Merkel cell polyomavirus small T antigen drives cell motility via Rho-GTPase-induced filopodia formation

Gabriėłė Stakaitytė¹,#, Nnenna Nwogu¹,#, Samuel J. Dobson¹, Laura M. Knight¹, Christopher W. Wasson¹, Francisco J. Salguero³, David J. Blackbourn⁴, G. Eric Blair¹, Jamel Mankouri¹,², Andrew Macdonald¹,², and Adrian Whitehouse¹,²,*

¹School of Molecular and Cellular Biology, Faculty of Biological Sciences, ²Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, ³School of Veterinary Medicine, ⁴School of Biosciences and Medicine, University of Surrey, Surrey, United Kingdom

*Correspondence to Adrian Whitehouse
Tel: +44 (0) 113 3437096
Email: a.whitehouse@leeds.ac.uk;

# Authors contributed equally to work

No. of pages: 27
No. of Figures: 9

Keywords: DNA viruses, tumour virus, cell motility, cell migration
ABSTRACT

Cell motility and migration is a complex, multi-step, and multi-component process, intrinsic to progression and metastasis. Motility is dependent on the activity of integrin receptors and Rho-family GTPases resulting in the remodelling of the actin cytoskeleton and formation of various motile actin-based protrusions. Merkel cell carcinoma (MCC) is an aggressive skin cancer with a high likelihood of recurrence and metastasis. Merkel cell polyomavirus (MCPyV) is associated with the majority of MCC cases, and MCPyV-induced tumourigenesis largely depends on the expression of the small tumour antigen (ST). Since the discovery of MCPyV, a number of mechanisms have been suggested to account for replication and tumourigenesis, but to date, little is known about potential links between MCPyV T antigen expression and the metastatic nature of MCC. Previously, we have described the action of MCPyV ST on the microtubule network and how this impacts on cell motility and migration. Here we demonstrate that MCPyV ST affects the actin cytoskeleton, to promote the formation of filopodia, through a mechanism involving the catalytic subunit of protein phosphatase 4 (PP4C). We also show that MCPyV ST-induced cell motility is dependent upon the activity of Rho-family GTPases Cdc42 and RhoA. In addition, our results indicate that the MCPyV ST-PP4C interaction results in the dephosphorylation of β1 integrin, likely driving the cell motility pathway. These findings describe a novel mechanism by which a tumour virus induces cell motility, which may ultimately lead to cancer metastasis and provides opportunities and strategies for targeted interventions for disseminated MCC.

IMPORTANCE

Merkel Cell Polyomavirus (MCPyV) is the most recently discovered human tumour virus. It causes the majority of cases of Merkel cell carcinoma (MCC), an aggressive skin cancer. However, the molecular mechanisms implicating MCPyV-encoded proteins in cancer development are yet to be fully elucidated. This manuscript builds upon our previous observations which demonstrate that the MCPyV ST antigen enhances cell motility, providing a potential link between MCPyV protein expression and the highly metastatic nature of MCC. Here we show that MCPyV ST remodells the actin cytoskeleton, promoting the formation of filopodia which is essential for MCPyV ST-induced cell motility and we also implicate the activity of specific Rho-family GTPases, cdc42 and RhoA in these processes. Moreover, we describe a novel mechanism for the activation of Rho GTPases and the cell motility pathway due to the interaction between MCPyV ST and the cellular phosphatase catalytic subunit PP4C, which leads to the specific dephosphorylation of β1 integrin. These findings may therefore provide novel strategies for therapeutic intervention for disseminated MCC.
INTRODUCTION

Cell motility is a complex, multi-step and multi-component process. The actin cytoskeleton is an important contributor to cell motility and is required for the formation of several types of actin-rich membrane protrusions (1). These protrusions contain actin filaments, which push the membrane forward leading to membrane deformation and extension. Actin filaments are formed by the polymerisation of globular monomeric actin (G-actin) into double stranded helical filamentous actin (F-actin), which is enhanced by the activity of several actin-binding proteins (2). Actin filaments can adopt different morphologies, such as filopodia and lamellipodia, depending on the number of filaments and the type and number of actin-binding proteins that associate with these filaments (2). Filopodia are thin, finger-like structures that are filled with tight parallel bundles of F-actin. They have been described as 'antennae' or 'tentacles' that migrating cells use to probe their microenvironment, thus serving as pioneers during protrusion (3). In contrast, lamellipodia are thin sheet-like protrusions filled with a branched network of actin. In both cases, the fast-growing barbed ends of actin filaments are orientated towards the plasma membrane and the elongation of these protrusions pushes the leading edge forward, promoting cell migration (1).

This complex cell motility process occurs via the remodelling of the actin cytoskeleton, which is controlled by the Rho-family GTPases, a large group of signalling molecules that act as signal mediators in the motility pathway. The Rho-family GTPases affect both the microtubule network and the actin cytoskeleton (4). Three of the most widely studied Rho-family GTPases are Cdc42, Rac1 and RhoA have all been implicated in the formation of actin containing plasma membrane protrusions. Cdc42 was originally implicated in filopodia formation (5), Rac1 in lamellipodia formation (6), and RhoA in stress fibre formation (7). While in many cases, this classification is still valid, recent research has shown that the functions of these Rho-family GTPases are intertwined, with cross-talk in the cell motility cascade, for example in filopodia formation (8). In addition to their important role in healthy cells, Rho-family GTPases also play a role in cancer development and metastasis (9-11). For instance, overexpression of RhoA has been observed in breast, colon, lung, and gastric cancers (12-14), amongst others.

Rho-family GTPases are signal mediators and are central to the cell motility pathway. The pathway starts, however, with cellular receptors, such as the trans-membrane integrin receptors (15). They are αβ heterodimers, with human integrins having 24 types of α subunits and 9 types of β subunits, although some types are found in specific tissues (e.g. α3β2 in platelets (16)) while others are widely expressed (e.g. α5β1) (17). Integrins have large extracellular domains that bind the extracellular matrix (ECM) and link to the actin cytoskeleton through short cytoplasmic tails (18). Their main function is to transmit signals from the ECM to the cell interior. In addition, integrins play a well-recognised role in cancer progression (19).

Cancers with high metastatic potential use the cell motility pathway to disseminate from the original tumour to distant secondary sites. One such cancer is Merkel cell carcinoma (MCC), a rare but aggressive malignancy of neuroendocrine origin that presents as reddish or purplish nodules...
on sun-exposed areas of skin (20). The number of reported cases of MCC has tripled in the past 20 years (21), and risk factors include advanced age, UV exposure, and immune suppression (20, 22). MCC has a poor 5-year survival rate, characterised by local recurrence, early spread to local lymph nodes and high likelihood of forming distant metastases (20).

Merkel cell polyomavirus (MCPyV) is a recently-discovered oncogenic virus that has been implicated as the causative agent of MCC in ~80% of cases in the north hemisphere (23). MCPyV infection is asymptomatic and ubiquitous in many populations, with up to 80% healthy adults infected (24). Like other polyomaviruses, MCPyV expresses the T antigen, whose spliced products, namely large T antigen and small T antigen (LT and ST), are required for viral replication and tumourigenesis (23). The mechanism for MCPyV tumourigenesis has been broadly established (23, 25, 26), upon loss of immunosurveillance, due to old age or immunosuppression, the virus can integrate into the cellular genome. Integration occurs prior to clonal expansion of tumour cells (25).

In addition to integration, another prerequisite for tumourigenesis is the truncation of MCPyV LT, as only the truncated form of this protein has been observed in MCPyV-positive MCC tumours. This is likely due to the fact that truncated MCPyV LT is replication deficient, as integrated MCPyV with a replication-competent MCPyV LT may initiate unlicensed replication which would ultimately lead to cytopathic cell death (26). The molecular mechanisms of the MCPyV life cycle and oncogenic properties have been extensively reviewed (27-31).

Both LT and ST are required for MCPyV-positive MCC cell survival and proliferation, and siRNA-mediated depletion of either leads to cell death (32). Taking SV40 as a model, MCPyV LT would be expected to be the main viral oncoprotein driving cellular transformation. However, in contrast to simian virus 40 (SV40), MCPyV LT cannot initiate cellular transformation (33), although it likely plays at least an accessory role, as it can bind to host factors that regulate cellular proliferation, such as the retinoblastoma protein (pRB) and Hsc70 (34). Conversely, MCPyV ST alone can transform rodent cells and induce serum-free proliferation of human cells, it is therefore considered to be the main transforming factor (33). However, there have been conflicting results on the contribution of MCPyV ST to MCPyV-positive MCC cell proliferation after initial transformation (35, 36), thus the role of MCPyV ST is not yet fully understood. What is known leads to the conclusion that MCPyV ST is a multi-functional protein. It promotes the hyperphosphorylation of 4E-BP1, deregulating cap-dependent translation (33), inhibits NF-κB-dependent gene transcription through NEMO (37), targets the host ubiquitin ligase SCF$^{F_{\text{wbb}}}$, leading to the stabilisation of MCPyV LT and several host oncoproteins (38) and also promotes transcriptional changes in glycolytic metabolic pathways (39). Importantly, recent studies using a panel of preterm transgenic mice co-expressing epidermis-targeted coexpression of ST and the cell fate-determinant atonal bHLH transcription factor 1 (ATOH1) lead to development of widespread cellular aggregates, with histology and marker expression mimicking human intraepidermal MCC, supporting the concept that ST is the major MCPyV-derived oncogenic driver in MCC (40).
MCC has a highly metastatic phenotype and correlates with poor MCC survival rates (41). This is also supported by recent studies showing that engraftment of MCC cell lines into SCID mice results in circulating tumour cells and metastasis formation (42). Aligned with this observation is that we have previously reported that MCPyV ST can enhance cell motility through microtubule dissociation. Expression of MCPyV ST upregulates the levels of stathmin, a microtubule-associated protein, and leads to microtubule destabilisation, which is necessary for a migratory phenotype (43).

Here we extend this analysis and show that MCPyV ST drives cell motility by disrupting the actin cytoskeleton. We demonstrate that MCPyV ST expression induces the formation of filopodia-like structures, through a mechanism dependent on the activity of the Rho-family GTPases Cdc42, RhoA, and, to a lesser extent, Rac1. This process is initiated by a MCPyV ST-PP4C interaction which results in the dephosphorylation of β1 integrin.
RESULTS

MCPyV ST expression affects the levels of actin-associated proteins. Cell motility regulation is a complex, multi-step process, an important aspect of which is the regulation of the actin cytoskeleton and associated proteins. We have previously used a SILAC-based quantitative proteomics approach (44, 45), to determine alterations in the host cell proteome upon inducible MCPyV ST expression in a HEK-293-derived cell line, i293-ST (43). Results demonstrated that MCPyV ST expression led to the upregulation of the microtubule-associated protein, stathmin, which affects microtubule dissociation (46). Moreover, results highlighted that proteins which regulated the actin cytoskeleton were also altered upon MCPyV ST expression. These proteins include Cofilin-1, Cortactin and Actin-related proteins 2/3 complex subunits which were upregulated by 5.1, 3.7 and 3.9 fold respectively.

To confirm increased levels of actin-associated proteins identified by the quantitative proteomic approach, i293-ST cells remained uninduced or were induced with doxycycline hyclate, or a MCPyV negative MCC cell line, MCC13 cells, transfected with EGFP or EGFP-ST. Immunoblotting of cell lysates confirmed an upregulation of actin-associated proteins Arp3, cortactin, and cofilin upon expression of MCPyV ST, both in i293-ST and in MCC13 cells (Fig. 1A). Densitometry showed a 4-fold increase in Arp3 in both cell lines, a 4-fold increase in i293-ST cells and a 3-fold increase in MCC13 cells of cortactin, and a 2-fold increase in i293-ST cells and a 4-fold increase in MCC13 cells of cofilin (Fig. 1B). Comparing these values against control cells, a significant increase in actin-associated protein levels is observed in MCPyV ST-expressing cells. This increase in protein levels probably occurs at the transcriptional level, as RT-qPCR showed significant changes in the mRNA levels of Arp3, cortactin, or cofilin upon MCPyV ST expression in i293-ST cells (Fig. 1C), which correlates with recent results showing that MCPyV ST can dynamically alter the transcriptome of human cells (39).

To investigate the differential expression of actin-associated proteins in the context of MCC, multicolour immunochemistry analysis was performed on formalin-fixed, paraffin-embedded (FFPE) sections of two primary MCC tumours. Sections were incubated with cortactin, cytokeratin 20 (CK20) (a marker widely used to distinguish MCC) and a MCPyV LT (CM42B) specific antibodies. An isotyped-matched control was also used as a negative control. Results show increased levels of cortactin expression coincident with CK20 and LT staining in regions of both tumours (Fig. 1D). Moreover, immunoblot analysis was performed on the cellular lysates of two independent MCC tumour samples comparing protein levels against a negative control non-tumour cadaveric skin sample. Results again demonstrate an increase in cortactin and Arp3 protein levels in MCC tumour samples compared to control (Fig. 1E and 1F). Notably, higher levels of actin-associated proteins were observed in MCC tumour sample 2 compared to MCC tumour sample 1, which correlates with higher levels of MCPyV ST in sample 2. Furthermore, immunoblot analysis was also performed on cellular lysates of the MCPyV-positive MCC cell line, WAGA, which were transduced with lentiviruses containing shRNA targeting ST or a scrambled control, as previously
described (33, 38). Results demonstrated that depletion of MCPyV ST led to a reduction in cortactin and Arp3 protein levels (Fig. 1G). Together these results demonstrate that levels of actin cytoskeleton-related proteins are altered upon MCPyV ST expression and in the context of MCC, implicating MCPyV ST in inducing cell motility and potentially MCC metastasis.

MCPyV ST expression induces the formation of actin-based protrusions. Observations of altered actin-associated protein levels upon MCPyV ST expression suggested the possibility of actin cytoskeleton-related phenotypic changes. To examine any changes in MCPyV ST-expressing cells, HEK-293 cells were transfected with EGFP or EGFP-ST. Cells were then fixed and stained with rhodamine phalloidin, an actin-binding compound to investigate any possible changes in the actin cytoskeleton. MCPyV ST-expressing HEK-293 cells showed an abundance of actin-based protrusions, compared to the much smoother cell periphery of control EGFP-expressing HEK-293 cells (Fig. 2A). Similar results were also observed in MCPyV ST-expressing MCC13 cells, showing an increased number of longer protrusions, although the difference in number was less pronounced. In addition, it was observed that MCC13 cells appeared to have more abundant levels of intracellular actin which may be due to the increase in actin-associated protein levels upon MCPyV ST expression (Fig. 2B). To quantify the increase in actin-based protrusions upon MCPyV ST expression, the protrusions were counted and their lengths measured using ImageJ software. Analysis showed an increase in the numbers and length of these actin-based protrusions in MCPyV ST-expressing HEK-293 cells (Fig. 2C), and an increase in longer actin-based protrusions in MCPyV ST-expressing MCC13 cells (Fig. 2D). Comparing the average length of protrusions from this analysis, a significant increase in length was observed in MCPyV ST-expressing cells. To confirm the actin cytoskeleton-related phenotypic changes observed in HEK-293 and MCC13 cells, similar experiments were performed in primary epidermal keratinocytes (Fig. 2E and F) and primary human dermal fibroblast cells (Fig. 2G and H). Results in both primary cell lines show an increase in the number and length of actin-based protrusions upon MCPyV ST expression. In addition, immunofluorescence studies suggest an increase in intracellular actin levels upon McPyV ST expression. Together these observations suggest that MCPyV ST expression leads to phenotypic changes in the actin cytoskeleton resulting in the formation of actin-based protrusions.

MCPyV ST expression induces the formation of filopodia. Visually, the thin, filamentous nature of the actin-based protrusions formed upon MCPyV ST expression appear to suggest that they are filopodia. However, to conclusively characterise and classify these protrusions, a number of actin-associated proteins were screened using immunofluorescence. HEK-293 cells were co-transfected with Flag-tagged MCPyV ST (ST-flag) (37), in combination with one of the following GFP-tagged constructs: cortactin, N-WASP, mDia2 or fascin, or EGFP-ST and one of the following
myc-tagged constructs: IRSp53 or IRSTK. Cells were then fixed and stained with rhodamine phalloidin to visualise actin-based structures.

Of particular interest were results utilising mDia2. In control cells, mDia2 was seen to be diffuse in the cytoplasm, while in cells expressing MCPyV ST, mDia2 relocalised to the cell periphery and into the actin-based protrusions (Fig. 3A). Similar results were observed for IRSp53, with this protein being observed to be diffuse in the cytoplasm in control cells but it relocalised to the cellular periphery and into the actin-based protrusions upon the expression of MCPyV ST (Fig. 3B). No change in N-WASP or IRSTK could be observed upon MCPyV ST expression (data not shown). As both mDia2 (47) and IRSp53 (48) are associated with filopodia formation, this was the first marker-associated indication that MCPyV ST expression induced filopodia formation. To further confirm that MCPyV ST induced the formation of filopodia, an additional filopodia marker, myosin X, was utilised, as myosin X is localised to the tips of filopodia (49). To this end, HEK-293 cells were transfected with EGFP or EGFP-ST and stained for myosin X. Punctate foci of myosin X staining can be observed at the tips of MCPyV ST-induced actin-based protrusions (Fig. 3C).

Together, these results confirm that the protrusions induced by MCPyV ST expression are filopodia.

The interaction between MCPyV ST and PP4C is important for inducing filopodia formation. We have previously shown the importance of the interaction between MCPyV ST and PP4C, with regards to MCPyV ST-induced cell motility using a deletion mutant of MCPyV, termed STΔ95-111. This MCPyV ST mutant ablates the interaction between MCPyV ST and protein phosphatase 2A (PP2A) Aβ and PP4C (43). We therefore investigated whether cellular phosphatases were required for MCPyV ST-induced filopodia formation. For this purpose, HEK-293 cells were first transfected with either EGFP, EGFP-ST, EGFP-R7A (a previously described PP2A Aα non-binding mutant) or EGFP-Δ95-111 (PP2A Aβ and PP4C non-binding mutant). Cells were then fixed and stained with rhodamine phalloidin to identify actin-based structures (Fig. 4A). Results suggest reduced filopodia formation upon the expression of EGFP-Δ95-111 similar to EGFP control, whereas EGFP-R7A induces filopodia similar to EGFP-ST. Quantitative analysis of filopodia confirmed that while expression of the MCPyV STΔ95-111 deletion mutant did not induce filopodia, the expression of the MCPyV ST R7A mutant induced filopodia formation to similar levels as wild type ST (Fig. 4B). However, this analysis was not sufficient to determine which MCPyV ST-phosphatase interaction was important in cell motility and filopodia formation, as the Δ95-111 deletion affects the interaction of MCPyV ST with both PP2A Aβ and PP4C.

Therefore, to conclusively determine which cellular phosphatase is responsible for MCPyV ST-induced cell motility and filopodia formation, additional alanine-scanning MCPyV ST mutants were utilised which distinguish the interaction between MCPyV ST and PP2A Aβ and PP4C, as previously characterised (50). However, these previous experiments utilised co-immunoprecipitation assays over-expressing tagged versions of PP2A and PP4C. Therefore, herein we have repeated the co-immunoprecipitation experiments to examine the interaction of
MCPyV ST-GFP mutants with endogenous PP2A Aβ and PP4C, using GFP-TRAP pulldowns.

Results show that wild type EGFP-ST interacts with both endogenous forms of PP2A Aβ and PP4C, in contrast EGFP-Δ95-111 and EGFP-ST102A ablates both PP2A Aβ and PP4C binding, whereas EGFP-ST102A only disrupts the interaction with PP4C (Fig. 4C). Therefore, to determine which cellular phosphatase is responsible for MCPyV ST-induced cell motility HEK-293 or MCC13 cells were transfected with either EGFP-ST102A, or EGFP-ST103A and cells imaged using the IncuCyte live cell imaging system (Fig. 4D). Cell motility was analysed using ImageJ software by tracing the tracks of individual cells, allowing quantification of the distance travelled, and results showed that in both cell lines expressing either EGFP-ST102A or EGFP-ST103A, a decrease in motility was observed in comparison to cells expressing EGFP-ST (p<0.001) (Fig. 4D). This suggests that the specific interaction of MCPyV ST with PP4C is required for MCPyV ST-induced cell motility. To confirm the association between MCPyV ST, PP4C, cell motility and filopodia formation, HEK-293 cells were transfected with EGFP-ST102A or EGFP-ST103A, fixed and stained with rhodamine phalloidin to observe actin-based structures (Fig. 4A). Together with quantitative analysis of filopodia formation (Fig. 4B), results showed that cells expressing EGFP-ST102A and EGFP-ST103A showed a marked decrease in filopodia formation when compared to cells expressing EGFP-ST. Comparing the average length of protrusions from this analysis, a significant increase in length was observed in MCPyV ST and R7A-expressing cells compared to EGFP-ST-Δ95-111, 102A and 103-expressing cells. Together this suggests that PP4C is required for MCPyV ST-induced filopodia formation. To confirm these observations, filopodia formation was also analysed in the absence or presence of a PP4C transdominant phosphatase-dead mutant, PP4-RL (51). Analysis and quantification of rhodamine phalloidin stained cells showed a reduced number of longer actin-based protrusions in MCPyV ST-expressing cells in the presence of PP4-RL (Fig. 4E and F). Again comparing the average length of protrusions from this analysis, a significant increase in length was observed in MCPyV ST-expressing cells alone compared to cells expressing PP4-RL. These results correlate with the data gathered from live cell imaging experiments and reveal the importance of the specific interaction of MCPyV ST with PP4C to induce cell motility and filopodia formation.

MCPyV ST-induced cell motility is dependent on the action of Rho-family GTPases.

The Rho-family GTPases are a superfamily of signalling molecules, some of which have been implicated in increased cell motility and metastasis in various cancers (9-11). There is also an undisputed role of the Rho-family GTPases in actin dynamics (52). Therefore to determine whether Rho-family GTPases are involved in MCPyV ST-induced cell motility, a selection of well characterised inhibitors targeting Rho-family GTPases were utilised at non-cytotoxic concentrations as measured by MTS assay (data not shown): ML141 (a Cdc42/Rac1 inhibitor), NSC23766 (a Rac1 inhibitor), Rhosin (a RhoA inhibitor) and ZCL278 (a Cdc42 inhibitor). Treatment of HEK-293-derived inducible cell lines, i293-EGFP and i239-EGFP-ST, along side...
MCC13 cells, enabled visualization of cell motility in live cells. i293-EGFP and i293-EGFP-ST cells were induced using doxycycline hyclate, while MCC13 cells were transfected with EGFP or EGFP-ST, and treated with each respective inhibitor for 24 h. Cells were then imaged using the IncuCyte live cell imaging system, taking an image every 30 min for a 24-h period (Fig. 5A and C). Distances travelled by individual cells were tracked using ImageJ software. No significant differences were observed among the average distances travelled by control cells not expressing MCPyV ST, in both untreated and treated cells with the Rho-family GTPase inhibitors (Fig. 5B and D), demonstrating that the concentrations of inhibitors were non-toxic. In contrast, decreased cell motility upon treating MCPyV ST-expressing cells with both Cdc42 inhibitors and the RhoA inhibitor is clearly apparent (p<0.001), while no significant decrease was observed when cells are treated with the Rac1 inhibitor (Fig. 5B and D). This suggests a role for Cdc42 and RhoA GTPases in facilitating MCPyV ST-induced cell motility.

MCPyV ST-induced filopodia formation is dependent on the activity of the Rho-family GTPases. It was also important to determine whether the observed effect of Rho-family GTPases on MCPyV ST-induced cell motility was mirrored in filopodia formation. HEK-293 cells were transfected with EGFP or EGFP-ST cells, and treated with the specific Rho-family GTPase inhibitors (ML141, NSC27366, Rhosin and ZCL278) for 24 h. Cells were then fixed and stained with rhodamine phalloidin to observe actin-based structures (Fig. 6A). Quantitative analysis of filopodia showed a decrease in filopodia formation when MCPyV ST-expressing cells were treated with Rho-GTPase inhibitors for Cdc42 and RhoA, however the Rac1 inhibitor showed little effect (Fig. 6B). Comparing the average length of protrusions from this analysis, a significant increase in length was observed in MCPyV ST-expressing cells compared to cells treated with the Cdc42 and RhoA inhibitors.

To further examine any differences on MCPyV ST-induced filopodia formation by Rho-family GTPases, HEK-293 cells were co-transfected with ST-Flag and Rho-family GTPase transdominant mutants: pcDNA-5-GFP-Cdc42-T17N, pcDNA5-GFP-Rac1-T17N, or pcDNA5-GFP-RhoA-T19N (Fig. 6C). These transdominant mutants are inactive and inhibit endogenous Cdc42, Rac1 and RhoA activity (53). Cells were then fixed and stained with rhodamine phalloidin to visualize actin-based structures. Quantitative analysis of filopodia showed a small decrease in filopodia upon co-expression of MCPyV ST with the Rac1 transdominant mutant; however, a marked reduction was observed when cells were co-transfected with either the Cdc42 or RhoA transdominant mutants, confirming the observation of the live cell imaging data (Fig. 6D). Again comparing the average length of protrusions from this analysis, a significant increase in length was observed in MCPyV ST-expressing cells compared to cells expressing the Cdc42 or RhoA transdominant mutants. This suggests a definitive role for Cdc42 and RhoA in MCPyV ST-induced filopodia formation. Consequently, we next aimed to directly measure the activity of Cdc42 and RhoA in MCPyV ST-expressing cells, employing an affinity precipitation assay to specifically
measure the amount of RhoA–GTP or Cdc42–GTP forms (54, 55). Transfected EGFP or EGFP-ST HEK-293 cell lysates were incubated with either PAK1 PBD or Rhotekin RBD Agarose beads, which selectively bind to the GTP-bound but not GDP-bound forms of Cdc42 and RhoA, respectively. The amount of active G protein was then detected by immunoblotting with Cdc42 and RhoA-specific antibodies. Expression of MCPyV ST resulted in elevated levels of active cdc42 and RhoA compared with control cells expressing GFP alone (Fig. 6E). Together these data further indicate that the Rho-family GTPases are involved in MCPyV ST-induced cell motility.

The activity of integrins is important for MCPyV ST-induced cell motility. Herein and previously (43), we report the importance of the MCPyV ST-PP4C interaction in cell motility and filopodia formation. In order to uncover a potential role of PP4C in MCPyV ST-induced cell motility, i293-GFP and i293-GFP-ST cells were transfected with HA-Cdc42 or HA-Rac1, then induced for 48 h. Cell lysates were probed for phosphorylated Cdc42/Rac1 (residue Ser 71) to determine if MCPyV ST expression affects the phosphorylation status of Rho-family GTPases. Surprisingly, results showed no change in the phosphorylation status of Cdc42/Rac1 (Fig. 7A) indicating that the MCPyV ST-PP4C interaction does not affect the phosphorylation status of Rho-family GTPases directly. Therefore, to uncover a possible target for the MCPyV ST-PP4C interaction, factors upstream of the Rho-family GTPases were investigated. Integrins are known to be important in various aspects of cell adhesion, polarity, and motility, where they initiate signalling cascades (15), and have been implicated in cancer progression (19). Moreover, their function can be regulated by their phosphorylation status (56). Therefore, MCPyV ST-induced cell motility was assessed using a range of concentrations of the integrin inhibitor, RGDS. RGDS is a tetrapeptide that has been shown to inhibit the binding of ligands to α5β1 and αvβ3 integrins (57). i293-GFP and i293-GFP-ST cells were induced using doxycycline hyclate, while MCC13 cells were transfected with EGFP or EGFP-ST. Cells were then treated with non-toxic concentrations of RGDS as measured by a cell viability (MTS) assay (data not shown) for a 24-h period prior to imaging using the IncuCyte life cell imaging system by taking an image every 30 min for a 24-h period (Fig. 7B and D). Distances travelled by individual cells were analysed and showed decreasing cell motility with increasing RGDS concentration (Fig. 7C and E). These results therefore suggest a role for integrins, in MCPyV ST-induced cell motility.

The activity of integrins is important to MCPyV ST-induced filopodia formation. To determine whether integrins have an essential role in MCPyV ST-induced filopodia formation. HEK-293 cells were transfected with EGFP or EGFP-ST, then treated with 100 μM RGDS for 24 h. Cells then fixed and stained with rhodamine phalloidin to visualise actin-based structures (Fig. 7F), coupled with quantitative analysis of filopodia (Fig. 7G). Results show that treatment of cells expressing MCPyV ST with RGDS display fewer filopodia compared to untreated cells expressing
MCPyV ST alone. These results further implicate a role for integrins in MCPyV ST-induced cell motility.

**The β1 integrin is dephosphorylated upon MCPyV ST expression.** Integrin activity can be regulated by phosphorylation (56). The importance of integrins in MCPyV ST-induced cell motility and filopodia formation, revealed above, suggested a possibility that integrin phosphorylation could be affected by MCPyV ST-PP4C interaction. In particular, the effect of RGDS on MCPyV ST-induced filopodia formation and cell motility suggested a role for α5β1 and/or αvβ3. HEK-293 cells do not express αvβ3 (58), thus α5 and/or β1 were of interest. More specifically, β1 phosphorylation status has been shown to be regulated at the Thr788/789 residues (59). Therefore, in order to investigate whether MCPyV ST expression had any effect on β1 phosphorylation, HEK-293 cells were transfected with EGFP, EGFP-ST, EGFP-ST102A or EGFP-ST103A. Cell lysates were probed for phosphorylated β1 integrin at Thr788/789. Results showed a dramatic reduction in the phosphorylation levels of β1 integrin at both these sites upon MCPyV ST expression. Notably, however, phosphorylation levels remain unchanged upon the expression of the PP4C-non-binding mutants EGFP-ST102A and EGFP-ST103A (Fig. 8). Densitometry results confirm that upon MCPyV ST expression, the phosphorylation levels of β1 at Thr788/789 decrease (Fig. 8A). In contrast, this reduction was inhibited upon the expression of the PP4C transdominant mutant, PP4-RL (Fig. 8B). These results suggest that the interaction of MCPyV ST and PP4C leads to reduced β1 integrin phosphorylation levels at Thr788/789, which in turn leads to downstream signalling that ultimately enhances filopodia formation and cell motility in MCPyV ST-expressing cells.
MCPyV ST is an oncogenic protein, sufficient to transform rodent cells to anchorage- and contact-independent growth and is also capable of inducing serum-free proliferation of human cells (33). Moreover, epidermis-targeted coexpression of ST and ATOH1 leads to development of widespread cellular aggregates, with histology and marker expression mimicking human intraepidermal MCC, using a panel of preterm transgenic mice. This supports the concept that ST is the major MCPyV-derived oncogenic driver in MCC (40). Notably, MCC has a highly metastatic phenotype which correlates with poor MCC survival rates (41). We have confirmed the existence of a link between MCPyV ST expression and cell motility and migration, both essential factors for primary tumour dissemination. Our observation are supported by recent studies showing that engraftment of MCC cell lines into SCID mice resulted in the appearance of circulating tumour cells and metastasis formation, with explanted tumours also exhibiting an upregulation of MCPyV ST-Antigen expression in all tumours (42).

Promoting motility and metastasis by virus oncoproteins has been reported previously. Human papillomavirus 16 (HPV16) E7, Epstein-Barr virus (EBV) EBNA1 and EBNA2, hepatitis B virus (HBV) X protein, and the SV40 ST have all been shown to induce metastasis, through a variety of mechanisms including disruption of cellular adhesion, cytoskeletal reorganisation and gene expression modulation (60-63). The utilisation of the actin cytoskeleton in many viral processes, including cell transformation, has also been reported (reviewed in (64)). In this report, we show that MCPyV ST also affects the actin cytoskeleton. Expression of MCPyV ST drives cell motility in a multistep process that involves the upregulation of a number of actin-associated proteins, forming filopodia-like structures, through relocalisation of filopodia-associated proteins.

The interaction of MCPyV ST with Ser/Thr cellular phosphatases PP2A Aα, PP2A Aβ and PP4C has been well documented (23, 37). Moreover, MCPyV ST interaction with PP4C, seems to be important in promoting cell motility (43). PP4C has been implicated in apoptosis, DNA mutation, and cell proliferation (65), as well as a number of cell signalling pathways (66). In addition, it has been found to be upregulated in some cancers (67) and we have previously reported its involvement in destabilising the microtubule network to promote cell motility (43). However, this is the first report of PP4C being implicated in actin dynamics, as MCPyV ST-induced filopodia formation is dependent upon the interaction.

We report that the Rho-family GTPases appear to be involved in MCPyV ST-induced cell motility. Other oncogenic viruses have been reported to affect the Rho-family GTPases. The best-known example is SV40 ST, whose activity leads to the rearrangement of filamentous actin networks, including Rac-induced lamellipodia formation, Cdc42 filopodia formation and loss of RhoA-dependent stress fibres. Levels of Rac1 and Cdc42 are increased in cells expressing SV40 ST, while levels of RhoA are decreased (63). Thus it is possible that a similar process is occurring in cells expressing MCPyV ST, except through interaction with PP4C instead of PP2A. Our results also show that MCPyV ST motility seems to be dependent on Cdc42 and RhoA, as is MCPyV ST-
induced filopodia formation. In addition, phosphorylation has been reported to negatively regulate the activity of RhoA (68, 69), and to affect the signalling of Cdc42 and Rac1 (70). However, our results suggest that MCPyV ST-PP4C interaction is not involved in modulating this effect directly.

Finally, we implicate integrins, including β1, in MCPyV ST-induced cell motility and filopodia formation. Integrins are cellular receptors that are known to be important in cell motility, particularly in Rho-family GTPase cycling (71). Initially, an integrin inhibitor was used to observe whether integrins were important in MCPyV ST-specific cell motility. RGDS is a tetrapeptide found on fibronectin, fibrinogen α, and von Willebrand factor (72, 73), and interacts with α5β1 and αvβ3 integrins (57). Our results indicate that with increasing concentrations of RGDS, a reduction in MCPyV ST-induced cell motility is observed, and that RGDS also affects MCPyV ST-induced filopodia formation. We investigated β1 integrin, as it is expressed by HEK-239 cells. A number of studies have shown the significance of phosphorylation in the activity of integrins, including β1 (56, 59, 74). Whilst the cellular phosphatase PP2A has been shown to dephosphorylate β1 integrin at Thr788/789 (59), our studies using the transdominant PP4C mutant and specific MCPyV ST mutants clearly show a role for PP4C. Interestingly, in the absence of MCPyV ST the PP4C transdominant mutant had no impact on integrin phosphorylation, indicating that PP4C function maybe specifically redirected in the presence of MCPyV ST. Parallels can be found in the repurposing of key host factors by virus oncoproteins, for example subversion of E6AP activity by the HPV E6 protein to ubiquity late cellular p53. Our results show decreased Thr788/789 phosphorylation upon MCPyV ST expression, and that this is dependent on the interaction of MCPyV ST with PP4C. We thus suggest a mechanism whereupon this interaction leads to the dephosphorylation of β1 integrin, which in turn activates the cell motility pathway. However, we cannot rule out the involvement of either other changes in the phosphorylation status of further integrins or indeed the other β1 integrin phosphorylation sites, including for example S785, which has been implicated in changes in cell motility in chicken cell lines (74).

Our overall findings suggest a possible mechanism (Fig. 9), whereby the interaction of MCPyV ST with PP4C leads to the dephosphorylation of one or more integrins, including β1. These changes may then contribute to the cell motility cascade, through the Rho-family GTPase modulators, leading to increased filopodia formation and cell motility. These findings highlight the importance of the MCPyV ST-PP4C interaction in promoting the metastatic phenotype of MCC. Therefore, this interaction may be a viable drug target. Currently, treatment of MCC depends on the disease stage, with surgical excision, lymph node dissection and adjuvant radiotherapy being the standard. Metastasised MCC is treated with various regimens of broad-spectrum chemotherapy, such as anthracyclines, cyclophosphamide, etoposide, and platinum derivatives, alone or in combination. Over half of MCC patients respond to chemotherapy, but the median survival is 21.5 months (75). Potential virus-related drug targets are being identified, particularly for MCPyV LT and ST. Type I interferon (IFN) reduces LT expression and inhibits cell viability in MCPyV-positive MCC cell lines (76), but has failed to induce a clinical response in patients (77).
YM155, an inhibitor of survivin, a cellular protein upregulated by MCPyV LT important for the survival of MCPyV-positive MCC cell lines, has shown a cytostatic effect in MCC xenograft tumours in mice (78, 79). Finally, the small-molecule tyrosine kinase inhibitor, pazopanib (80), is currently undergoing phase II clinical trials. The field remains open for novel drugs.

In summary, we describe a novel mechanism by which a human tumour virus induces cell motility and cancer metastasis, as such it provides new opportunities for therapeutic interventions for disseminated MCC.
MATERIALS AND METHODS

Plasmids and antibodies. Expression vectors for EGFP-ST and EGFP-STΔ95-111 have been previously described (37, 43). EGFP-ST-R7A, EGFP-STΔ95-111, and EGFP-ST103A were produced using the Q5 site directed mutagenesis kit (New England Biolabs) according to the manufacturer’s protocol and have been previously characterised (50). Sequence verified mutants were cloned into EGFP using EcoRI and BamHI restriction sites. EGFP-mDia2 was provided by Shuh Narumiya and pcDNA5-IRS53-myc was provided by Laura Machesky. Transdominant mutants pcDNA3-GFP-Cdc42-T17N, pcDNA3-GFP-Rac1-T17N, and pcDNA3-GFP-RhoA-T19N were purchased from Addgene. MCPyV ST-tagging shRNA plasmids were kindly provided by Dr Masa Shuda, Pittsburgh. Antibodies for Arp3, cortactin, and coflin were purchased from Genetex and used at 1:500 dilution, the p-Cdc42/Rac1 antibody (Cell Signaling Technologies) and the p-ß1-Thr788/789 antibody (Abcam), Myosin X (kindly provided by Michelle Peckham, Leeds) were used at 1:100 dilution, antibodies for Flag, myc and HA (Sigma-Aldrich) and GFP (Living Colours) were used at 1:200 dilution. All antibodies used for immunofluorescence were diluted 1:200.

Chemicals. Rho-family GTPase inhibitors ML141 (Sigma Aldrich), NSC23766 (Santa Cruz Biotech), ZCL278 (Tocris Bioscience), and Rhosin (Merck Milipore) were used at 15 μM, 50 μM and 30 μM in i293-GFP and i293-GFP-ST and at 30 μM, 75 μM and 60 μM in MCC13s. The integrin inhibitor RGDS (Tocris Bioscience) was used at a range of concentrations (see Results) on both 293-derived cells and MCC13 cells. Cell toxicity was measured using a MTS-based CellTiter 96 AqueousOne Solution Proliferation assay (Promega), as previously described (81).

Mammalian cell culture. HEK-293 Flip-In cell line was purchased from Invitrogen. i293-ST (37), i293-GFP, and i293-GFP-ST cell lines were derived from HEK-293 Flip-Ins using manufacturer’s protocol as previously described (37). HEK-293s (ECACC) and derivative cells were grown in 10% were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin as previously described (82). The MCPyV positive MCC cell lines, WaGa, was grown in RPMI 1640 (Sigma) supplemented with 10% FBS. MCC13 cell line (ECACC) were maintained in RPMI 1640 media supplemented with 15% FBS and 1% penicillin/streptomycin. Primary normal human epidermal keratinocytes (Promocell) were cultured in serum free keratinocyte media (Gibco 17005-34) with 5ng/ml EGF supplemented. Primary Normal Adult Dermal Fibroblast cells (ATCC) were cultured using a Fibroblast Growth Kit–Serum-Free kit (ATCC). ST-FLAG, EGFP and EGFP-ST expression was induced from i293-ST [34], i293-GFP, and i293-GFP-ST cells respectively with 2 μg/ml Doxycycline hyclate for up to 48 hours. Cells were plated into 6-well plates and transfections routinely used 1 μg plasmid DNA and Lipofectamine 2000 (Life Technologies) or 5 μg plasmid DNA and nucleofection (Lonza) following the manufacturer’s instructions.

Multicolour immunohistochemistry. Formalin-fixed, paraffin-embedded (FFPE) sections from primary MCC tumours were purchased from Origene and analysed as previously described (83). Primary antibodies were: CK20 (Dako, dilution 1:50), MCPyV LTA CM2B4 (Santa Cruz...
Biotechnology, dilution 1:125) and Anti-Cortactin (Abcam, dilution 1:250). An isotype-matched irrelevant antibody was used as a negative control on sections of tissues in parallel, a rabbit polyclonal isotype control antibody (Abcam) was used to match the cortactin primary antibody. Sections were incubated with appropriate secondary antibodies labelled with different fluorochromes (Alexa Fluor 488 IgG2B, 633 IgG2A, Invitrogen, and IgG (H+L)-TRITC, Jackson ImmunoResearch). All slides were mounted with Immuno-Mount and images were captured with a Zeiss LSM 510 confocal microscope.

**Immunoprecipitation assays and Immunoblotting.** Co-immunoprecipitations, in addition to subsequent protein analysis by SDS-PAGE and western blot, were performed as previously described (84). Tumour and skin samples were homogenised in 5 volumes of suspension buffer (0.1M NaCl, 10mM Tris.Cl [pH 8.0], 1mM EDTA and 0.1 mg/ml AEBSF protease inhibitor [Roche, Germany]), as previously described (85). In contrast, cells were lysed in a modified RIPA buffer (50 mM Tris-Cl pH 7.6, 150 mM NaCl, 1% NP40), supplemented with protease inhibitor cocktail (Roche) [73]. For phosphorylation studies, cells were lysed in a modified buffer (20 MM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM Na2O2P2B, 1 mM EDTA, 1 mM Ta3VO4, 10% glycerol, 1% Triton). Proteins were separated by SDS-PAGE, before transfer onto nitrocellulose membrane (Hybond C extra, Amersham Biosciences). Membranes were probed with the appropriate primary and HRP-conjugated secondary antibodies. Proteins were detected using EZ-ECL enhancer solution (Geneflow) as previously described [74]. Densitometry was performed using ImageJ software.

**Live cell imaging.** Cell motility was analysed using an Incucyte kinetic live cell imaging system as directed by the manufacturer. HEK293 cells or i293-GFP/i293-GFP-ST cells were seeded at a density of 25,000 cells per well of a 6 well plate, MCC13 cells were seeded at a density of 100,000 cells per well of a 6 well plate. After 12h, the cells were transfected with 1 μg of DNA per well and/or induced using doxycycline hyclate. For transfected cells, media was changed after 6h (HEK-293 or derivatives) or 12h (MCC13). If appropriate, cells were treated with inhibitors for 24h pre-imaging. Imaging was performed for a 24h period, with images taken every 30 minutes. Cell motility was then tracked and analysed using ImageJ software.

**Immunofluorescence.** Immunofluorescence was carried out as previously described (86). If appropriate, cells were treated with inhibitors for 24h pre-fixing. Cells were viewed on a Zeiss LSM700 confocal microscope under an oil-immersion 63x objective lens. Images were analysed using the LSM imaging software. Filopodia were counted using ImageJ software.

**Activation Assay for RhoA and Cdc42.** The activation of RhoA or cdc42 was determined using pull-down assays for activated RhoA or activated cdc42, as previously described (87), using RhoA and cdc42 Activation Assay kits (Cell Biolabs), as directed by the manufacturer’s instructions. For the analysis of RhoA activation, cell lysates were incubated with Rhotekin RBD Agarose beads, which have a high affinity for GTP-RhoA. For the analysis of cdc42 activation, cell lysates were incubated with PAK1 PBD Agarose beads, which have a high affinity for GTP-cdc42. Affinity-
precipitated activated GTP-bound RhoA or cdc42 levels were then analysed by immunoblotting
using RhoA and cdc42-specific antibodies.

**qRT-PCR.** RNA was extracted from uninduced and induced i293-ST cells using TRIzol
(Invitrogen) (88). RNA was DNase treated using the Ambion DNase-free kit, as per the
manufacturer’s instructions, and RNA (1μg) from each fraction was reverse transcribed with
SuperScript II (Invitrogen), as per the manufacturer’s instructions, using oligo(dT) primers
(Promega). 10ng of cDNA was used as template in SensiMixPlus SYBR qPCR reactions
(Quantace), as per manufacturer’s instructions, using a Rotor-Gene Q 5plex HRM Platform
(Qiagen), with a standard 3-step melt program (95°C for 15 seconds, 60°C for 30 seconds, 72°C for
20 seconds) as previously described (89). With GAPDH as internal control mRNA, quantitative
analysis was performed using the comparative ΔΔcT method as previously described (90).

**ACKNOWLEDGEMENTS.** We are grateful to members of the Whitehouse laboratory for helpful
discussions. The authors would like to thank Masa Shuda, Shuh Narumiya, Laura Machesky and
Michelle Peckham for expression constructs and antibody reagents. The work was funded in parts
by a BBSRC DTP studentship (BB/J014443/1), MRC DTG studentship (95505126) and Royal
Society University Research Fellowship to JM (UF100419).
REFERENCES


FIGURE 1. MCPyV ST expression results in the upregulation of several actin-associated proteins. (A) (i) i293-ST cells remained uninduced or were incubated for 48 h in the presence of doxycycline hyclate or (ii) MCC13 cells were transfected with 1 μg EGFP or EGFP-ST for 12 h. Cell lysates were then probed with Arp3-, cortactin- and coflin-specific antibodies. GAPDH was used as a measure of equal loading, the 2T2 hybridoma was used to confirm MCPyV ST expression. (B) Densitometry quantification of the western blots was carried out using the Image J software and is shown as a percentage of relative densitometry to the loading control, GAPDH (n=3). (C) Total RNA was extracted from uninduced or induced i293-ST cells after 24 hours and relative transcript levels analysed by qRT-PCR using GAPDH as a reference. Fold increase was determined by ΔΔcT and statistical significance analysed using a non-paired t-test. Data from 3 independent experiments is presented as fold increase versus uninduced control, * = p<0.001. (D) FFPE sections of two primary MCC tumours were stained with CK20, MCPyV LT and cortactin-specific antibodies or an isotype negative control. Sections were then incubated with Alexa Fluor-labelled secondary antibodies and analysed using a Zeiss LSM 510 confocal laser scanning microscope. (E) Immunoblot analysis was performed on the cellular lysates of two independent MCC tumour samples and a negative control non-tumour cadaveric skin sample using Arp3- and cortactin-specific antibodies. GAPDH was used as a measure of equal loading, the 2T2 hybridoma was used to confirm MCPyV ST expression. (F) Densitometry quantification of the western blots was carried out using the Image J software and is shown as a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance, * = p<0.01. (G) The MCPyV-positive MCC cell line was transduced with lentivirus expressing a scrambled shRNA or ST-targetted shRNA. Upon ST depletion cell lysates were probed with Arp3- and cortactin-specific antibodies. GAPDH was used as a measure of equal loading, the 2T2 hybridoma was used to confirm MCPyV ST expression. (H) Densitometry quantification of the western blots was carried out using the Image J software and is shown as a percentage of relative densitometry to the loading control, GAPDH (n=3).

FIGURE 2. MCPyV ST expression results in an increase in the numbers and length of actin-based protrusions. (A) HEK-293  (B) MCC13 (E) Primary Epidermal keratinocytes (G) Primary Dermal Fibroblast cells were transfected with 1-5 μg of either EGFP or EGFP-ST. Cells were fixed after 24 h and stained with rhodamine-phalloidin. Slides were then analysed using a Zeiss LSM 700 confocal laser scanning microscope. (C), (D), (F) and (H) The number and length of actin-based protrusions in each cell line were analysed for 100 cells per condition using ImageJ software. Protrusion length is presented as a percentage of total number of protrusions.
FIGURE 3. Screening of actin-associated proteins suggests MCPyV ST expression induces filopodia formation. (A) HEK-293 cells were co-transfected with 1 μg of EGFP-mDia2 and empty control vector or co-transfected with 1 μg of EGFP-mDia2 and ST-Flag. 24 h later, cells were fixed, permeabilised and GFP fluorescence analysed by direct visualisation, in addition cells were stained with rhodamine-phalloidin and a Flag-specific antibody. (B) HEK-293 cells were co-transfected with 1 μg of EGFP and IRSp53-myc or co-transfected with 1 μg of EGFP-ST and IRSp53-myc. 24 h later cells were fixed, permeabilised and GFP fluorescence analysed by direct visualisation, in addition cells were stained with rhodamine-phalloidin and a Myc-specific antibody. (C) HEK-293 cells were transfected with 1 μg of EGFP or EGFP-ST. 24 h later cells were fixed, permeabilised and GFP fluorescence analysed by direct visualisation, in addition cells were stained with rhodamine-phalloidin and a Myosin X-specific antibody. The expanded box shows myosin X-staining at the tips of filopodia. All slides were analysed using a Zeiss LSM 700 confocal laser scanning microscope.

FIGURE 4. MCPyV ST interaction with cellular phosphatases is required for filopodia formation. (A) HEK-293 cells were transfected with 1 μg EGFP, EGFP-ST, EGFP R7A, EGFP-STΔ95-111, EGFP-ST102A or EGFP-ST103A. 24 h later cells were fixed and GFP fluorescence analysed by direct visualisation, in addition cells were stained with rhodamine-phalloidin. All slides were analysed using a Zeiss LSM 700 confocal laser scanning microscope. (B) The number and length of actin-based protrusions were analysed for 50 cells per condition using ImageJ software. Protrusion length is presented as a percentage of total number of protrusions. (C) HEK-293 cells were transfected with 1 μg of EGFP, EGFP-ST, EGFP-STΔ95-111, EGFP-ST102A or EGFP-ST103A. 24 h later cell lysates were incubated for either GFP-TRAP agarose beads. Pulldowns were then immunoblotted with PP2A Aβ and PP4C-specific antibodies. A GFP-specific antibody was used to confirm the expression of the EGFP-tagged MCPyV ST constructs. (D) HEK 293 cells or MCC13 cells were transfected with 1 μg EGFP, EGFP-ST, EGFP-ST102A or EGFP-ST103A. After 24 h, cell motility was analysed using an Incucyte kinetic live cell imaging system. Images were taken for 30 minutes for a 24 hour period. Movement is represented as average distance travelled compared to control EGFP-transfected cells was measured in μM (n=25 per condition) and significance was tested using a 3-tailed Student’s t-test, * = p<0.01. (E) HEK-293 cells were transfected with 1 μg of EGFP or EGFP-ST in the absence or presence of the PP4C transdominant mutant, PP4-R1. 24 h later cells were permeabilised, fixed and GFP fluorescence analysed by direct visualisation, in addition cells were stained with rhodamine-phalloidin and an HA-tag-specific antibody. All slides were analysed using a Zeiss LSM 700 confocal laser scanning microscope. (F) The number and length of actin-based protrusions were analysed for 50 cells per condition using ImageJ software. Protrusion length is presented as a percentage of total number of protrusions.
FIGURE 5. Live cell imaging shows a dependence of MCPyV ST-induced cell motility on Cdc42 and RhoA. (A) i293-EGFP and i293-EGFP-ST cells were induced using doxycycline hyclate or (C) MCC13 cells were transfected with 1µg of EGFP and EGFP-ST. Cells were then treated with 1 µg/µl DMSO, 15 µM of ML141, 50 µM of NSC23766 or ZCL278, or 30 µM of Rhosin. After 24 h, cell motility was analysed using an Incucyte kinetic live cell imaging system. Images were taken for 30 minutes for a 24 h period. The movement of cells were then tracked using Image J software. (B) and (D) The average distance travelled was measured in µm (n=25 per condition) and significance was tested using a 3-tailed Student's t-test, * = p<0.01.

FIGURE 6. MCPyV ST-induced filopodia formation is dependent on the activity of Rho-family GTPases. (A) HEK-293 cells were transfected with 1 µg EGFP or EGFP-ST and treated with 1 µg/µl DMSO, 15 µM of ML141, 50 µM of NSC23766 or ZCL278, or 30 µM of Rhosin for 24 h. After 24 h cells were fixed and GFP fluorescence analysed by direct visualisation, in addition cells were stained with rhodamine-phalloidin. (C) HEK-293 cells were transfected with 1 µg EGFP-ST or cotransfected with 1 µg ST-Flag and pcDNA5-GFP-Cdc42-T17N, pcDNA5-GFP-Rac1-T17N, or pcDNA5-GFP-RhoA-T19N. 12 hours later cells were fixed, permeabilised and stained with rhodamine-phalloidin and a Flag-specific antibody. All slides were analysed using a Zeiss LSM 700 confocal laser scanning microscope. (B) and (D) The number and length of actin-based protrusions were analysed for 50 cells per condition using ImageJ software. Protrusion length is presented as a percentage of total number of protrusions. (E) HEK-293 cells were transfected with 1 µg EGFP or EGFP-ST and after 24 hours cell lysates were incubated with either PAK1 PBD or Rhotekin RBD Agarose beads. Pulldowns were then immunoblotted with Cdc42 and RhoA-specific antibodies and the 2T2 hybridoma was used to confirm MCPyV ST expression.

FIGURE 7. Integrin inhibitor RGDS reduces MCPyV ST-induced cell motility and filopodia formation. (A) i293-GFP or i293-GFP-ST were transfected with 1 µg HA-Cdc42 or HA-Rac1 and then induced with doxycycline hyclate and for 6 h. Cell lysates were probed for phosphorylated Cdc42/Rac1 at the S71 residue. HA-tag and GAPDH-specific antibodies were used to measure equal loading, 2T2 was used to probe for MCPyV ST expression. (B) i293-EGFP and i293-EGFP-ST cells were induced using doxycycline hyclate or (D) MCC13 cells were transfected with 1µg of EGFP and EGFP-ST. After 24 hours cells were treated with 1 µg/µl DMSO or 1 µM, 10 µM, 50 µM, or 100 µM RGDS. After 24 h, cell motility was analysed using an Incucyte kinetic live cell imaging system. Images were taken for 30 minutes for a 24 h period. The movement of cells were then tracked using Image J software. (C) and (E) The average distance travelled was measured in µm (n=25 per condition) and significance was tested using a 3-tailed Student’s t-test, p<0.001. (F) HEK-293 cells were transfected with 1 µg EGFP or EGFP-ST, then treated with 100 µM RGDS. After 24 h cells were fixed and GFP fluorescence analysed by direct visualisation, in addition cells...
were stained with rhodamine-phalloidin. (G) The number and length of actin-based protrusions were analysed for 50 cells per condition using ImageJ software. Protrusion length is presented as a percentage of total number of protrusions.

**FIGURE 8.** MCPyV ST expression reduces the phosphorylation levels of β1 integrin at Thr 788/789 residues. (A) (i) HEK-293 cells were transfected with 1 µg EGFP, EGFP-ST, EGFP-ST102A or EGFP-ST103A. After 24 hours, cell lysates were probed for phosphorylated Thr 788/789 residues of β1 integrin. GAPDH was used to measure equal loading. 2T2 was used to probe for MCPyV ST expression. (ii) Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance, * = p<0.01. (B) (i) HEK-293 cells were transfected with 1 µg EGFP, EGFP-ST in the absence or presence of PP4C transdominant mutant, PP4-RL. After 24 hours, cell lysates were probed for phosphorylated Thr 788/789 residues of β1 integrin. GAPDH was used to measure equal loading. 2T2 was used to probe for MCPyV ST expression. (ii) Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance, * = p<0.01.

**Figure 9.** Schematic representation of MCPyV ST-induced cell motility. The MCPyV ST-PP4C interaction leads to the dephosphorylation of β1 integrin. This change in phosphorylation status leads to the initiation of the cell motility pathway, mediated via the Rho-family GTPases.
FIGURE 2

A

Phase  |  GFP   | Phalloidin  | Merge
--- | --- | --- | ---
EGFP  | ![Image](image1) | ![Image](image2) | ![Image](image3)
EGFP-ST | ![Image](image4) | ![Image](image5) | ![Image](image6)

B

Phase  |  GFP   | Phalloidin  | Merge
--- | --- | --- | ---
EGFP  | ![Image](image7) | ![Image](image8) | ![Image](image9)
EGFP-ST | ![Image](image10) | ![Image](image11) | ![Image](image12)

C

% of total number of protrusions vs. Protrusion length, μm

D

% of total number of protrusions vs. Protrusion length, μm

E

GFP  | Phalloidin  | Merge
--- | --- | ---
EGFP  | ![Image](image13) | ![Image](image14)
EGFP-ST | ![Image](image15) | ![Image](image16)

F

% of total number of protrusions vs. Protrusion length, μm

G

GFP  | Phalloidin  | Merge
--- | --- | ---
EGFP  | ![Image](image17) | ![Image](image18)
EGFP-ST | ![Image](image19) | ![Image](image20)

H

% of total number of protrusions vs. Protrusion length, μm
FIGURE 3

A

EGFP  
mDia2   Phalloidin  Flag-ST  Merge

Flag

Flag-ST

B

GFP   Phalloidin  IRSp53  Merge

EGFP

EGFP-ST

C

GFP    Phalloidin  Myosin X  Merge

EGFP

EGFP-ST
FIGURE 5

A  
EGFP  EGFP-ST

DMSO
ML141
NSC23766
Rhosin
ZCL278

B

Distance travelled, µm

EGFP  EGFP-ST

DMSO  15 µM  50 µM  30 µM  50 µM
ML141
NSC23766
Rhosin
ZCL278

C  
EGFP  EGFP-ST

DMSO
ML141
NSC23766
Rhosin
ZCL278

D

Distance travelled, µm

EGFP  EGFP-ST

DMSO  30 µM  100 µM  60 µM  100 µM
ML141
NSC23766
Rhosin
ZCL278
FIGURE 6

A

GFP  Phalloidin  Merge

EGFP  
EGFP-ST  
EGFP-ST ML141  
EGFP-ST NSC27366  
EGFP-ST RhoA

B

% of total number of protrusions

Protrusion length, μm

EGFP  EGFP-ST  EGFP-ST ML141  EGFP-ST NSC27366  EGFP-ST RhoA  EGFP-ST ZCL278

C

GFP  Phalloidin  ST-Flag  Merge

Mock  ST-Flag  Cdc42 TD  Rac1 TD  RhoA TD

D

% of total number of protrusions

Protrusion length, μm

Mock  ST-Flag  ST-Flag/Cdc42-TD  ST-Flag/Rac1-TD  ST-Flag/RhoA-TD

E

-  +  MCPyV ST

cdc42  PAK1 PBD Pulldown  Input Lysate

cdc42  RhoA

-  +  MCPyV ST

2T2  Rhotekin RBD Pulldown  Input Lysate
FIGURE 8

A (i)

Phospho Thr 877/798  
β1 integrin

2T2

GAPDH

(ii)

Densitometry normalised  
GAPDH

EGFP

EGFP-ST

EGFP-ST102A

EGFP-ST103A

B (i)

Phospho Thr 877/798  
β1 integrin

2T2

HA tag

GAPDH

(ii)

Densitometry normalised  
GAPDH

EGFP

EGFP-PP4C-TD

EGFP-ST

EGFP-PP4C-TD