Phosphorylation of WAVE2 by MAP kinases regulates persistent cell migration and polarity

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Summary

The WAVE family of proteins has long been implicated in the stimulus-dependent generation of lamellipodia at the leading edge of migrating cells, with WAVE2 in particular implicated in the formation of peripheral ruffles and chemotactic migration. However, the lack of direct visualisation of cell migration in WAVE2 mutants or knockdowns has made defining the mechanisms of WAVE2 regulation during cell migration difficult. We have characterised three MAP kinase phosphorylation sites within WAVE2 and analysed fibroblast behaviour in a scratch-wound model following introduction of transgenes encoding phospho-defective WAVE2. The cells exhibited an increase in migration speed, a decrease in the persistence of migration, and disruption of polarisation of the Golgi apparatus. All these effects could be mimicked by acute knockdown of endogenous WAVE2 expression with RNAi, indicating that phosphorylation of WAVE2 by MAP kinases regulates cell polarity during migration.

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Introduction

The directional migration of eukaryotic cells drives a diverse array of fundamental biological processes, from colony formation in amoebae (Ibarra et al., 2005) to the morphogenesis of highly differentiated multicellular organisms (Dormann and Weijer, 2003). Furthermore, the migration of distinct cell types within a multicellular organism underpins the immune inflammatory response, neuronal connectivity and wound healing, enabling the organism to adapt to and survive in its environment (Ridley et al., 2003). In order to achieve the required levels of sensitivity and control, these processes are subject to exquisite levels of regulation. How cells integrate chemical and mechanical cues into coordinated migration has been the subject of intense study in recent years.

A common feature of migrating cells is the acquisition and maintenance of a polarised morphology, characterised by a broad, protrusive membrane (or lamella) at the leading edge and a narrow contractile tail at the rear. Protrusion at the leading edge is widely believed to be powered by the polymerisation of soluble actin monomers into insoluble filaments. Filamentous (F) actin is observed in several dynamic intracellular structures generated by the activity of various classes of actin nucleation-promoting factor (NPF) and subsequently is modulated by a gamut of accessory proteins (Pollard, 2007). Broad protrusive structures include the lamellipodium – a region 1-3 μm from the leading edge containing branched F-actin structures displaying rapid turnover and often folding back in the form of a ruffle, and the lamella – a region, rich in myosin II, of slower F-actin turnover 3-15 μm from the leading edge (Ponti et al., 2004). Members of the WASP/WAVE family of proteins are NPFs that act as hubs translating numerous signalling inputs into the production of branched F-actin arrays, by means of their ability to activate the F-actin-nucleating Arp2/3 complex. WASP and N-WASP can respond to signals in the form of GTP-bound Cdc42, phosphoinositides, kinases, phosphatases, Src-homology 3 (SH3) domains and other protein ligands (Bompard and Caron, 2004). Their NPF activity has been implicated in the production of the F-actin required for formation of filopodia, invadapodia and podosomes and also for some endocytic events (Dawson et al., 2006).

There are three WAVE isoforms (1, 2 and 3) in mammals. WAVE2 is reported to be required for the formation of lamellipodia and for cell migration downstream of the Rho-family GTPase Rac1 (Miki et al., 1998; Yan et al., 2003; Yamazaki et al., 2003; Kurisu et al., 2005; Kheir et al., 2005; Kawamura et al., 2004). As WAVE2 has no Rac1-binding motif, the molecular linkage between cause and effector remains unclear. WAVE proteins exist in a multimeric complex with NAP, PIR, HSC300, Abi1 and Abi2 (Gautreau et al., 2004; Eden et al., 2002). Three models have emerged to explain the mechanism of regulation of WAVE2 by Rac; the first posits that Rac1 binding to PIR relieves the inhibitory effects of the complex on WAVE activity (Eden et al., 2002), the second that WAVE activity is not directly regulated by the members of the complex but that Rac binding to PIR concentrates WAVE at the membrane of the leading edge (Innocenti et al., 2004), and the third that IRSp53 mediates Rac1 signals independently of other members of the complex through binding to a poly-proline rich region in WAVE2 (Suetsugu et al., 2006).

A commonly used assay to probe aspects of cell migration and polarity involves the generation of an artificial 'scratch' wound in a confluent monolayer of cultured fibroblasts. Cells

at the scratch edge extend lamellipodial protrusions into the void and migrate directionally to 'heal' the wound. An early event in this process is the polarisation of the cell such that the microtubule organising centre (MTOC) and Golgi apparatus are localised between the nucleus and the leading edge of the cell (Magdalena et al., 2003a; Nobes and Hall, 1999). This is achieved by a combination of capture of microtubule plus-ends at the leading edge to maintain MTOC position (Gundersen et al., 2004) and acto-myosin-driven rearward movement of the nucleus (Gomes et al., 2005). Thus, microtubules and the actin cytoskeleton collaborate to dictate multiple features of cell migration.

In addition to its role downstream of Rac1, the use of dominant-negative constructs has implicated WAVE2 in the polarisation of the Golgi apparatus following scratch wounding of fibroblasts (Magdalena et al., 2003a). As this is a Rac1-independent process (Nobes and Hall, 1999), the signalling pathways that converge on WAVE2 to regulate polarisation are of great interest. Recent work has demonstrated that, like WASP, WAVE proteins are subject to regulation by phosphorylation (Ardern et al., 2006; Stuart et al., 2006; Leng et al., 2005). Here, we identify MAP kinase target residues in WAVE2 that are phosphorylated in response to growth factors, serum stimulation and scratch wounding of fibroblasts. In our efforts to determine the cellular requirement of these phosphorylations, we have demonstrated that WAVE2 regulates the parameters of cell speed, persistence and direction of migration and cell polarity in a phospho-specific manner downstream of MAP kinases.

Results

Erk and Jnk phosphorylate WAVE2 at distinct sites

A previous study identified that an undetermined WAVE isoform was subject to Erk-dependent phosphorylation following growth factor stimulation and that this resulted in a mobility shift of the WAVE protein revealed by SDS-PAGE (Miki et al., 1999). In order to identify the WAVE isoform and the target sites, we characterised this mobility shift in detail. We observed that WAVE2, but not WAVE1, underwent a mobility shift upon SDS-PAGE in response to 10% serum (Fig. 1A) or platelet-derived growth factor (PDGF) stimulation of NIH 3T3 fibroblasts (Fig. 2C). We could not detect WAVE3 expression in this cell line when lysates were analysed next to a recombinant protein control (data not shown). The shift in WAVE2 mobility was inhibited by the Erk pathway inhibitor

Uo126 (Fig. 1A) and was also observed in murine embryonic fibroblasts and C2C12 muscle myoblasts (data not shown). The shift in mobility of WAVE2 was caused by phosphorylation as it was completely reversed by treatment with alkaline phosphatase (Fig. 1C). Use of WAVE2 truncation mutants (Fig. 1B) shows that several shifted species are observed between the Scar-homology domain (SHD) and the VCA domain, in a region we have termed W2-ΔSHD/ΔVCA. The presence of several species indicates that multiple phosphorylation events occur in this region (Fig. 1C). Analysis of the SHD showed no shift caused by phosphorylation, whereas the VCA domain did display an increase in mobility following dephosphorylation
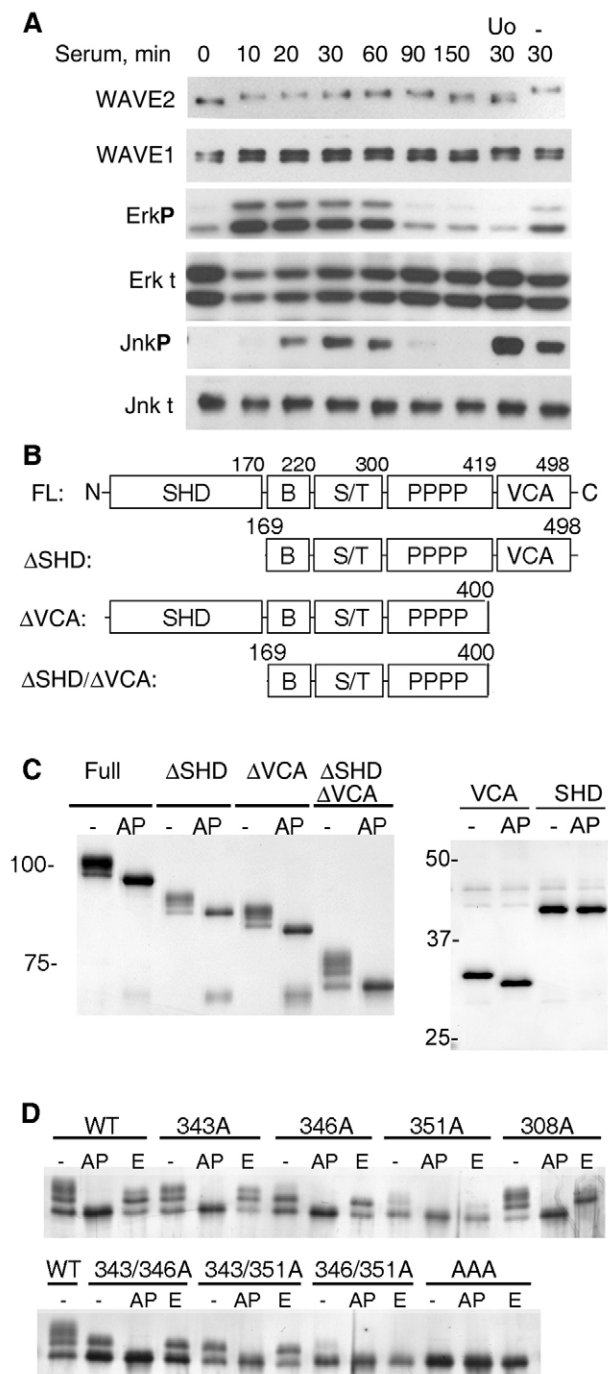
Fig. 1. WAVE2 undergoes a mobility shift assessed by SDS-PAGE. (A) NIH 3T3 fibroblasts were serum starved and stimulated with 10% serum for the indicated times. Cells were incubated with 10 μM Uo126 or DMSO control where indicated. Lysates were immunoblotted for the indicated protein species. ErkP, phospho-Erk; Erk t, total Erk. (B) Domain structure of WAVE2 and truncation constructs. (C) GST-WAVE2 constructs were purified from Cos-7 cells growing in 10% serum and treated with alkaline phosphatase (AP) where indicated before resolution by SDS-PAGE and silver staining. (D) GST-ΔSHD/ΔVCA constructs containing the indicated alanine (A) mutations were purified and treated as above. 308A is included as an example of a Ser-to-Ala mutation that does not affect the mobility of WAVE2. Note the disappearance of faint upper species in the 343A mutant. An aliquot of each phosphatase-treated sample was incubated with recombinant Erk2 (E). AAA, 343A–346A–351A triple mutant.
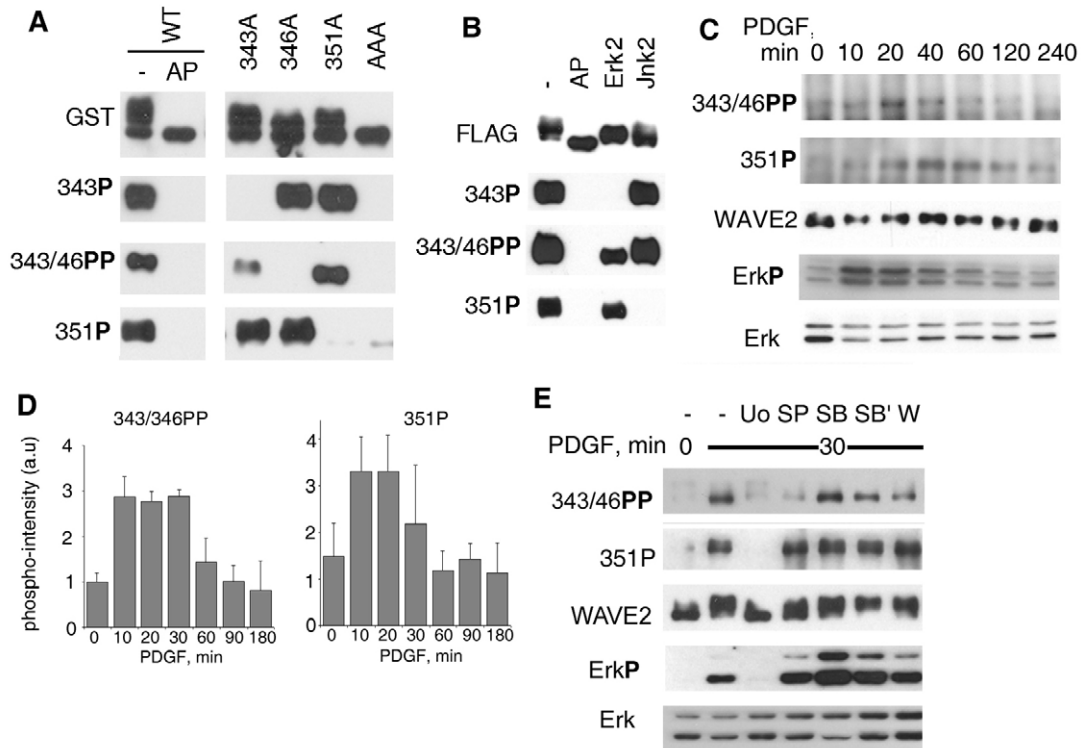
Fig. 2. Phosphorylation of WAVE2 by Erk and Jnk. (A) GST-ΔSHD/ΔVCA constructs were purified and treated as described in Fig. 1 before immunoblotting with antibodies raised against the indicated phospho-peptides. WT, wild type. (B) FLAG-tagged full-length WAVE2 was expressed in, and immunoprecipitated from, Cos-7 cells and either untreated (–), phosphatase treated (AP) or phosphatase treated and then incubated with recombinant Erk2 or Jnk2 as indicated. Samples were analysed by immuno-blotting with the indicated antibodies. (C) Serum-starved NIH 3T3 fibroblasts were stimulated with PDGF-ββ for the indicated times, before lysis. Immunoprecipitated WAVE2 was immunoblotted as indicated. Lysates were immunoblotted with antibodies against phospho-Erk (ErkP) to monitor activation of the Erk pathway. (D) Serum-starved NIH 3T3 cells were stimulated with PDGF-ββ for the indicated times, lysed and WAVE2 phosphorylation monitored by phospho-specific immunoblotting. Densitometry of phospho-WAVE2 signals compared to total WAVE2 signal after stripping was averaged for three separate experiments. (E) NIH 3T3 cells were preincubated with the indicated inhibitors [Uo126 at 10 μM (Uo), SP600125 at 10 μM (SP), SB202190 at 10 μM (SB), SB203580 at 10 μM (SB′), wortmannin at 100 nM (W)] before stimulation with PDGF for 30 minutes, lysis and immunoprecipitation of WAVE2. Lysates were immunoblotted with antibodies against ErkP to assess inhibition of this pathway. The blots shown are representative of numerous experiments.

(Fig. 1C). Contrary to recent findings (Nakashini et al., 2007), further analysis of this phosphorylation event has shown that it is not mediated by mitogen-activated protein (MAP) kinases and is not induced downstream of growth factor stimulation (C.M.D. et al., unpublished). We therefore concentrated our efforts to determine the specific residues responsible for the mobility shifts within the ΔSHD/ΔVCA region, and performed scanning mutagenesis on all serine and threonine residues in this part of the protein. We identified three residues, Ser343, Thr346 and Ser351, that, when mutated, altered the shifting pattern. Mutation of all three residues to alanine prevented any detectable mobility shift of the W2-ΔSHD/ΔVCA protein (Fig. 1D and data not shown).

Phosphorylation of WAVE2 in vivo

During the preparation of this manuscript, another report also identified Thr346 and Ser351 as Erk substrates in vitro (Nakanishi et al., 2007), but this did not characterise phosphorylation of endogenous WAVE2 or establish a cellular function for these modifications. To characterise WAVE2 phosphorylation further, we raised phospho-specific antibodies

against the three sites individually. Owing to the close proximity of Ser343 and Thr346, an antibody was also raised against a doubly phosphorylated peptide corresponding to dual occupation of these sites. The p343, p351 and p343p346 antibodies specifically recognised the corresponding phospho-WAVE2 states on GST-W2-ΔSHD/ΔVCA isolated from Cos-7 cells, as demonstrated by a loss of signal following WAVE2 dephosphorylation or mutation (Fig. 2A). The antibody raised against p346 was not functional, but the p343p346 antibody demonstrated an absolute requirement for phosphorylation at Thr346 and a partial requirement for phosphorylation at Ser343 (Fig. 2A). Together, these results show that Ser343, Thr346 and Ser351 are true cellular phosphorylation targets.

Ser343, Thr346 and Ser351 all fit the simplest consensus sequence for MAP kinase phosphorylation sites (S/T, P) (Johnson and Lapadat, 2002) and both Erk and Jnk are activated following serum stimulation. To determine whether these sites were Erk or Jnk targets, recombinant WAVE2 was isolated from Cos-7 cells and dephosphorylated with alkaline phosphatase before performing in vitro kinase assays. Treatment with recombinant Jnk or Erk revealed the site

specificity of these kinases, namely that Ser343 is a substrate for Jnk and not Erk, Ser351 is a substrate for Erk and not Jnk, whereas Thr346 is a substrate for both kinases (Fig. 2B).

We next characterised the phosphorylation of endogenous WAVE2 protein. PDGF stimulation of serum-starved NIH 3T3 fibroblasts caused phosphorylation at sites Thr346 and Ser351 (Fig. 2C). Phosphorylation of Thr346 and Ser351 peaked 10-30 minutes after stimulation (Fig. 2D). We were unable to detect phosphorylation of Ser343 in the endogenous protein with the phospho-specific antibody p343 (data not shown), suggesting that the signal generated by the p343p346 antibody was mainly caused by phosphorylation at Thr346. Inhibition of the Erk pathway abolished stimulus-dependent phosphorylation of Ser351, whereas inhibitors of the Erk or Jnk pathways prevented phosphorylation of Thr346 (Fig. 2E). These results tally with the in vitro data and suggest that both Erk and Jnk pathways are required for the optimal phosphorylation of Thr346, whereas Ser351 is a specific target of Erk activity.

The function of WAVE2 phosphorylation

We assessed the effect of phosphorylation of these three sites on the ability of WAVE2 to polymerise actin in vitro in the presence of purified Arp2/3 complex (Welch and Mitchison, 1998). FLAG-WAVE2-WT or FLAG-WAVE2 bearing single phospho-site mutations or a triple Ser343Ala, Thr346Ala, Ser351Ala mutation (AAA) were purified from Cos-7 cells and subsequently treated with Erk and Jnk kinases. No differences in activity were seen between wild-type and mutant proteins (supplementary material Fig. S1), suggesting that phosphorylation of WAVE2 on these sites does not impinge on the inherent ability of WAVE2 to stimulate the activity of the Arp2/3 complex.

Another reported function of WAVE2 is in the formation of lamellipodia (Yan et al., 2003; Kheir et al., 2005). We investigated the effect of introducing wild-type and phospho-defective transgenes on the ability of fibroblasts to ruffle in response to PDGF. Populations of NIH 3T3 cells stably expressing either EGFP, EGFP-tagged wild-type (WT) WAVE2
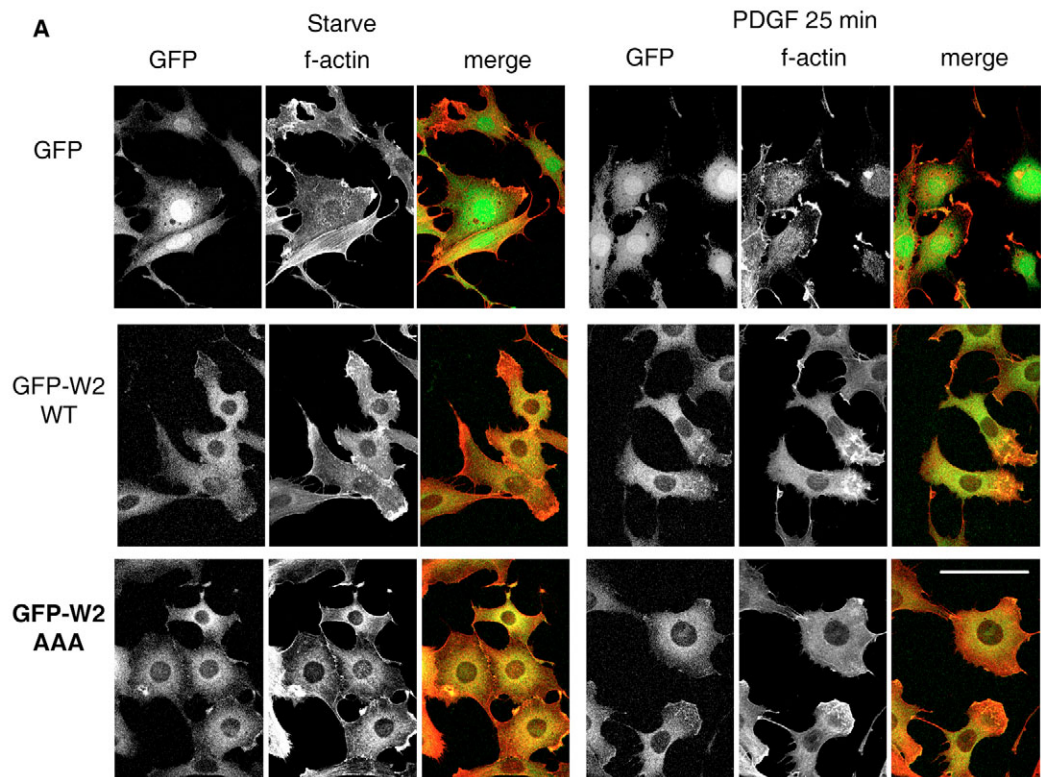
Fig. 3. Phospho-defective WAVE2 expression does not affect ruffling in response to PDGF. (A) NIH 3T3 cells stably expressing EGFP, EGFP-WAVE2-wild-type (W2 WT) or EGFP-WAVE2-AAA (W2 AAA) were plated on fibronectin-coated glass coverslips overnight in 0.5% serum. PDGF was added where indicated for 25 minutes before cells were fixed, permeabilised and stained with phalloidin (F-actin). Bar, 30 μm. (B) Experiments were performed as for A, then six random microscopic fields were imaged and the number of cells with one or more ruffles were counted. Dark bars (+) represent cells with ruffles, and light-grey bars (−) represent those without ruffles. Statistical analysis with Fisher's exact test revealed no significant differences between cell lines in either starved or stimulated conditions.
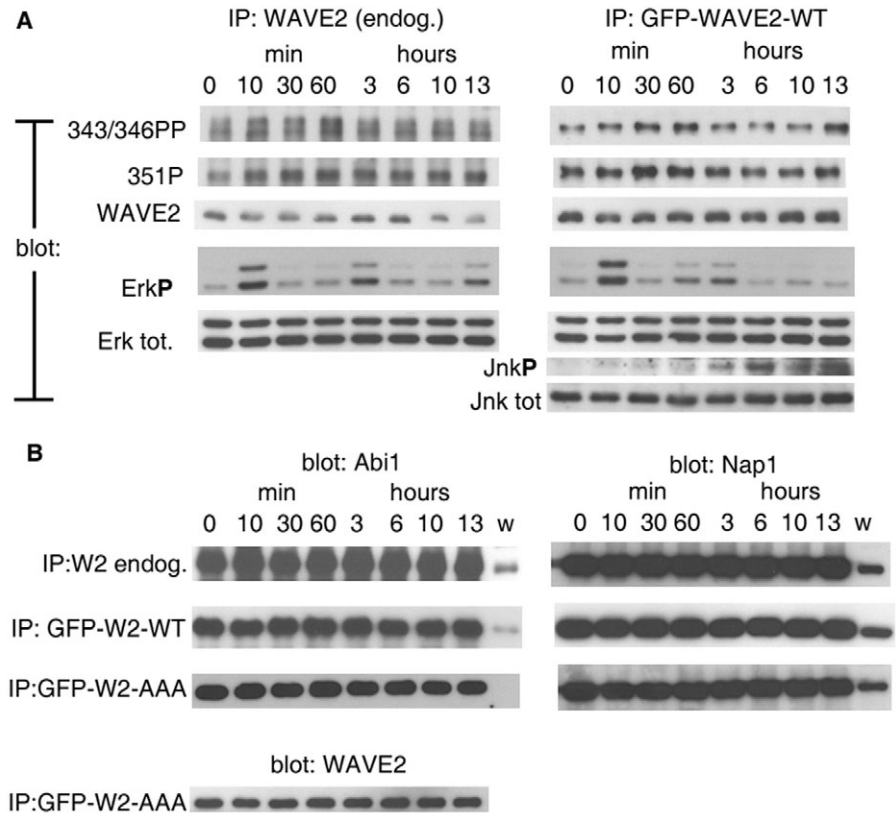
or a phospho-defective WAVE2 mutant (AAA) were established following lentiviral infection of the appropriate transgene followed by fluorescence-activated cell sorting (FACS; data not shown). Low-level expression of WAVE2 was obtained (see below), and introduction of neither wild-type nor AAA WAVE2 affected the ability of cells to ruffle in 0.5% serum-starved conditions or following treatment with PDGF (Fig. 3). In addition, knockdown of endogenous WAVE2 did not affect ruffling in this assay, with both dorsal and peripheral ruffling observed after both 7.5 minutes (data not shown) and 25 minutes (supplementary material Fig. S2), suggesting that other pathways are available to the cells in these conditions. It is possible that sufficient WAVE2 remains following knockdown to effect ruffle formation or that WAVE1 can compensate in NIH 3T3 cells.

We could see no obvious difference in the subcellular localisation of WAVE2-WT or the AAA mutant protein either before or after PDGF stimulation (Fig. 3; supplementary material Fig. S3). The low level of expression of GFP-WAVE2 is essential to prevent the widespread disruption of actin dynamics seen at high levels of expression (Hahne et al., 2001), but this prevents the obtaining of high-resolution fluorescence images to investigate subcellular localisation in live-imaging studies. Unfortunately, the phospho-specific antibodies generated in this study do not stain specifically in immunofluorescence studies (data not shown).


Fig. 4. WAVE2 phosphorylation following scratch-wounding. (A) Native NIH 3T3 cells (left panels) or NIH 3T3 cells expressing EGFP-WAVE2-wild-type (right panels) were grown to confluence and scratched. Cultures were left for the indicated times before lysis and immunoprecipitation of endogenous WAVE2 or EGFP-WAVE2 with the indicated antibodies. Immunoprecipitates were blotted as shown. Lysates were taken and blotted for phospho-Erk and phospho-Jnk. (B) In addition to the immunoprecipitates shown in A, NIH 3T3 cells expressing EGFP-WAVE2-AAA were also wounded. Immunoprecipitates were resolved next to a sample of lysate (w) and immunoblotted as indicated.

Phosphorylation of WAVE2 regulates migration speed and directional persistence of fibroblasts

We next measured the effect of phosphorylation of WAVE2 on cell migration. We used scratch-wounding of confluent NIH 3T3 monolayers to induce a robust, directional migration response. First, we monitored WAVE2 phosphorylation following scratch-wounding, and observed similar kinetics of phosphorylation of residues 343/346 and 351 compared with the kinetics observed under serum/PDGF stimulation, but with levels peaking slightly later at >60 minutes (Fig. 4A). Interestingly, the levels of phospho-WAVE were seen to rise again after 13 hours of wounding, which corresponds to the time of initial contact between the two edges of the wound in this assay.

Stimulation of Erk and Jnk activity was also induced by scratch wounding, and interestingly Erk activation largely preceded WAVE2 phosphorylation, whereas Jnk activation peaked at 6 hours. Erk activation appeared to be cyclical, with peaks in activity detected at 10 minutes and 3 hours post wounding. The differences in the temporal phosphorylation profile of MAP kinases and WAVE2 suggest the possibility that phosphorylation of WAVE2 is mediated by small subpopulations of MAP kinases, whose activation is not detectable by phospho-western analysis.

Transgenic WAVE2 displayed kinetics of phosphorylation similar to those of endogenous WAVE2 (Fig. 4A), indicating that the stable cell lines are a suitable system for studying the effects of WAVE2 phosphorylation. In order to determine whether phosphorylation of WAVE2 affected its association with other members of the WAVE complex, we immunoblotted WAVE2 immunoprecipitates for NAP1 and Abi1 during a wound-healing response. No difference in association with these members of the complex could be seen either during the time course or when a phospho-defective WAVE2-AAA protein was examined (Fig. 4B).

To analyse cell behaviour during migration, we quantified cell speed and the persistence of direction [namely, the straight-line distance (D) between start and finish points divided by the total (T) distance travelled by the cell: D/T] of transgenic cell lines scratch-wounded in parallel and filmed over a period of 16 hours (Fig. 5A and supplementary material Movies 1-3). Cells expressing the phospho-defective WAVE2-AAA mutant
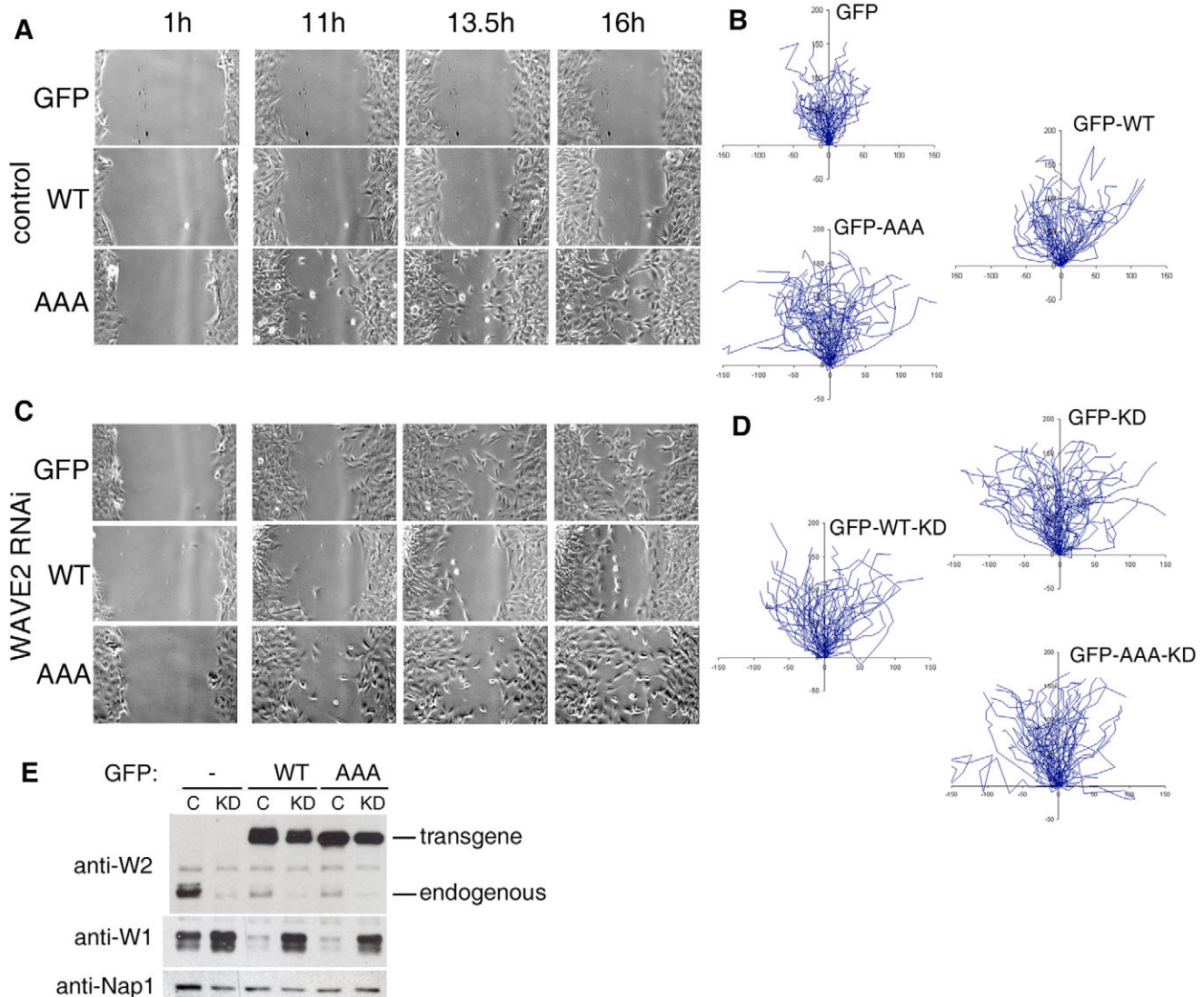
Fig. 5. Phosphorylation of WAVE2 is required to maintain the directional persistence of cell migration. (A) NIH 3T3 cells stably expressing EGFP, EGFP-WAVE2-wild-type (WT) or EGFP-WAVE2-AAA (AAA) were treated with control siRNA (C) and grown to confluence in a chamber slide. Monolayers were scratch-wounded and cell migration was filmed using time-lapse microscopy. Frames were taken from a representative movie (see supplementary material Movies 1-3 for originals) showing migration at the indicated time points. (B) Cells were tracked every 30 minutes from 8-16 hours post-wounding and their paths overlayed. Paths are oriented such that the start point is normalised to the origin and the wound face runs parallel to the *x*-axis. All tracks from several fields in this representative experiment are shown. (C,D) Experiment performed as for A and B except that cells were treated with WAVE2 siRNA 48 hours before wounding (see supplementary material Movies 4-6 for originals). (E) Cells from the experiment described in panels A-D were plated in parallel and lysates immunoblotted as indicated.

moved faster than those expressing WAVE2-WT or controls and exhibited spastic cell movement with several changes in direction. This can be visualised by superimposing the paths of wound-edge cells tracked in several fields within this experiment (Fig. 5B). Quantitation of this phenotype revealed that WT-expressing cells displayed a slight, but insignificant, increase in their relative speed (1.09-fold the control value; *P*>0.05; see Table 1 for detailed quantitation and statistical significance of all cell migration data) but migrated with a persistence similar to that of control cells (1.02-fold the control value; *P*>0.05). However, cells expressing the phospho-defective WAVE2 mutant displayed a marked increase in relative speed that was 1.37-fold the control levels (*P*<0.001)

and a significant decrease in persistence that was 0.83 times that of the control levels (*P*<0.001), indicating that phosphorylation of WAVE2 mediates persistent directionality following stimulation of cell migration. Closer examination of protrusions during the first 3 hours of wound-healing (a time-course that covers the major phosphorylation profile of WAVE2) shows no obvious differences in membrane dynamics between control-, wild-type- or mutant-expressing cells (see supplementary material Movies 7-9), suggesting that WAVE2 phosphorylation does not directly affect formation of lamellae/lamellipodia in response to wounding. It is possible that subtle differences are present that are not distinguishable by phase-contrast microscopy.

Table 1. Quantitation and statistical significance of cell migration data

	Transgene product	mean RPers.	±s.e.m.	Newman-Kuels P value versus control	Newman-Kuels P value between conditions		
Persistence							
a	GFP	1.00	0.02	–	–		
b	GFP-W2-WT	1.02	0.02	>0.05	<0.001 (b,c)		
c	GFP-W2-AAA	0.83	0.02	<0.001			
d	GFP (cont)	1.00	0.02	–	–		
e	GFP (KD)	0.93	0.02	<0.01	<0.001 (e,f)		P>0.05 (e,g)
f	GFP-W2-WT (KD)	1.03	0.02	>0.05		P<0.001 (f,g)	
g	GFP-W2-AAA (KD)	0.90	0.02	<0.001			

	Transgene product	mean Rspeed	±s.e.m.	Newman-Kuels P value versus control	Newman-Kuels P value between conditions		
Speed							
a	GFP	1.00	0.03	–	–		
b	GFP-W2-WT	1.09	0.03	>0.05	<0.001 (b,c)		
c	GFP-W2-AAA	1.37	0.04	<0.001			
d	GFP (cont)	1.00	0.03	–	–		
e	GFP (KD)	1.51	0.04	<0.001	<0.01 (e,f)		>0.05 (e,g)
f	GFP-W2-WT (KD)	1.34	0.04	<0.001		>0.05 (f,g)	
g	GFP-W2-AAA (KD)	1.41	0.04	<0.001			

All cell lines/knockdown conditions were run concurrently in separate wells of a chamber slide. Cont, control siRNA; KD, WAVE2 siRNA. For each experiment, the persistence values of the control (EGFP-transfected cells treated with control siRNA–a is the control for b and c; d is the control for e-g) were averaged and the relative persistence (RPers.) of each tracked cell calculated by dividing its persistence value by the control mean. This controlled for variations in absolute persistence between experiments and enabled us to pool data from three separate experiments. A similar approach was used to determine relative speeds (Rspeed). P values are derived from ANOVA with Newman-Kuels pos*t*-tests to determine the significance of differences between conditions (shown in parentheses). See experimental procedures for details of statistical analysis. n=109 (a-c); n=134 (d-g).

RNAi-mediated knockdown of endogenous WAVE2 (Fig. 5E) yielded a similar phenotype to that caused by overexpression of WAVE2-AAA, specifically a 1.51-fold increase in cell speed (P<0.001) and decrease in persistence to 0.93 (P<0.05) times that of control values (Fig. 5C,D; Table 1; supplementary material Movie 4). These findings suggest that WAVE2 is not required for cell migration, as has been suggested by others (Yan et al., 2003), but is required to stabilise the direction of migration, and that overexpression of WAVE2-AAA seems to exert dominant-negative effects on this aspect of cell behaviour. We noted that introduction of transgenes encoding wild-type or phospho-mutant WAVE2 led to a dramatic decrease in the expression levels of endogenous WAVE2 and also WAVE1 (Fig. 5E). This might reflect the ability of the transgene to compete for other members of the WAVE complex (such as Abi1 and Nap1), leading to displacement and degradation of endogenous WAVE proteins (Kunda et al., 2003). This expression pattern might explain the generation of a strong, apparently dominant-negative, phenotype by the WAVE2-AAA mutant. As both GFP-expressing cells (which have normal WAVE1 levels) and WAVE2-WT-expressing cells (which have low WAVE1 levels) have similar properties, it appears that WAVE1 is not required for normal persistence/speed of migration.

As the transgene encoding WAVE2 was of human origin and largely resistant to the RNA oligomer used for knockdown (Fig. 5E), we were able to test the ability of the WAVE2 constructs to reconstitute normal migration following depletion of endogenous protein. WAVE2-WT rescued the RNAi-induced decrease in migration persistence (to 1.02-fold the control value), whereas WAVE2-AAA did not (0.90-fold the control value, P<0.001) (Fig. 5C,D; Table 1; supplementary material Movies 5, 6). These findings support the idea that WAVE2 phosphorylation regulates persistent fibroblast migration. In addition, despite the levels of Nap1 and Abi1 being reduced slightly following WAVE2 knockdown (presumably owing to reduction of WAVE2, leaving uncomplexed Nap1 and Abi susceptible to degradation), Nap1 and Abi1 were restored to control levels following knockdown in the presence of either WT or AAA WAVE2 (supplementary material Figs S2, S4). Furthermore, knockdown of endogenous WAVE2 led to restoration of WAVE1 levels to close to those of controls (Fig. 5E and supplementary material Fig. S4), suggesting that the significant differences in persistence observed between cells expressing WT and AAA-WAVE2 were specifically due to the phosphorylation state of WAVE2.

Neither WAVE2-WT nor AAA could rescue the increase in migration speed caused by RNAi-induced knockdown, suggesting that cell speed is very sensitive to changes in the composition of WAVE protein complexes within the cell generated by RNAi treatment.

Phosphorylation of WAVE2 regulates polarisation of the Golgi apparatus

Following scratch-wounding, fibroblasts polarise their Golgi apparatus to lie between the nucleus and the leading edge of the cell in an actin-dependent process (Gundersen et al., 2004; Magdalena et al., 2003a; Magdalena et al., 2003b; Nobes and Hall, 1999). This might facilitate membrane traffic to the leading edge and turnover of adhesions (Rodriguez et al., 2003; Prigozhina and Waterman-Storer, 2004; Bershadsky and Futerman, 1994). Previous reports have shown that overexpressing dominant-negative WAVE2 constructs impairs this polarisation response (Magdalena et al., 2003a). We therefore wanted to investigate the possibility that the function of WAVE2 in polarisation of the Golgi apparatus is regulated by phosphorylation. First, we confirmed the importance of WAVE2 in regulating the polarisation of the Golgi apparatus

Fig. 6. WAVE2 regulates polarisation of the Golgi apparatus. (A) Histogram showing the number of NIH 3T3 cells with polarised Golgi apparatus following treatment with siRNA targeting either: nothing (cont), WAVE1 (W1), WAVE2 (W2) or both. Data from three separate experiments were collated. *** indicates a *P* value <0.0001 (Fisher's exact test) for the null hypothesis that there is no difference between WAVE- and control-knockdown cells. Percentage figures indicate the percentage of cells with a polarised Golgi apparatus. P, polarised Golgi; NP, nonpolarised Golgi. (B) Typical immunoblot analysis of protein levels following treatment with siRNA. (C) Visualisation of the Golgi apparatus (antibody against GM130) in green, F-actin (phalloidin) in red and nuclei (DAPI) in blue following control siRNA (cont) or WAVE2 siRNA. The wound edge is oriented vertically at the right of each panel. The lower panel shows GM-130 staining alone. Bar, 20 μm.
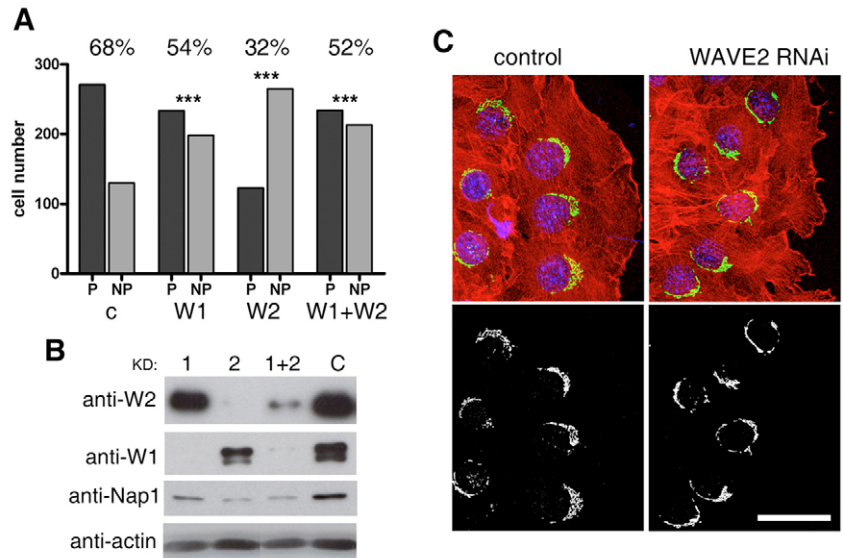
using RNAi-mediated knockdown of endogenous WAVE2. The percentage of cells exhibiting a polarised Golgi apparatus dropped from 68% (*n*=401) in control cells to 32% (*n*=388, *P*<0.0001) in cells lacking WAVE2. Knockdown of WAVE1 resulted in a milder, but significant, disruption of polarisation of the Golgi apparatus, with the number of polarised cells dropping to 54% (*n*=431, *P*<0.0001; Fig. 6A).

This disruption of the polarisation of the Golgi apparatus by WAVE2 knockdown appeared to be dose dependent as dual knockdown of WAVE2 and WAVE1 resulted in less efficient WAVE2 knockdown (Fig. 6B) and only caused a decrease in polarisation similar to that caused by knockdown of WAVE1 alone (52%, *n*=447, *P*<0.0001). Strikingly, lack of polarisation was often accompanied by the presence of a fragmented Golgi apparatus, similar to that seen in cells located away from the wound edge (Fig. 6C, Fig. 7A), indicating a disruption in the relay of the polarisation signal to the Golgi apparatus.

Of relevance to this study, it has been demonstrated that polarisation of the Golgi apparatus is a Rac-independent process (Nobes and Hall, 1999), suggesting that WAVE2 is responding to alternative signals. Overexpression of the transgene encoding WAVE2-WT had little effect on polarisation of the Golgi apparatus at the wound edge, whereas polarisation of the Golgi apparatus in cells expressing WAVE2-AAA dropped significantly (Fig. 7A,B). This suggests that the non-phosphorylatable version of WAVE2 partially interferes with the normal function of WAVE2 in polarising the Golgi apparatus, suggesting that phosphorylation of WAVE2 contributes to its function in regulating polarisation of the Golgi apparatus. Inhibitors of the MAP kinase pathway also disrupted polarisation of the Golgi apparatus, suggesting that MAP kinases and WAVE2 could lie on a pathway regulating the stimulus-dependent orientation of the Golgi apparatus in migrating cells (Fig. 7C,D).

Discussion

The data presented in this paper suggest that WAVE2 acts to enhance the persistence of cell migration, following phosphorylation by MAP kinases. Efficient polarisation of the Golgi apparatus following scratch-wounding also requires

WAVE2 and can be disrupted by a phospho-defective WAVE2 construct.

We have shown that MAP kinases phosphorylate WAVE2 on three sites in vitro – Ser343, Thr346 and Ser351. By using phospho-specific antibodies, we have confirmed that Thr346 and Ser351 are MAP kinase targets in vivo following cell treatment with a number of motogenic stimuli. Phosphorylation of Ser343 was detected on overexpressed protein but was not detected on endogenous WAVE2, suggesting that it is not physiologically relevant, or that it is phosphorylated at low levels on endogenous protein, or that it occurs in response to an untested stimulus. The biochemical impact of the three phosphorylation events we have identified remains unclear. In vitro actin-polymerisation assays on wild-type and phospho-defective WAVE2 revealed no differences in the kinetics of activation of the Arp2/3 complex, and immunoprecipitation of wild-type and mutant proteins from stably expressing cell lines revealed no obvious differences in the pattern of co-precipitating proteins either by silver staining or immunoblotting for other members of the WAVE2 complex such as Nap and Abi1. Thus, further experiments will have to be performed to establish the biophysical effects of phosphorylation on WAVE2 function. However, we were able to show clear cellular functions for these phosphorylation events.

Overexpression of a phospho-defective WAVE2 construct led to a decrease in migratory persistence in scratch-wounding models, an effect that could be mimicked by RNAi-mediated knockdown of endogenous WAVE2. Our findings using WAVE2 knockdown cells are in partial agreement with studies of WAVE2-deficient mouse embryo fibroblasts obtained from transgenic mice in scratch-wounding models, which also displayed decreased persistence (Suetsugu et al., 2003). However, that study showed a decrease in cell speed in WAVE2-null cells, whereas we observe a dramatic increase following knockdown or introduction of a transgene encoding a phospho-defective protein. These differences might reflect the acute nature of our RNAi-based system compared with that using transgenic MEFs, which might have made aberrant lineage decisions when developing in the absence of WAVE2.
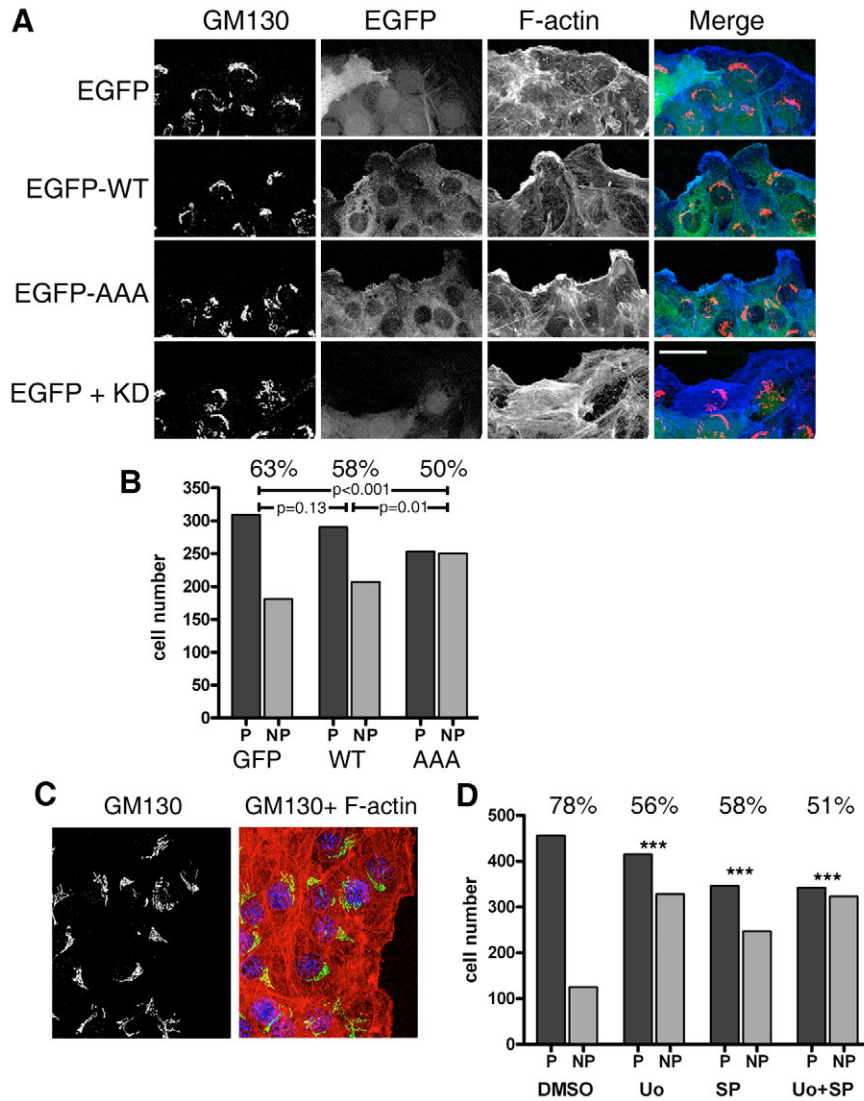
Fig. 7. Phosphorylation of WAVE2 regulates polarisation of the Golgi apparatus. (A) Sample images taken 3 hours post wounding of cells expressing the indicated transgene (EGFP-WT refers to wild-type WAVE2 and EGFP-AAA refers to the triple alanine mutant of WAVE2). EGFP + KD indicates EGFP control cells treated with WAVE2 siRNA. The Golgi apparatus was visualised with an antibody against GM130, and F-actin with phalloidin. Bar, 20 μm. (B) The polarisation of the Golgi apparatus was quantified in cells overexpressing either EGFP (GFP), EFGP-WAVE2-WT (GFP-WT): *P* values derived by Fisher's exact test are indicated below the percentage figures indicating the percentage of cells with a normally polarised Golgi apparatus. (C) Visualisation of disruption of the Golgi apparatus following treatment with Uo126 and SP600125, each at 10 μM (Uo126+SP). (D) Quantitation of the effects of 10 μM Uo126 (Uo) and SP600125 (SP) on polarisation of the Golgi apparatus. Cell number refers to wound-edge cells with a polarised Golgi apparatus. *** indicates a *P* value <0.0001 for inhibitor-treated cells compared with DMSO-treated controls. P, polarised Golgi; NP, nonpolarised Golgi.

It is also possible that the low level of WAVE2 remaining following knockdown in NIH 3T3 fibroblasts is sufficient to fulfil a requirement for WAVE2 in migration. However, our findings that disruption of WAVE2 function leads to an increase in cell speed suggest that WAVE2 is not required for cell migration per se, at least in NIH 3T3 fibroblasts. Other work has demonstrated increased migration speeds in cells in which lamellipodia have been inhibited by the overexpression of tropomyosin (Gupton et al., 2005), leaving just the lamellum, a myosin-II-rich region of slower turnover of F-actin (Ponti et al., 2004). Interestingly, these cells showed an increase in their migration persistence, revealing a role for lamellipodia in directing, but not powering, cell migration. While we do not observe any obvious differences in lamellipodial protrusions in WAVE2 knockdown or phospho-mutant cells, it is possible that lamellipodial function is altered at a biochemical level. Direct imaging has enabled us to show that the decrease in directional migration of WAVE2-deficient fibroblasts is not due to the inability of cells to migrate, but instead we show that WAVE2 confers a kind of control mechanism downstream of MAP kinase signalling by which directional migration is maintained. Many WAVE2-knockdown and AAA-transgenic cells are seen to break away from the sheet and move as independent cells. It is possible that this is a consequence of numerous changes in direction that pull them away from the rest of the monolayer. It is also conceivable that interfering with WAVE2 function acts to break apart the monolayer of cells and that, once in 'free space', the cells are less constrained and more able to change direction. We favour the former view, having seen control cells break away from the monolayer and still move with more persistence than mutant or knockdown cells (data not shown).

As we observed that polarisation of the Golgi apparatus is also dependent on WAVE2 function and is in part regulated by WAVE2 phosphorylation, it is tempting to speculate that WAVE2 function links these processes. Disruption of the Golgi apparatus with brefeldin A prevents formation of lamellipodia (Bershadsky and Futerman, 1994), and Rac-induced lamellipodia are delocalised from a single leading edge following a block in membrane traffic from the trans-Golgi network, resulting in a loss of persistent migration (Prigozhina and Waterman-Storer, 2004). Therefore, it is possible that mis-polarisation of the Golgi apparatus by loss of WAVE2 or loss of WAVE2 phosphorylation might disrupt the formation of

stable lamellipodia or induce their formation in a nonpolarised fashion, which potentially could explain the decrease in migration persistence in cells expressing WAVE2-AAA. However, a reciprocal level of control by which lamellipodial dynamics impinge upon the organisation of the Golgi apparatus cannot be ruled out, especially as polarisation of the Golgi apparatus can be blocked by the F-actin-disrupting agent cytochalasin D (Magdalena et al., 2003a; Magdalena et al., 2003b) (data not shown), as can reorganisation of the MTOC and nuclear repositioning following scratch-wounding (Gomes et al., 2005). It is of course possible that WAVE2 might have distinct functions in persistent migration and polarisation of the Golgi apparatus, and that further research will need to be performed in order to determine which of these hypotheses is correct. Finally, what is clear from this research is that phosphorylation of WAVE2 constitutes a novel form of regulation that influences fundamental aspects of cell motility.

Materials and Methods
Reagents and cells
All reagents were obtained from Sigma unless otherwise stated. NIH 3T3 fibroblasts were grown in DMEM (Invitrogen) supplemented with 10% foetal calf serum (Invitrogen) and penicillin/streptomycin.

Antibodies
Antibodies against WAVE2 (D16), WAVE1 (L19), Abi and secondary HRP-linked secondary antibodies were obtained from Santa Cruz Biotechnology. Antibody against Nap1 was obtained from Upstate Biotechnology. Antibodies against phospho-Erk (and total ERK) and Jnk were obtained from Cell Signalling. Antibody against GM-130 was obtained from BD Transduction Labs, and antibody against giantin from Covance. Alexa 488- and 568-labelled secondary antibodies and phalloidin were obtained from Molecular Probes. Phospho-specific antibodies were raised against the following phospho-peptides: non-phospho: CGG–VGFG**S**PG**T**PPPP**S**PPSF; Ser343: CGG–VGFG **S(***P***)** PGTP; Ser351: CGG–PPPP **S(***P***)** PPSF; and Ser343+Thr346: CGG–VGFG **S(***P***)** PG **T(***P***)** PPPP.

Peptides were conjugated to keyhole limpet haemocyanin (KLH) and used to immunise two rabbits (Eurogentec, fast program). Serum was negatively purified against the non-phospho-peptide coupled to epoxy sepharose 6B (GE Healthcare) according to the manufacturer's instructions (10 mg/ml). Flow-through was positively purified against the relevant phosphopeptide used for immunisation, coupled to epoxy sepharose 6B.

Immunoprecipitation of WAVE2
Cells were trypsinised, washed and plated at 75% confluence in starvation medium (DMEM containing 0.25% serum) for 18-24 hours. Cells were serum stimulated by the addition of prewarmed growth medium (10% FCS) or stimulated with PDGF-ββ (Upstate Biotechnology) at 1 ng/ml in starvation medium. Following stimulation, cells were washed in PBS, lysed (Cory et al., 2003) and incubated with 1 μg antibody against WAVE2 with protein G sepharose. Densitometry of immunoblots of WAVE2 immunoprecipitates was performed on non-saturated exposures using a Biorad GS-690 and Molecular Analyst software.

Expression constructs
cDNA encoding human WAVE2 was a generous gift from Laura Machesky, University of Birmingham, UK. WAVE2 was amplified by PCR and cloned into either pEF-Bos downstream of GST, pCMV5 (accession no. AF239024) downstream of the FLAG epitope, or pEGFP-C1 (Clontech). ΔSHD WAVE2 was derived by PCR from residue 169 to the C-terminus. ΔSHD/ΔVCA was derived by digesting the ΔSHD construct with ApaI.

Lentiviral vectors were generated by transfer of EGFP-WAVE2 cassettes into a pSEW sin vector backbone (a generous gift of Adrian Thrasher, Institute of Child Health, London) lacking the WPRE region. GFP control vector contained the WPRE region and was a gift from A. Thrasher. Virus was generated by transfection of 293-T as described previously (Demaison et al., 2002). NIH 3T3 cells were infected using virus at a multiplicity of infection of 0.8, grown for 1 week and then FACS sorted using a FACSCalibur (BD Biosciences). Identical gates were used for the WAVE2 WT and AAA mutant cells, which were sorted to >98.5% positivity.

Phosphatase/kinase reactions
Cos-7 cells expressing GST- or FLAG-tagged proteins were lysed and incubated with glutathione sepharose or antibodies against FLAG attached to sepharose beads. Precipitates were washed three times with lysis buffer and once with 0.5 M LiCl, 20 mM Tris pH 8 before incubation with 40 units calf intestinal phosphatase (New England Biolabs) for 30 minutes at 37°C in the manufacturer's buffer. Following further washing, precipitates were resuspended with 100 ng recombinant Jnk2 or Erk2 (Upstate Biotechnology) according to the manufacturer's instructions.

RNAi knockdown of WAVE1 and WAVE2
The following RNA oligonucleotides were synthesised by Dharmacon (*option C): WAVE2: GACGUUGCCUAGCGAUACAdTdT; WAVE1: UCACACAGCUUGA-UCCUAAdTdT; control: siCONTROL Non-Targeting siRNA #1 D-001210-01-20. For knockdown experiments, RNA was suspended at 160 nM in 250 μl optimem-1 (Invitrogen). 5 μl lipofectamine 2000 was separately mixed with 250 μl optimem-1 for 5 minutes before mixing with the RNAi solution for a further 20 minutes at room temperature. NIH 3T3 cells were plated together with RNAi mix at 4×10^5 cells per 5 cm dish in a final volume of 2 ml of DMEM, 10% FCS lacking penicillin/streptomycin. The final siRNA concentration was 16 nM. Cells were left overnight before replating and left a further 24 hours before use in experiments. An aliquot of knockdown cells was plated separately and lysed at the time of the experiment. Lysates were immunoblotted with antibodies against WAVE1, WAVE2 and Nap1 to assess the levels of protein expression.

Migration assay
Cells were plated at 1×10^5 per cm^2 on glass-bottomed four-well chamber slides (Iwaki cat. no. 5720-004) and allowed to grow to confluence overnight in growth medium. Culture medium was replaced by CO$_2$-independent medium (Invitrogen) supplemented with 10% FCS, penicillin/streptomycin and cells were wounded with a plastic rod sharpened to a 0.5 mm point. Cells were visualised using a Leica DMIRE2 microscope with automated stage at 37°C. Images were taken at intervals of 30 minutes and cell nuclei tracked manually using ImageJ software (Rasband, W.S., ImageJ, NIH, Bethesda, MA; http://rsb.info.nih.gov/ij/; 1997-2006.) for 8 hours before wound closure, usually 8-16 hours following wounding. If the wound had not closed in 16 hours, cells were tracked from 8-16 hours. All cells at the leading edge were tracked unless they underwent division or became obscured.

Assay of polarisation of the Golgi apparatus
Cells were plated on glass coverslips as described above and wounded the following day for 3 hours at 37°C before fixation with 4% formaldehyde. Cells were permeabilised with 0.25% triton X-100 for 5 minutes before staining with antibodies against either GM130 or giantin to visualise the Golgi apparatus. Nuclei were visualised with DAPI or by propidium iodide staining. Images were taken using a Leica confocal microscope. Polarisation of the Golgi apparatus was assessed in cells at the wound edge in five fields of view (n>80) in each of three separate experiments. For cells to be scored as polarised, >90% of the Golgi (by eye) had to lie between the nucleus and the cell-edge exposed to the wound void (Magdalena et al., 2003a).

Statistics
All statistics were performed using Graphpad Prism (Graphpad Software). The significance of relative migration persistence and relative speeds compared with control values was performed using one-way ANOVA followed by a Newman-Keuls post-test to determine the significance of differences between groups. The significance of polarisation of the Golgi apparatus compared with that of controls was determined using a two-tailed Fisher's exact test, where the Golgi apparatus was described as polarised or not polarised. All quoted errors refer to the standard error of the mean.

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