

The Arp2/3 activator WASH regulates $\alpha 5\beta 1$ -integrin-mediated invasive migration

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Accepted 29 June 2011

Journal of Cell Science 124, 3753–3759

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doi: 10.1242/jcs.080986

Summary

The actin cytoskeleton provides scaffolding and physical force to effect fundamental processes such as motility, cytokinesis and vesicle trafficking. The Arp2/3 complex nucleates actin structures and contributes to endocytic vesicle invagination and trafficking away from the plasma membrane. Internalisation and directed recycling of integrins are major driving forces for invasive cell motility and potentially for cancer metastasis. Here, we describe a direct requirement for WASH and Arp2/3-mediated actin polymerisation on the endosomal membrane system for $\alpha 5\beta 1$ integrin recycling. WASH regulates the trafficking of endosomal $\alpha 5\beta 1$ integrin to the plasma membrane and is fundamental for integrin-driven cell morphology changes and integrin-mediated cancer cell invasion. Thus, we implicate WASH and Arp2/3-driven actin nucleation in receptor recycling leading to invasive motility.

Key words: Arp2/3 complex, Invasion, Actin assembly, Integrin recycling, Membrane trafficking

Introduction

The Arp2/3 complex initiates actin networks that facilitate clathrin-mediated vesicle invagination and scission from the plasma membrane as well as assembling actin tails that push newly formed vesicles through the cytoplasm (Kaksonen et al., 2006). However, less is known about how actin networks function downstream of vesicle internalisation. Actin nucleation-promotion factors (NPFs), such as the WASP-family proteins (Veltman and Insall, 2010) drive Arp2/3-complex-mediated actin assembly both in cell motility and in membrane trafficking. Recently, a highly conserved NPF called Wiskott–Aldrich syndrome protein and SCAR homolog (WASH) was implicated in many aspects of endosomal trafficking (Derivery et al., 2009; Gomez and Billadeau, 2009; Linardopoulou et al., 2007). We describe a central role of WASH in integrin-mediated invasive migration. WASH resides on perinuclear endosomes where it activates the Arp2/3 complex and regulates recycling of internalised $\alpha 5\beta 1$ integrin back to the plasma membrane, promoting invasive migration into fibronectin-containing matrix. This recycling of $\alpha 5\beta 1$ integrin promotes invasive behaviour of many cancer cell types (Caswell et al., 2007; Muller et al., 2009; White et al., 2007) linking WASH and Arp2/3-mediated actin polymerisation on endocytic vesicles to cancer cell invasion.

Results and Discussion

WASH localises to multiple endocytic compartments and can associate with the retromer recycling machinery (Derivery et al., 2009; Duleh and Welch, 2010; Gomez and Billadeau, 2009; Harbour et al., 2010). We hypothesised that WASH and Arp2/3-mediated actin assembly might mediate the retrieval of endocytic cargo and thus affect cell motility. Endogenous WASH colocalised

with the early endosomal markers EEA1, Rab4 and Rab5 in many cell types, including A2780 ovarian carcinoma cells, which use recycling of $\alpha 5\beta 1$ integrin to effect invasive motility (Fig. 1A,B; supplementary material Fig. S1A; data not shown). In addition, WASH was significantly associated with the multivesicular body/late endosome (MVB/LE) markers CD63 and Rab7 in both HeLa and A2780 cells (Fig. 1A,B and data not shown). WASH showed little overlap with the lysosomal sialic acid transporter sialin (Fig. 1A,B) or marker proteins for the Golgi complex (data not shown). Patches of short filamentous actin accompany fission and fusion of early Rab5-positive endosomes (Morel et al., 2009). Filamentous actin and the Arp2/3 complex colocalised with WASH on early endosomal compartments (supplementary material Fig. S1A–C and Movie 1). Approximately half ($51 \pm 13\%$) of large perinuclear EEA1-positive structures also contained Arp2/3 complex (supplementary material Fig. S1C). After WASH depletion, less than half the number of EEA1 endosomes colocalised with Arp2/3 ($14 \pm 1\%$ with siW1 and $24 \pm 4\%$ with siW4; siRNA1 targeting WASH and siRNA4 targeting WASH, respectively). Thus, WASH colocalises with EEA1- and Rab4-positive endosomes and also with CD63-positive MVB/LEs, where it recruits the Arp2/3 complex and mediates actin polymerisation.

WASH depletion was reported to have either a subtle or no effect on retention of transferrin (Derivery et al., 2009; Duleh and Welch, 2010), raising a question of whether specific cargo(s) depended on WASH for recycling or transport, or whether WASH played only a subtle supportive role for all cargoes through a particular route. We observed a small but significant increase in transferrin retention in HeLa, A2780 ovarian cancer and B16F10 mouse melanoma cells depleted of WASH

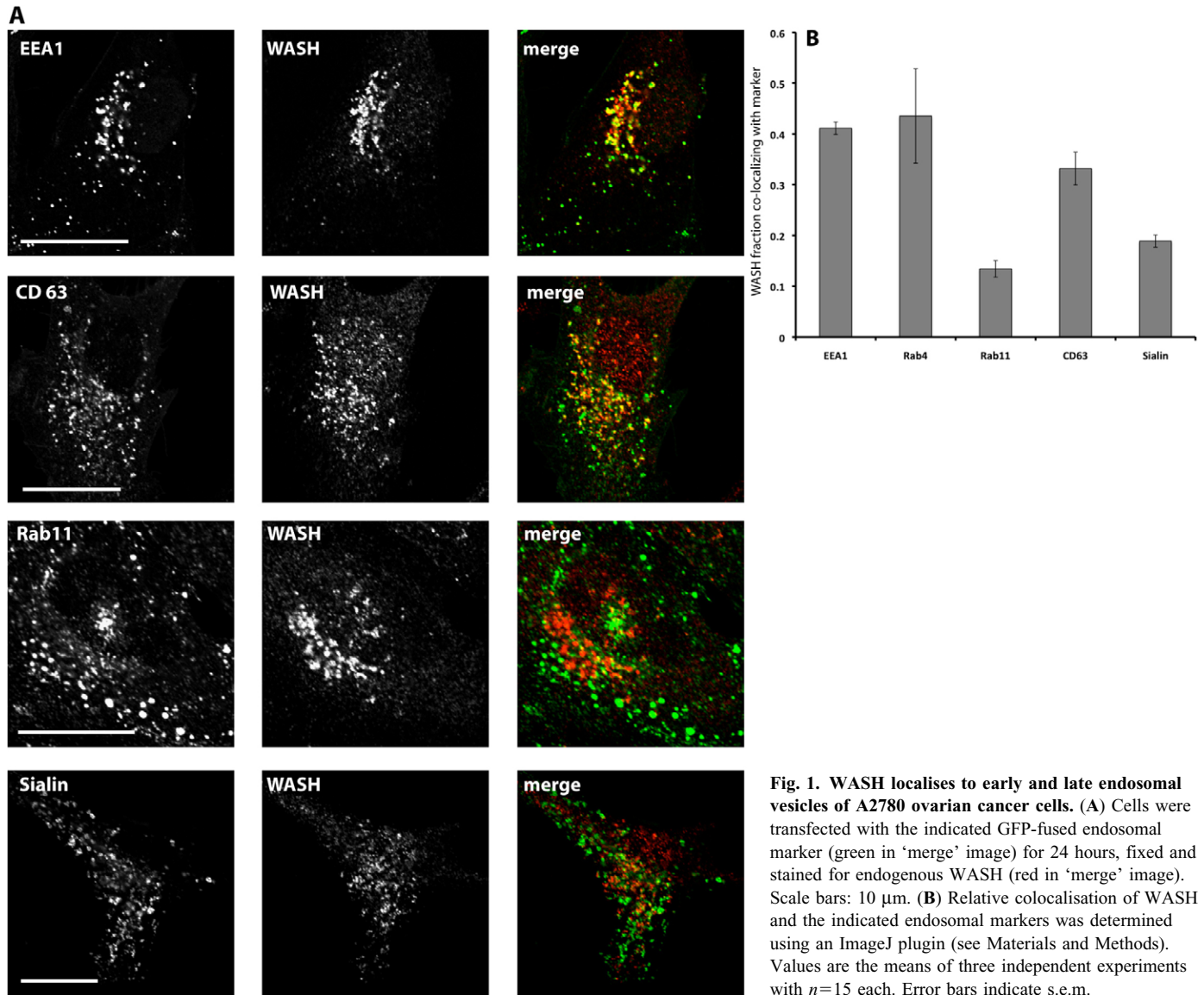


Fig. 1. WASH localises to early and late endosomal vesicles of A2780 ovarian cancer cells. (A) Cells were transfected with the indicated GFP-fused endosomal marker (green in 'merge' image) for 24 hours, fixed and stained for endogenous WASH (red in 'merge' image). Scale bars: 10 μ m. (B) Relative colocalisation of WASH and the indicated endosomal markers was determined using an ImageJ plugin (see Materials and Methods). Values are the means of three independent experiments with $n=15$ each. Error bars indicate s.e.m.

(supplementary material Fig. S1D–F), but no effect on uptake rates (data not shown). Furthermore, transferrin-containing vesicles colocalised with WASH and budded or tubulated from WASH-rich vesicles in cells where endogenous human WASH had been replaced by GFP–murine-WASH (GFP–mWASH; supplementary material Movies 2 and 3). Our studies suggest a partial role for WASH in transferrin receptor recycling from endosomes to the plasma membrane, in agreement with the findings of Derivery et al. (Derivery et al., 2009).

Integrin recycling is inhibited in WASH-depleted cells

The subtle nature of the effect of WASH depletion on transferrin receptor recycling might be due to cycling through sorting endosomes and the passive flow of the transferrin receptor through the endocytic system (Collinet et al., 2010; Maxfield and McGraw, 2004). Cargos that use a more selective route, such as integrins, could have a more dramatic requirement for WASH. We thus examined WASH function in $\alpha 5\beta 1$ integrin trafficking in

invasive ovarian (A2780) cancer cells, where trafficking is well characterised and regulates invasive motility (Caswell et al., 2007; Muller et al., 2009; White et al., 2007). Indeed, WASH puncta in A2780 ovarian cancer cells colocalised prominently with $\alpha 5\beta 1$ integrin (Fig. 2A) and WASH knockdown (WASH-kd) cells accumulated $\alpha 5\beta 1$ integrin in exaggerated perinuclear clusters (Fig. 2A), indicating that WASH might be required for trafficking of $\alpha 5\beta 1$ integrin. WASH depletion did not affect EEA1, Rab11 or sialin colocalisation with $\alpha 5$ integrin, but instead resulted in a significant increase in colocalisation of $\alpha 5\beta 1$ integrin with the MVB/LE tetraspanin CD63 (Fig. 2B,C; supplementary material Fig. S2). This suggests that WASH is important for $\alpha 5\beta 1$ integrin recycling and WASH depletion leads to accumulation of $\alpha 5\beta 1$ integrin in a pre-lysosomal MVB/LE compartment. This might seem somewhat unexpected, because the Rab11-positive compartment also is a major recycling compartment, but is consistent with at least one recent report that late endosomes and multivesicular bodies are involved in trafficking back to the plasma membrane (Tu et al., 2010).

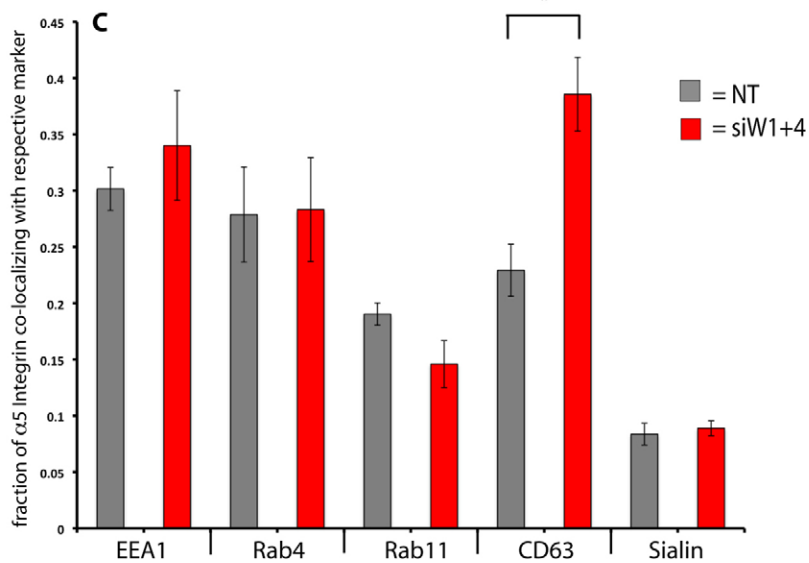
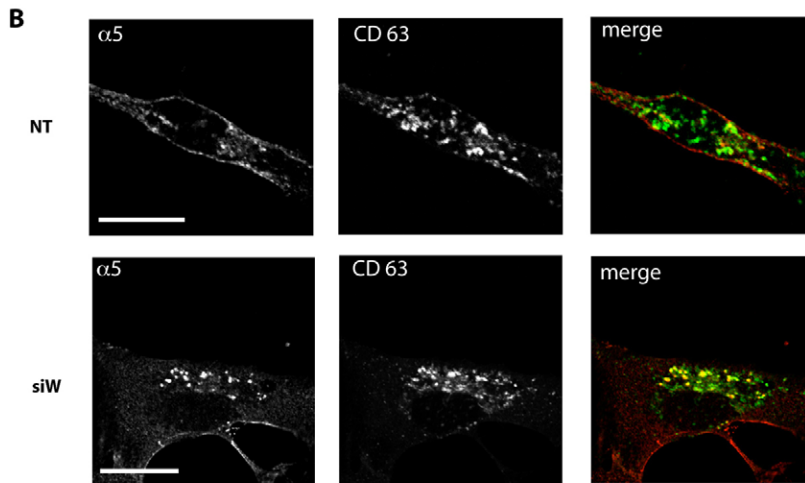
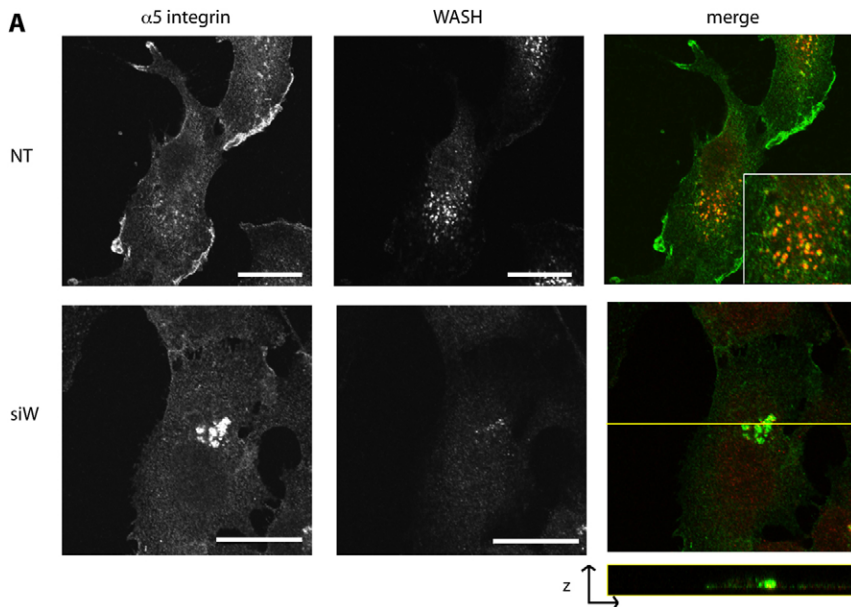


Fig. 2. WASH depletion in A2780 cells results in an increased association of $\alpha 5\beta 1$ integrin with MVB/LEs. (A) Nontargeting siRNA (NT) and WASH-kd A2780 cells were immunostained for $\alpha 5$ integrin and WASH. Images represent z-stacks of 19 slices of 0.13 μm ; $n=3$ experiments. Scale bars: 10 μm . (B) Control and WASH-kd cells with a pool of two oligonucleotides (siW1+4) were transfected with GFP-CD63 and stained for $\alpha 5$ integrin. Scale bars: 10 μm . (C) Endosomal distribution of $\alpha 5\beta 1$ integrin. Colocalisation was quantified using an ImageJ plugin (see Materials and Methods). * $P<0.05$. Error bars indicate s.e.m.

To directly visualize integrin colocalising with WASH recycling back to the plasma membrane, we rescued WASH-kd cells with mCherry–mWASH in conjunction with a photoactivatable (pa) GFP– $\alpha 5$ -integrin. We then photoactivated the integrin with a pulse of laser light (405 nm) at a ‘single point’ corresponding to a mCherry–WASH-positive vesicle (Fig. 3A). Fluorescence intensity was

rapidly lost from the photoactivated vesicle and accompanied by increased fluorescence intensity at an adjacent region of the plasma membrane (Fig. 3A,B; supplementary material Movie 4). Quantification of a number of movies indicated that the exit rate of photoactivated integrin from the WASH compartment was substantially slower when WASH-depleted cells were rescued

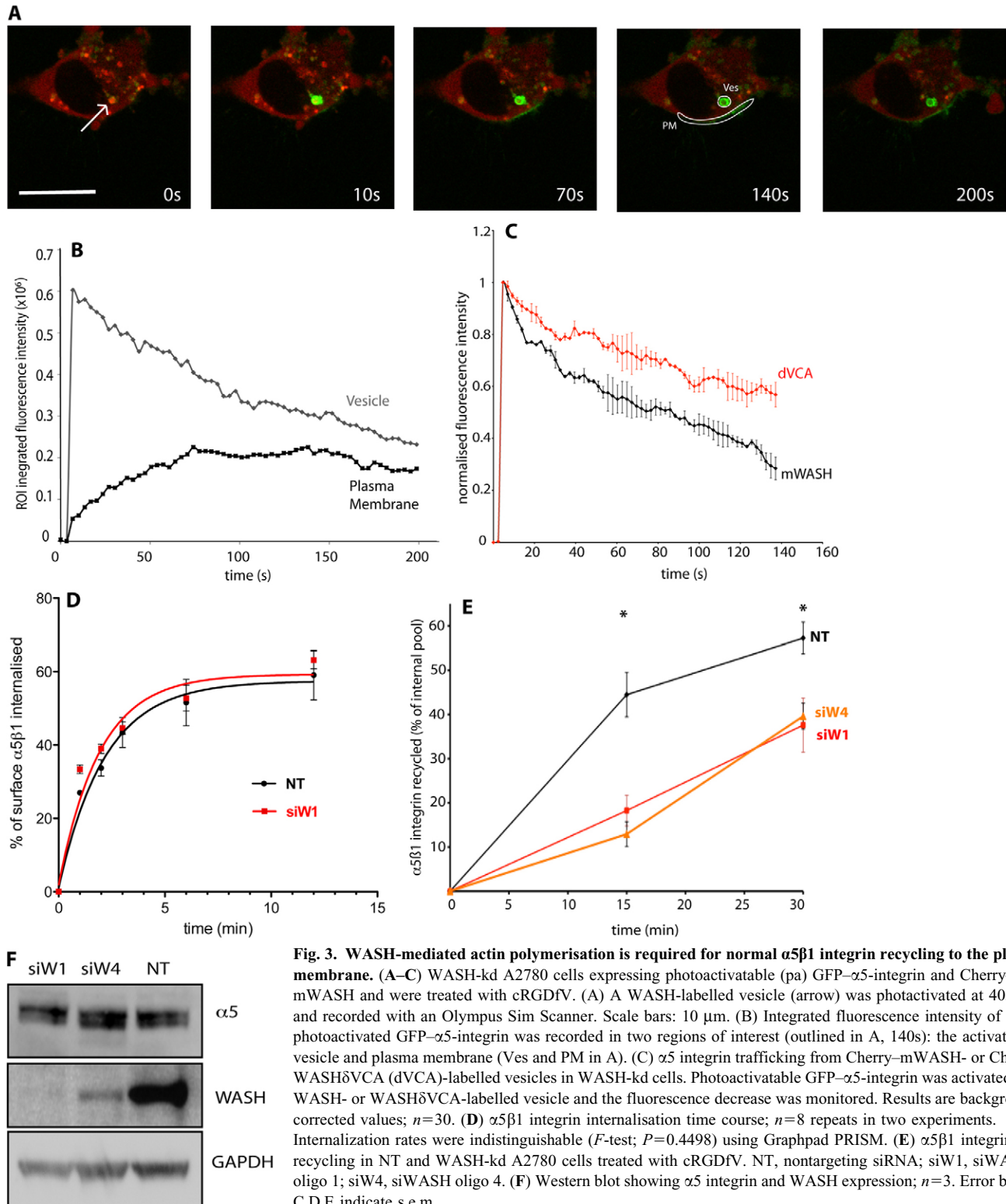


Fig. 3. WASH-mediated actin polymerisation is required for normal $\alpha 5\beta 1$ integrin recycling to the plasma membrane. (A–C) WASH-kd A2780 cells expressing photoactivatable (pa) GFP– $\alpha 5$ -integrin and Cherry–mWASH and were treated with cRGDFV. (A) A WASH-labelled vesicle (arrow) was photoactivated at 405 nm and recorded with an Olympus Sim Scanner. Scale bars: 10 μ m. (B) Integrated fluorescence intensity of photoactivated GFP– $\alpha 5$ -integrin was recorded in two regions of interest (outlined in A, 140s): the activated vesicle and plasma membrane (Ves and PM in A). (C) $\alpha 5$ integrin trafficking from Cherry–mWASH- or Cherry–WASH δ VCA (dVCA)-labelled vesicles in WASH-kd cells. Photoactivatable GFP– $\alpha 5$ -integrin was activated on a WASH- or WASH δ VCA-labelled vesicle and the fluorescence decrease was monitored. Results are background-corrected values; $n=30$. (D) $\alpha 5\beta 1$ integrin internalisation time course; $n=8$ repeats in two experiments. Internalization rates were indistinguishable (F -test; $P=0.4498$) using Graphpad PRISM. (E) $\alpha 5\beta 1$ integrin recycling in NT and WASH-kd A2780 cells treated with cRGDFV. NT, nontargeting siRNA; siW1, siWASH oligo 1; siW4, siWASH oligo 4. (F) Western blot showing $\alpha 5$ integrin and WASH expression; $n=3$. Error bars in C,D,E indicate s.e.m.

with mWASH lacking the Arp2/3-interacting 'VCA' (for verprolin, central, acidic) region (WASH δ VCA) than when normal mWASH was used (Fig. 3C). Thus, WASH-mediated actin polymerisation is directly implicated in integrin recycling. When a cell region devoid of WASH-positive structures was laser activated, little or no photoactivation resulted (although subsequent activation of a larger

region confirmed expression of paGFP- α 5-integrin) (data not shown), indicating that the photoactivation of paGFP- α 5-integrin shown in Fig. 3A was occurring at WASH vesicles and not at the plasma membrane above and below. Importantly, WASH δ VCA co-immunoprecipitated with endogenous WASH complex (supplementary material Fig. S5B).

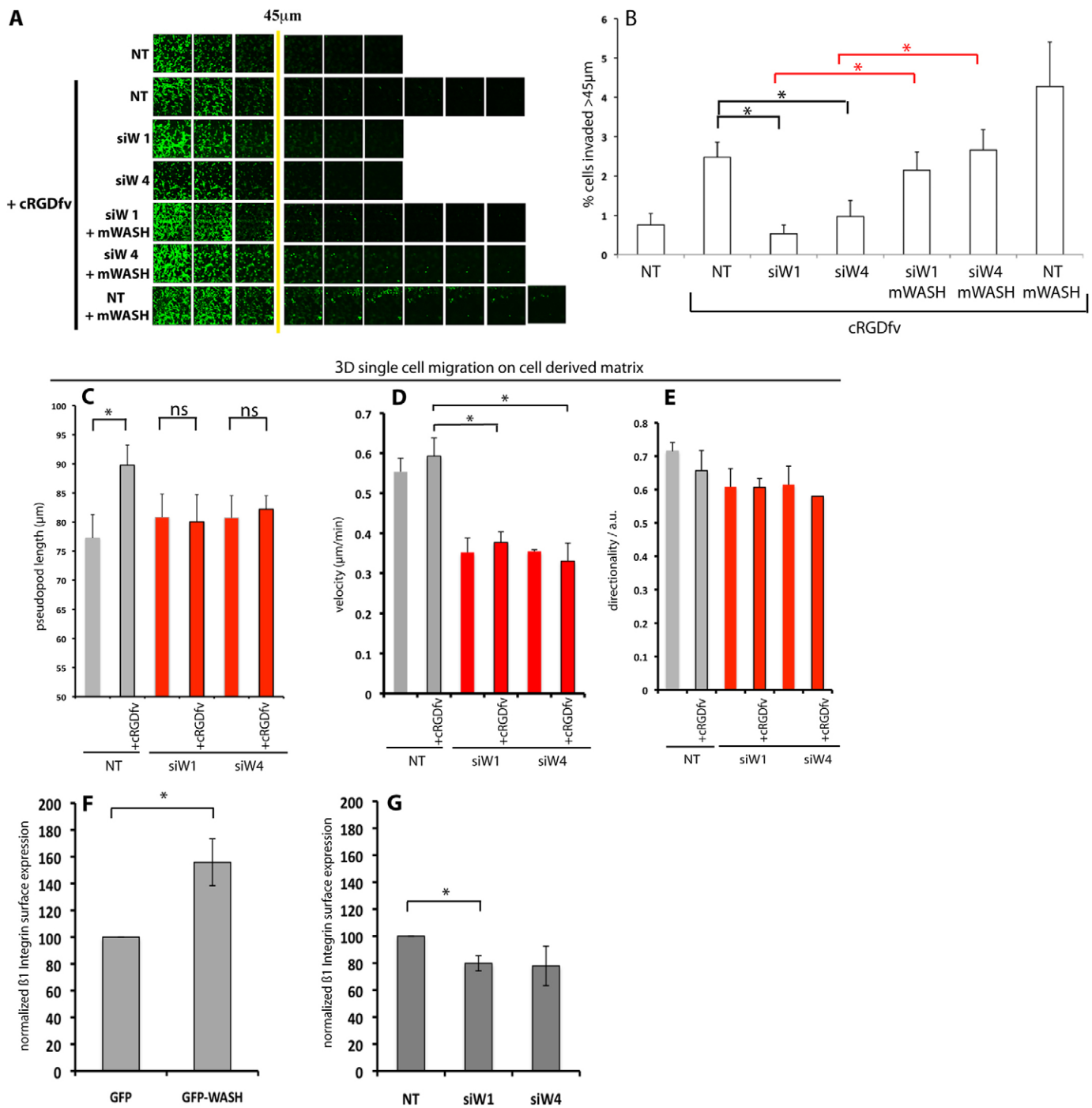


Fig. 4. WASH-depleted A2780 cells are severely impaired in integrin-mediated 3D invasion. (A,B) NT-, WASH-kd- and mWASH-expressing cells invaded Matrigel containing 100 µg/ml fibronectin, for 3 days. The cells that migrated beyond 45 µm were counted; $n \geq 3$ experiments, $*P < 0.05$. (C–E) NT and Wash-kd cells were plated on cell-derived matrices for 3 hours before imaging for 24 hours. Speed, directionality and pseudopodium length were calculated using ImageJ manual tracking and a chemotaxis plugin; $n \geq 3$ experiments each with ≥ 150 cells. NT, nontargeting siRNA; siW1, siWASH oligo 1; siW4, siWASH oligo 4. $*P < 0.05$; ns, not significant ($P > 0.05$). (F) A2780 cells expressing GFP-WASH or GFP were analysed by FACS for surface expression of β 1 integrin; $n = 3$. (G) β 1 integrin cell surface expression in A2780 cells with cRGDfv, measured by FACS; $n = 3$ (siW1 and NT); $n = 2$ (siW4). Error bars indicate s.e.m.

Endocytosis kinetics for $\alpha 5\beta 1$ integrin were similar in control and WASH-kd cells (Fig. 3D). However, after a chase, the rate of $\alpha 5\beta 1$ integrin recycling back to the surface was significantly reduced in WASH-kd cells (Fig. 3E). Importantly, $\alpha 5$ integrin expression levels were comparable in WASH-depleted cells and nontargeting siRNA (NT) controls ($90 \pm 25\%$ of control, $n=3$; Fig. 3F). Therefore, WASH-mediated actin assembly directly regulates integrin recycling back to the plasma membrane from internal endocytic vesicles. WASH could function mechanistically in either, or a combination, of the two following ways. In the first, normal transit of $\alpha 5\beta 1$ integrin from early endosomes to the ERC is blocked by loss of WASH, but the integrin continues to traffic on to the MVB/LE where it accumulates. In the second, recycling back to the plasma membrane by CD63-positive MVB/LE intermediates might be WASH dependent.

WASH is essential for integrin-mediated invasive migration in three-dimensional matrix

Integrin recycling is crucial for invasive migration. Inhibition of $\alpha v\beta 3$ integrin using either soluble ligands such as osteopontin, specific inhibitors such as cRGDFV, or expression of mutant p53 promotes invasion through a mechanism requiring $\alpha 5\beta 1$ integrin recycling (Caswell et al., 2008; Muller et al., 2009; White et al., 2007). WASH depletion strongly and specifically opposed cRGDFV-driven invasion of A2780 ovarian cancer cells, indicating that WASH-mediated $\alpha 5\beta 1$ integrin trafficking is an important part of the pro-invasive machinery (Fig. 4A,B). The invasion defect in Wash-kd cells was completely rescued upon expression of mWASH (Fig. 4A,B), whereas WASH overexpression increased $\alpha 5\beta 1$ integrin cell surface expression and invasion (Fig. 4A,B,F,G). WASH depletion did not, in most experiments, lead to a statistically significant reduction of the cRGDFV-driven increased $\alpha 5\beta 1$ integrin cell surface expression (compare Fig. 4G with supplementary material Fig. S3D) but we would only expect an $\sim 15\%$ decrease, which is near the limits of our signal-to-noise ratios. Invasive A2780 cells, with enhanced integrin recycling, extend long pseudopodial processes in the direction of migration (Caswell et al., 2008; Caswell et al., 2007). WASH function supports the extension of invasive pseudopodia in cRGDFV-driven invasion as evidenced by shorter pseudopodia in WASH-kd cells (Fig. 4C). WASH-kd also reduced the speed of migration on cell-derived matrix but had little effect on directionality (Fig. 4D,E). However, Wash-kd did not detectably alter two-dimensional (2D) migration or ruffling (supplementary material Fig. S3 and Movie 5). WASH knockdown also blocked invasion of MDA-MB-231 breast cancer cells, suggesting that mutant p53-driven invasion, which also depends on $\alpha 5\beta 1$ integrin recycling (Muller et al., 2009) requires WASH (supplementary material Fig. S4). Thus, WASH plays a specific role in 3D-migration mediated by trafficking of integrins, rather than directly influencing the actin polymerisation machinery involved in locomotion in both 2D and 3D contexts.

The endocytic system that contributes to regulated cell motility and cancer cell invasion is directly linked to dysregulated endocytic traffic (Lanzetti and Di Fiore, 2008). Cells invading in a 3D extracellular matrix are dependent on localised trafficking of receptors into their pseudopodia for advancement (Caswell et al., 2007). We propose that WASH specifically regulates endocytic recycling events, including delivery of integrins at the front of invasive cells, resulting in an integrin-dependent invasion defect in Wash-kd cells, but no effect on 2D motility. The role of

integrin recycling in invasive 3D migration is greater than for general random migration in 2D (Caswell et al., 2008; Caswell et al., 2007). Our data suggest that WASH might be a relevant target for metastasis in cancers and this is supported by a previous study showing upregulation of WASH in invasive breast cancer (Leirdal et al., 2004). In conclusion, we demonstrate, for the first time, a role for WASH in integrin recycling leading to invasive migration. This is to our knowledge the first functional demonstration that the role of WASH in endocytic recycling has strong biological consequences and suggests a wider role of WASH in receptor trafficking that will impact on motility and invasion. Our results also highlight the importance of actin assembly pathways and NPFs in regulation of invasive migration through recycling of receptors, rather than (or in addition to) their possible role in actin-based protrusion.

Materials and Methods

Materials

WASH polyclonal rabbit antibodies were made against CVNTKSEPEDEAEEG (aa145–160) and CMRRKGISGKGPSTG (aa424–439; Biogenes, Berlin, Germany). Antibodies were purified using a MicroLink peptide coupling kit from ThermoScientific (Rockford, IL, USA) and characterised by western blotting before use (supplementary material Fig. S5). Other antibodies are described in supplementary material Table S1. Human and mouse *WASH* cDNA clones (ImaGenes, Berlin, Germany), GFP- δ VCA human WASH and GFP-mWASH were made using the pEGFP-C1 vector (Clontech, Invitrogen, Paisley, UK). Human and mouse RNAi oligonucleotides were obtained from Qiagen (Crawley, UK) as follows: Human WASH siW1 (Hs_WASH1_1) 5'-CCGCCACAGGATCCAGAGCAA-3' and siW4 (Hs_FLJ00038_1) 5'-CAAGCTGGTCATGAGGCGCAA-3'. shRNA retroviral vectors were pSM2-V2HS_18085 for WASH and RHS4971 non-targeting shRNA control vector both from OPENBIOSystems (ThermoScientific, AL, USA). Lifecare expression constructs were a kind gift from Michael Sixt and Roland Wedlich-Soeldner (Riedl et al., 2008). Transferrin labelled with I¹²⁵ was obtained from Perkin Elmer. All other reagents were from Sigma (Dorset, UK) or Invitrogen (Paisley, UK).

Cell culture and transfection

Cells were grown and transfected as previously described (Li et al., 2010) or using the Amara technology program A-23 for A2780 cells. WASH-kd was assayed alongside other cytoskeletal proteins (supplementary material Fig. S5A). For stable transfection of MDA-MB-231 cells, retroviral pSM2 vectors were transfected by standard methods (Scott et al., 2004) using Phoenix cells. Invasion assays were performed and analysed as previously described (Li et al., 2010). For A2780 cells, 6 mg/ml Matrigel was supplemented with 40 μ g/ml fibronectin as previously described (Caswell et al., 2007).

Microscopy and FACS

Standard methods were used (Machesky and Hall, 1997) for immunofluorescence. Cells were imaged using an Olympus FV1000 (Leica, Milton Keynes, UK) or Nikon A1 and ImageJ software. Identical exposure times and processing were used in all experiments where comparisons were made. Image J software was used for quantification of colocalisation in experiments where the confocal images underwent two rounds of local contrast enhancement (image blurring, subtraction of the blurred image and subsequent contrast enhancement) and threshold adjustment. The number of pixels in the yellow channel was then expressed as a percentage of the red pixels in the merged image. Cells were detached in Gibco cell dissociation buffer for FACS, fixed in 4% PFA at 4°C and labelled with anti- $\beta 1$ -integrin and anti-mouse Alexa Fluor 594 antibodies. Cell-derived matrix was prepared and analysed as previously described (Bass et al., 2007; Cukierman et al., 2001).

Transferrin receptor recycling

Cells in which *WASH* had been knocked down by siRNA treatment on days 0, 2 and 4 of the experiment were plated at 1×10^4 – 4×10^4 cells/ml on day 4 and grown for 2 days before being serum-starved in Roswell Park Memorial Institute medium (RPMI) or DMEM for 30 minutes. After 30 minutes, 10 μ g/ml Alexa-Fluor-488–transferrin was added. After 60 minutes the cells were placed on ice, washed with ice-cold PBS twice, and 8 μ g/ml unlabelled transferrin in serum-free medium (SFM) was added for up to 120 minutes. Cells were lysed in duplicate, with 100 μ l lysis buffer with protease inhibitors. mWASH-rescued cells were transfected 24 hours before addition of transferrin, and treated as above. Alexa-Fluor-488–transferrin was determined by fluorimetry (using a fluorimeter from Photon Technology International, West Sussex, UK).

Experiments involving I^{125} -transferrin recycling were essentially carried out as described previously (Caswell et al., 2008). Cells were incubated in 2 ml ice-cold SFM and 10 mM HEPES containing $1 \mu\text{l}$ I^{125} -transferrin/ml (~300 ng transferrin/ml) for 60 minutes at 4°C. The cells were incubated with SFM and 10 mM HEPES at 37°C for 8 minutes to allow the transferrin to internalise. Cells were acid washed for 3 minutes at pH 3 and any remaining cell surface I^{125} -transferrin was competed away with SFM plus 4 $\mu\text{g/ml}$ unlabelled transferrin at 4°C for 3 minutes. Chase samples were incubated for the indicated time in SFM, 10 mM HEPES and 4 $\mu\text{g/ml}$ unlabelled transferrin at 37°C. All samples were lysed in 1 M NaOH. Data were analysed using SigmaPlot 11.0 (Systat Software Inc, Germany).

Integrin receptor trafficking assays

Integrin internalisation and recycling assays were carried out as described previously (Roberts et al., 2001), with the following modifications: A2780 cells were not serum-starved; receptor internalisation conditions for recycling were 30 minutes at 37°C; cRGDFv (160 nM) was added to the cells during the internalisation and recycling periods. To measure cell-surface expression of $\alpha 5\beta 1$ integrin, cells were surface labelled with 0.2 mg/ml NHS-biotin for 30 minutes at 4°C and lysed (Caswell et al., 2008).

Acknowledgements

We thank David Strachan and Kurt Anderson for developing the ImageJ plugins used to measure colocalisation, and Gabriela Kalna for help with statistical analysis.

Funding

This work was supported by Cancer Research UK core funding to L.M.M., R.H.I. and J.C.N.; and Medical Research Council UK senior non-clinical fellowships [grant numbers G117/569, G117/537 to L.M.M. and R.H.I.] Deposited in PMC for release after 6 months.

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.080986/-/DC1>

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