or a non-targeting control (si-Con) (movie 1). Transfected cells were imaged by time-lapse fluorescence confocal microscopy. Movies were captured with 0.85 sec frame intervals over a period of 2.85 min.