Supervillin couples myosin-dependent contractility to podosomes and enables their turnover

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SUMMARY

Podosomes are actin-rich adhesion and invasion structures. Especially in macrophages, podosomes exist in two subpopulations, large precursors at the cell periphery and smaller podosomes (successors) in the cell interior. To date, the mechanisms that differentially regulate these subpopulations are largely unknown. Here, we show that the membrane-associated protein supervillin localizes preferentially to successor podosomes and becomes enriched at precursors immediately prior to their dissolution. Consistently, podosome numbers are inversely correlated with supervillin protein levels. Using deletion constructs, we find that the myosin II-regulatory N-terminus of supervillin (SV 1-174) is crucial for these effects. Phosphorylated myosin light chain (pMLC) localizes at supervillin-positive podosomes, and time-lapse analyses show that enrichment of GFP-supervillin at podosomes coincides with their coupling to contractile myosin IIA-positive cables. We also show that supervillin binds only to activated myosin IIA, and a dysregulated N-terminal construct (SV 1-830) enhances pMLC levels at podosomes. Thus, preferential recruitment of supervillin to podosome subpopulations may both require and induce actomyosin contractility. Using siRNA and pharmacological inhibition, we demonstrate that supervillin and myosin IIA cooperate to regulate podosome lifetime, podosomal matrix degradation and cell polarization. In sum, we show here that podosome subpopulations differ in their molecular composition and identify supervillin, in cooperation with myosin IIA, as a critical factor in the regulation of podosome turnover and function.
INTRODUCTION

Protease-driven invasive cell migration depends on contact with the extracellular matrix (ECM) and degradation of matrix material (Friedl and Wolf, 2009). Both properties are combined in podosomes and invadopodia, which constitute a subset of cell-matrix contacts with an inherent ability to locally degrade the ECM. These structures, collectively called “invadosomes”, promote matrix degradation through release of ECM-lytic enzymes such as matrix metalloproteinases or ADAMs (Destaing et al., 2011; Linder, 2009; Linder et al., 2011; Saltel et al., 2011). Podosomes are formed constitutively in monocytic cells such as macrophages (Linder et al., 1999), dendritic cells (Burns et al., 2001) and osteoclasts (Destaing et al., 2003), and are also generated in other cell types such as endothelial cells (Moreau et al., 2003; Osiak et al., 2005; Tatin et al., 2006) or smooth muscle cells (Gimona et al., 2003). Current evidence is that these or similar structures exist in vivo, where they potentially regulate invasive cell migration in the context of transendothelial diapedesis (Carman et al., 2007) or vascular remodelling (Linder et al., 2011; Murphy and Courtneidge, 2011; Rottiers et al., 2009; Saltel et al., 2011).

Podosomes consist of an inner core of F-actin and actin-associated proteins, including Arp2/3 complex (Linder et al., 2000a), cortactin (Bowden et al., 2006), gelsolin (Chellaiah et al., 2001), and Tks5 (Crimaldi et al., 2009; Thompson et al., 2008). Podosome cores are surrounded by a ring structure of adhesion plaque proteins such as talin or vinculin (Tarone et al., 1985; Zambonin-Zallone et al., 1989). Podosomes are highly dynamic structures with a lifetime of 2-12 min (Destai

Especially in monocytic cells, podosomes are formed constitutively and exist in two subpopulations: (i) larger structures near the cell edge, called precursors, which undergo fission and fusion processes, and (ii) smaller podosomes in the cell interior (Evans et al., 2003; Kopp et al., 2006). Following the established nomenclature of calling larger podosomes “precursors” (Evans et al., 2003; Kopp et al., 2006), we propose to call the subpopulation of smaller podosomes “successors”. At the leading edge of a migrating cell, successors are found behind precursors, and many of the successors form after fission of precursors.

Current knowledge about the differential regulation of podosome subpopulations is limited. The kinesin KIF1C influences fission of precursors (Kopp et al., 2006) while the kinesin KIF9 contacts mostly successors (Cornfine et al., 2011). However, neither protein is a podosome component, and actual molecular differences between the two podosome subpopulations have not been described so far.

We show here that the membrane- and cytoskeleton-associated protein supervillin localizes preferentially to successor podosomes, but is also recruited to precursors upon their dissolution. Supervillin is a member of the villin/gelsolin family that associates tightly with cholesterol-rich membrane signaling domains (Nebl et al., 2002; Pestonjamasp et al., 1997). A multi-domain protein, supervillin interacts with many cytoskeletal proteins, including F-actin, myosin II, the long form of myosin light chain kinase (L-MLCK), and as many as five microtubule-dependent motors (Chen et al., 2003; Smith et al., 2010; Takizawa et al., 2007; Takizawa et al., 2006). Supervillin induces L-MLCK-dependent myosin II contractility via its first N-terminal 171 amino acids, while residues 342-571 negatively regulate focal adhesions in COS7 cells (Takizawa et al., 2007; Takizawa et al., 2006). Interestingly, supervillin localization at invadopodia is associated with increased matrix degradation by MDB-MB-231 cells (Crowley et al., 2009), and supervillin binds directly to both cortactin and Tks5 (Crowley et al., 2009; Smith et al., 2010). Our data now identify supervillin as the first podosome component to localize differentially to podosome subpopulations and show that supervillin couples myosin-dependent contractility to podosomes, thus enabling their turnover.
MATERIALS AND METHODS

Cell isolation and cell culture
Human peripheral blood monocytes were isolated from buffy coats (kindly provided by Frank Bentzien, University Medical Center Hamburg-Eppendorf) and differentiated into macrophages as described previously (Linder et al., 1999).

Transfection of cells
Cells were transiently transfected using the Microporator (Peqlab, Erlangen, Germany). For transfection of primary human macrophages, the following parameters were used: 1000 V, 40 ms, 2 pulses, 0.5 µg DNA per 1x10^5 cells.

Expression vectors
GFP-supervillin, mRFP-supervillin, GFP-SV 171-1792, GFP-SV Δ343-570, GFP-SV 1-830, GFP-SV 1-174, GFP-SV 1010-1792, GFP-SV 830-1792, GFP-SV 571-830, GFP-SV 174-343, GFP-SV 343-570 were generated as described (Chen et al., 2003; Fang et al., 2010; Takizawa et al., 2007; Takizawa et al., 2006; Wulffkuhle et al., 1999). Lifeact-GFP was a kind gift of Dr. Michael Sixt ( Martinsried, Germany) (Riedl et al., 2008), GFP-myosin IIA was a kind gift of Dr. Robert Adelstein (Bethesda, USA) (Wei and Adelstein, 2000). GFP-L-MLCK was a kind gift by Anne Bresnick (New York, USA) (Poperechnaya et al., 2000).

SiRNA-induced knockdown
For siRNA-induced knockdown, specific targeting siRNA was generated as follows: 5’-GAUCUGAACUCGUUUGAGCTT-3’ (myosin IIA, Eurofins MWG, Ebersberg, Germany) (Vicente-Manzanares et al., 2009); 5’-GGCCAAACCUGCCGAAUAAA -3’ (myosin IIA, Eurofins MWG, Ebersberg, Germany) (Ivanov et al., 2008); 5’-CAGCCAUAAGGAAUCUAUAUUGCU-3’ (supervillin Stealth siRNA, Invitrogen, Oregon, USA) (Crowley et al., 2009); 5’-UAUUAAGGUAGAAAGGUUGAUUCGC-3’ (supervillin, Invitrogen, Oregon, USA) (Smith et al., 2010); 5’-CAGUUCUAUGGAGCGACAGCUACA-3’ (gelsolin, Stealth siRNA, Invitrogen) (Crowley et al., 2009); a pool of 5’-GGGAUGACGAUGCCAAGUA-3’ and 5’-GCAUGAUCACGGCU-3’ (L-MLCK and S-MLCK, Dharmacon, Thermoscientific); 5’-AGGUAGUGUAACCAGCCUUGU-3’ (firefly luciferase negative control siRNA, Dharmacon, Thermo Scientific (catalogue no. D-001210-02-20, Lafayette, ...
CO, USA) (Kopp et al., 2006). Primary human macrophages were transfected with siRNA (50 nM) twice at 0 h and 72 h, and evaluated after a further incubation period of 5 h.

U2OS cells (ATCC, Manassas, VA) were treated with control or supervillin-specific siRNA for 48 hours and lysed with 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.2% SDS, 50 mM Tris, pH 8.0, with a protease inhibitor cocktail (Sigma #8340). Lysates were sonicated, clarified and examined for depletion of supervillin by immunoblotting with H340 and HSV715 anti-supervillin antibodies (Suppl. Figure 1B).

**Immunofluorescence and microscopy**

Cells were fixed for 10 min in 3.7% formaldehyde and permeabilized for 5 min in ice-cold acetone. F-actin in podosome cores was stained with phalloidin coupled to Alexa Fluor568, Alexa Fluor 488, or Alexa Fluor647 (Invitrogen). Vinculin was stained using mouse monoclonal antibody (V9264, Sigma, St. Louis), myosin IIA with rabbit polyclonal antibody (M8064, Sigma), and gelsolin with mouse monoclonal antibody (G37820, BD Transduction Laboratories, Heidelberg, Germany). An affinity-purified rabbit polyclonal antibody against supervillin (H340) has been described previously (Nebl et al., 2002; Oh et al., 2003). The HSV715 antibody was generated against human supervillin aa 715-728 by GenScript (Piscataway, NJ) and affinity-purified as described (Nebl et al., 2002). Cells were stained for supervillin by fixation in -20°C methanol for 30 s, post-fixed with 3.7% formaldehyde for 20 min, and permeabilized with 0.1% Triton X-100. Phosphorylated myosin light chain (pMLC) was detected using an antibody raised against a peptide corresponding to aa 12-27 in human MLC, KKRQRAT(pS)NVFAMFD, after conjugation to Keyhole Limpet Hemocyanin (ab2480, Abcam, Cambridge, UK). Secondary antibodies were Alexa Fluor568- or 488-labeled goat anti-mouse or goat anti-sheep (Invitrogen). Coverslips were mounted in Mowiol (Calbiochem, Darmstadt, Germany) containing p-phenylenediamine (Sigma-Aldrich) as anti-fading reagent and sealed with nail polish.

Microscopy was performed as described (Kopp et al., 2006). Images of fixed samples were acquired with a confocal laser-scanning microscope (Leica DM IRE2 with a Leica TCS SP2 AOBS confocal point scanner) equipped with an oil-immersion plan Apo 63x NA 1.4 objective. Acquisition and processing of images was performed with Leica Confocal Software (Leica, Wetzlar, Germany).

To detect pMLC at podosomes, a protocol was created in Volocity (Improvision, Coventry, UK) based on fluorescence intensity thresholds and object size. The ROI tool was
used to restrict the selected objects to the podosome area. Levels of pMLC at podosomes are expressed as ratios of pMLC area and podosomal area.

**Live Cell Imaging**

Images were acquired with a spinning disc confocal system (Spinning disc CSU22, Yokogawa, Japan) fitted on a Zeiss Axiovert 200M microscope with a temperature- and CO2-controllable environmental chamber (Solent Scientific, Regensworth, UK), oil immersion plan Apo 63x NA 1.4 objective and a CCD camera (EM-CCD C-9100-2, Hamamatsu, Japan). Acquisition and processing of images was performed with Volocity Software (Improvision). Cells were seeded on glass bottom dishes (Ibidi, Martinsried, Germany) at a density of $2 \times 10^5$ cells/dish and incubated 5 h or 72 h before the start of the experiment.

To evaluate podosome lifetime, cells were transfected with respective siRNAs. After 2 days, cells were re-transfected with siRNAs, as well as Lifeact-GFP and seeded on glass bottom dishes. After further incubation for 24 h, cells were imaged using time-lapse microscopy. Podosome lifetime was evaluated using Volocity (Improvision). Individual podosomes were tracked manually. Only those podosomes were evaluated that formed during imaging. At least ten podosomes were evaluated per cell.

**Immunoblotting**

Immunoblotting was performed by standard procedures, using primary antibodies: rabbit polyclonal myosin IIA (M8064, Sigma), mouse monoclonal myosin light chain kinase (MLCK, M7905, Sigma), mouse monoclonal actin (MAB1501, Millipore, Billerica, USA), and mouse polyclonal GFP, a kind gift of J. Faix (Medical University Hannover, Hannover, Germany). Secondary antibodies were horse-radish-peroxidase-coupled anti-mouse or anti-rabbit IgG (Dianova, Hamburg, Germany). Protein bands were visualized by using Super Signal Pico or Femto kit (Pierce, Rockford, USA) and X-Omat AR film (Kodak, Stuttgart, Germany).

**Reverse Transcriptase Reaction**

$6 \times 10^6$ cells were cultured for 7 d, and total RNA was isolated using 1 ml TRIzol Reagent (Invitrogen, Carlsbad, USA). DNA was removed by DNAse digestion (Novagen, Madison, WI). For cDNA synthesis, 1 µg of random primer (Promega) was annealed to 2 µg of RNA for 5 min at 70°C, and first strand synthesis was performed using Moloney murine leukaemia virus reverse transcriptase (Promega). Second strand synthesis was performed using an
oligonucleotide primer pair corresponding to nucleotides 3517-3569 and 4071-4094 of the SVIL coding gene, respectively. As a control for quantitative removal of residual DNA, oligonucleotide primers specific for an exon in the human $\beta$-actin gene were used, corresponding to nucleotides 1161–1142 and 716–735, respectively.

**Immunoprecipitation**

Immunoprecipitations of GFP-fusion proteins were performed using the µMACS GFP Tagged Protein Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach), according to the manufacturer’s instructions. For lysis, preparation of columns and washing, the following buffers were used: lysis buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 8.0, with Complete Mini protease inhibitor (Roche Diagnostics); buffer 1 (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 8.0); and wash buffer 2 (20 mM Tris HCl, pH 7.5).

**Matrix labelling and degradation**

Porcine gelatin (ROTH, Karlsruhe, Germany) was fluorescently labeled with NHS-rhodamine (ThermoScientific, Rockford, IL) (Chen, 1996). Coverslips were coated with labeled gelatin solution, fixed in 0.5% glutaraldehyde (ROTH) and washed with 70% ethanol and medium. Cells were seeded on coated coverslips with a density of $8 \times 10^5$ cells/coverslip. Cells previously transfected with siRNA and incubated for the times indicated were seeded on coated coverslips with a density of $8 \times 10^5$ cells/coverslip and incubated for further 5 h, followed by fixation and staining.

**Statistics**

ImageJ software was used to analyze rhodamine-labeled gelatin fluorescence intensity (Abramoff et al., 2004). Values of matrix degradation were determined by loss of fluorescence intensity, with the intensity of adjacent undegraded areas set to 100%. For comparability, laser intensity was not changed between measurements. For each value, 30 cells were evaluated in each of 3 experiments. Statistical analyses were performed with Graphpad Prism 5 software, using the Student’s two-tailed $t$ test, or ANOVA for multi-sample comparisons. Results are presented as means ± s.e.m., $P < 0.05$ was considered as statistically significant, $P < 0.01$ as highly statistically significant.
RESULTS

GFP-supervillin localizes to a subpopulation of podosomes

In a screen for differences in molecular composition between podosome subpopulations in primary human macrophages, we found that GFP-fused supervillin (GFP-SV) showed the most prominent differences. GFP-SV localized preferentially at successor podosomes in the inner regions of unpolarized cells (Fig. 1A-C) or at the rear of the podosome field of polarized macrophages (Fig. 1D-F). GFP-SV also was enriched in the cortex at the trailing edges of polarized cells (Fig. 1D-F).

A more detailed analysis showed that GFP-supervillin localizes to a distinct cap-like structure over the F-actin-rich cores of successor podosomes. Fluorescence intensity measurements of individual podosome cores (Fig. 1G-J) revealed that the intensities of GFP-supervillin and F-actin stained with Alexa Fluor 568-phalloidin showed similar profiles (Fig. 1J). In 3D reconstructions of optical z-stacks (Fig. 1K-M; Suppl. Video 1), GFP-supervillin (green) was found mostly at the top of the podosome core, although it partially co-localized with F-actin (red, overlap in yellow). This analysis was confirmed by co-staining of vinculin, a component of the podosome ring structure (Suppl. Fig. 1A-F). The highest intensities for GFP-supervillin-based fluorescence were found between the two maxima of vinculin-based fluorescence (Suppl. Fig. 1; Suppl. Video 1). By contrast, the supervillin-related protein gelsolin showed no differential localization to podosome subpopulations and was present at both precursors and successors (Suppl. Fig. 2). GFP-supervillin expression significantly enhanced the number of polarized cells (Fig. 1N), suggesting a role for supervillin in the development of a migratory phenotype (and see below). Quantification showed significant enrichment of GFP-supervillin at successor vs. precursor podosomes (Fig. 1O). Collectively, these data show that GFP-supervillin is enriched at successor podosomes, where it forms a cap structure on top of the F-actin-rich core, with only minor localizations at precursor podosomes and at the leading edge.

Endogenous supervillin localizes to macrophage podosomes

Supervillin has not previously been described in macrophages. Supervillin was detected on Western blots of macrophage lysates as an appropriately sized, ~205-kDa endogenous protein with the specific H340 antibody against the supervillin N-terminus (Nebl et al., 2002; Oh et al., 2003) (Fig. 1P). RT-PCR of macrophage mRNA using supervillin-specific primers also generated a band of the expected size (521 bp; Fig. 1Q). Mass spectrometric analyses of podosome-enriched macrophage fractions (Materials and Methods;
not shown) identified 13 supervillin-specific peptides in the podosome-containing fraction and none in the corresponding fraction from macrophages in which podosomes had been disrupted by prior addition of the Src kinase inhibitor PP2 (Gringel et al., 2006; Linder et al., 2000b). The presence of endogenous supervillin at macrophage podosomes was further confirmed by immunofluorescence staining with a newly developed antibody (HSV715) specific for amino acids 715-728 in human supervillin. Endogenous supervillin localized to F-actin-rich podosome cores and secondarily at subcortical actin filaments (Fig. 1R-T), confirming the results obtained with GFP-supervillin (Fig. 1A-M).

Measuring endogenous supervillin-based fluorescence intensity at randomly chosen successor and precursor podosomes, we found that successors have a significantly higher mean value of supervillin-based fluorescence (Suppl. Fig. 3). This difference is not as large as in cells expressing GFP-supervillin (Suppl. Fig. 3J vs. Fig. 1O), suggesting that supervillin overexpression may accentuate the difference between precursor and successor podosomes.

**Supervillin becomes enriched at dissolving precursor podosomes**

To study the dynamics of supervillin and to clarify its localization in living cells, macrophages were transfected with constructs encoding GFP-supervillin and either mRFP-β-actin or mRFP-Lifeact to label F-actin and analyzed by video microscopy. In polarized, i.e. migratory, cells, GFP-supervillin was largely absent from precursor podosomes at the leading edge (Fig. 2A; Suppl. Video 2), comparable to results with fixed cells (Fig. 1D-F). However, when cells retracted their leading edge and precursor podosomes were dissolved, the dissolving podosomes acquired GFP-supervillin, i.e. the gradual disappearance of the mRFP-β-actin (Fig. 2B-E, F-I) or mRFP-Lifeact signal (Fig. 2N-Q, R-U) was accompanied by a progressive enrichment of GFP-supervillin (Fig. 2B-E, J-M; N-Q, V-Y; Suppl. Videos 2, 3).

Consistent with a role in podosome dissolution, overexpression of GFP-supervillin led to a pronounced decrease in podosome numbers in macrophages transfected for 48 h or 72 h (Fig. 3). This effect was only marginally evident at 24 h (Fig. 3C vs. Fig. 3B), suggesting that podosome counts were relatively normal in our live cell imaging experiments (Fig. 2), when expression levels of GFP-supervillin averaged only ~2-fold that of endogenous supervillin (not shown).

**The myosin IIA-binding region is essential for supervillin effects on podosomes**

Based on overexpression of GFP-tagged supervillin fragments, multiple regions of supervillin contribute to the regulation of macrophage podosome numbers (Fig. 3). Deletion of the myosin IIA- and L-MLCK-binding N-terminal 170 amino acids in GFP-SV 171-1792
completely abrogated the reduction of podosome numbers observed with full-length GFP-supervillin (Fig. 3A; Fig. 3D vs. Fig. 3C). GFP-tagged supervillin residues 1-174 (GFP-SV 1-174), by themselves, only modestly decreased podosome numbers (Fig. 3E), suggesting that this sequence is necessary but not sufficient for this effect.

Other deletion constructs suggest contributory regulation by the TRIP6-binding, focal adhesion-targeting sequence in SV 343-571, by other supervillin N-terminal sequences, and by the supervillin C-terminus. Although expression of GFP-tagged SV 343-571, by itself, has only a delayed effect on podosome numbers (suppl. Fig. 4A), a supervillin construct lacking this region (GFP-SV Δ343-570) causes an even more pronounced reduction in podosome numbers than does full-length GFP-supervillin (Fig. 3F). GFP-SV 174-343 and GFP-SV 571-830, each of which also contains an F-actin binding site (Chen et al., 2003), also decrease podosome formation (Fig. 3G; suppl. Fig. 4B). A potential co-regulatory role for both N- and C-terminal sequences is inferred from the lack of an effect of either the entire supervillin N-terminus (GFP-SV 1-830; Suppl. Fig. 4C) or the gelsolin/villin homology region (GFP-SV 1010-1792; Suppl. Fig. 4D) on podosome numbers.

Of the implicated domains, only the myosin IIA- and L-MLCK-binding sequence, SV 1-174, appeared to regulate the targeting of supervillin to successor podosomes (Fig. 4). Successor podosome localization was preserved in SV 1-830 (Fig. 4A-C) and SV Δ343-570 (Fig. 4D-F), indicating that the minimal sequence(s) responsible for this phenomenon i) were present in the N-terminal half of the protein, but ii) not in the region 343-570, which is responsible for regulation of focal adhesions (Takizawa et al., 2006). Strikingly, GFP-SV 171-1792, which lacks the myosin IIA / L-MLCK-binding region, was not differentially localized, but was present at both precursor and successor podosomes (Fig. 4G-I). GFP-SV 1-174 by itself did not show a pronounced co-localization to podosomes, but induced the formation of F-actin- and myosin IIA-rich cables (Fig. 4J-L and Suppl. Fig. 5). Other GFP-tagged constructs containing isolated N-terminal domains (SV 174-343, SV 343-570, SV 571-830) localized to all podosomes and other F-actin structures, including cortical cables (Suppl. Fig. 6A-I), probably due to their inherent F-actin binding activities.

To test whether overexpression of an isolated F-actin binding region could exert dominant-negative effects on the distribution of the full-length protein, we examined the effects of co-overexpressing GFP-SV174-343 with mRFP-supervillin (Suppl. Fig. 7). GFP-SV174-343 localizes to both precursor and successor podosomes (Suppl. Fig. 7C), which contained much-reduced levels of mRFP-supervillin (Suppl. Fig. 7B vs. 7F). Competition for binding was less apparent at actin cables in the cell interior (Suppl. Fig. 7B), where the actin-
bundling sites in full-length mRFP-supervillin (Chen et al., 2003) probably out-compete GFP-SV 174-343 for binding to the aligned actin filaments.

Taken together, these results suggest that supervillin-mediated loss of podosomes requires the myosin IIA- and L-MLCK-binding sequence (SV 1-174), and that this sequence is important for the association of supervillin with successor podosomes.

**Supervillin and myosin IIA regulate podosome lifetime and matrix degradation**

To explore possible roles for both supervillin and myosin IIA in podosome regulation and function, we performed siRNA-based knockdown experiments in macrophages and analyzed podosome lifetime and matrix degradation (Fig. 5). Knockdown of either protein (to 17% of endogenous supervillin levels or 5% of endogenous myosin IIA levels), using validated siRNAs (see Materials and Methods) (Suppl. Fig. 8), resulted in a pronounced ~2-fold increase in lifetimes of both successors (Fig. 5A) and precursors (Fig. 5B). Strikingly, the combined knockdown of both proteins did not show an additive effect, with podosome lifetimes being statistically indistinguishable from the single knockdowns (Fig. 5A,B) (Note: because precursors can split and fuse, only precursors that formed and dissolved within the experimental period were evaluated). Although there is no change in the percentages of cells with >100 podosomes (Suppl. Fig. 9A), average numbers do drop from ~400 podosomes/cell in luciferase siRNA-treated cells to ~340 and ~270 podosomes/cell after treatment with supervillin- or myosin IIA siRNA, respectively (Suppl. Fig. 9B). The variability in the relative changes in podosome numbers over time also decreases somewhat (Suppl. Fig. 9C). Taken together, these results suggest that supervillin and myosin IIA function together in a pathway that stimulates podosome disassembly with a lesser effect on podosome formation.

By contrast, we found that supervillin and myosin IIA act synergistically to promote podosomal matrix degradation (Fig. 5C). Cells transfected with control siRNA against firefly luciferase or with specific validated siRNAs were scored into groups with low (0-25%) vs. high (26%-100%) (Fig. 5C) levels of degradation of the underlying matrix. Unlike in previous studies of matrix degradation by tumor cells (Crowley et al., 2009), knockdown of supervillin alone did not significantly alter levels of matrix degradation (Fig. 5C-E). Reduction of myosin IIA or the supervillin-related protein gelsolin did reduce the percentages of highly degradative cells (Fig. 5C,F,G). Knockdown of supervillin in combination with either myosin IIA or gelsolin (Fig. 5C,H,I), or the triple knockdown of all three proteins (Fig. 5C,J), further reduced the extent of matrix degradation, with knockdowns of the single proteins all being significantly different from the double or triple knockdowns. The bar diagram (Fig. 5C)
illustrates that the number of cells that degrade much less (≤ 25% of control) is highly and significantly increased from ~5% in control cells to ~45% in the double and triple knockdowns. These additive effects suggest that supervillin regulates a pathway that is functionally redundant with both myosin IIA and gelsolin and that all three proteins contribute to podosomal matrix degradation.

**Dissolving successor podosomes acquire GFP-myosin IIA**

We next investigated the potential co-regulatory roles of supervillin and myosin IIA in podosome turnover in more detail. Consistent with a role for the myosin IIA / L-MLCK-binding region in supervillin-dependent regulation of podosomes, myosin IIA localization partially overlapped with F-actin and supervillin at successor podosomes and at cable-like structures between these podosomes (Fig. 6A-D). In the cell periphery, where precursors are located, myosin IIA was present in dash-like accumulations, which intercalated between precursors but did not appear to be in direct contact with them (Fig. 6A-D). In polarized cells, GFP-supervillin co-localized with myosin IIA cables at the trailing cell edges (Fig. 6E-H). Supervillin-associated myosin IIA cables connecting successors were especially prominent in polarized cells (Fig. 6E-H). Such cable-like staining of myosin IIA was not found in control cells expressing GFP alone (not shown; Kopp et al., 2006). Thus, modest overexpression of supervillin induces the formation or stabilization of myosin IIA-containing cables between successor podosomes, suggesting the existence of contractile forces.

In live-cell imaging, small accumulations of GFP-myosin IIA often appeared in proximity to mRFP-tagged supervillin (mRFP-SV) at successor podosomes, especially at the rear of the podosome field (Fig. 6 I-O, Suppl. Video 4). Importantly, the appearance of these myosin IIA accumulations often preceded or coincided with podosome dissolution (Fig. 6I-M). During cell migration, successor podosomes enriched in supervillin and myosin IIA at the rear of the podosome field dissolved, and podosomes closer to the leading edge of the cell began to acquire mRFP-supervillin. Especially in cells expressing higher levels of myosin IIA, contractile myosin IIA cables were connected to successor podosomes (Fig. 6N,O; Suppl. Videos 5,6).

Interestingly, GFP-tagged L-MLCK localized to both successor and precursor podosomes in fixed and living cells (Suppl. Fig. 10; Suppl. Video 7). No changes in GFP-L-MLCK signals were discernible prior to dissolution of podosomes from either subpopulation. These observations suggest that L-MLCK, which is present at all podosomes, and supervillin at successor podosomes may recruit and/or activate myosin IIA, causing the dissolution of these structures.
Supervillin N-terminal regions induce myosin contractility

To interrogate the order of these interactions, we co-immunoprecipitated cellular myosin IIA (Fig. 6P,Q) and L-MLCK (Fig. 6P) with GFP-fused supervillin constructs from macrophage lysates. As reported using recombinant polypeptides (Takizawa et al., 2007), GFP-SV 1-174 binds to both myosin IIA and to L-MLCK (Fig. 6P). Strikingly, myosin IIA contractility was required for binding to supervillin because inhibition of the myosin ATPase activity with blebbistatin (10 µM) prior to cell lysis abolished the co-precipitation of myosin IIA with both GFP-SV 1-174 and GFP-SV -1-830 (Fig. 6Q). These results confirm that SV 1-174 interacts with cellular L-MLCK and myosin IIA and suggest that supervillin binds selectively to contractile myosin IIA filaments.

Because supervillin increases myosin II contractility during cell spreading (Takizawa et al., 2007), we examined the levels of phosphorylated myosin light chain (pMLC), a measure of myosin contractility (Matsumura, 2005; Vicente-Manzanares et al., 2009), in macrophages treated with supervillin-specific siRNA or luciferase siRNA (Fig. 7). Fluorescence-based measurements of pMLC associated with podosomes (Materials and Methods) showed that supervillin knockdown significantly decreased pMLC levels, as compared to controls (Fig. 7A,B,G). Cells expressing GFP alone, GFP-supervillin or GFP-SV 1-174 showed no significant differences in pMLC levels at podosomes, whereas overexpression of GFP-SV 1-830 resulted in a pronounced increase in podosomal pMLC (Fig. 7C-G), despite lower levels of expression as compared with GFP alone or GFP-SV 1-174 (not shown). Myosin IIA (white) also was more compactly associated with individual podosomes in cells expressing GFP-SV 1-830 (Fig. 7H-J; Suppl. Video 8), as compared to podosomes with GFP-supervillin (Fig. 7K-M; Suppl. Video 9). These data indicate that endogenous supervillin increases basal pMLC levels at podosomes and that SV 1-830 elevates the levels of pMLC at podosomes.

Supervillin localization at successor podosomes and supervillin-induced cell polarization both require myosin IIA and MLCK

To test whether myosin contractility increases the localization of supervillin at successor podosomes, siRNA-mediated knockdowns of myosin IIA, MLCK, or a combination were performed, using luciferase siRNA as a control (Fig. 8A-L; Suppl. Fig. 8). MLCK knockdown was accompanied by a large reduction in pMLC (Suppl. Fig. 8E), indicating an important regulatory role for MLCK in macrophage myosin II activation. The preferential
localization of GFP-supervillin to successor podosomes in control cells was significantly reduced by lowered levels of myosin IIA, MLCK, or both proteins (Fig. 8S). The difference between the single myosin IIA knockdown and double myosin IIA / MLCK knockdown conditions was not statistically significant. Differential supervillin recruitment to podosomes also was inhibited in cells treated with 2 µM blebbistatin (Fig. 8M-S), suggesting that MLCK-mediated activation of myosin IIA facilitates differential recruitment of supervillin to successor podosomes. This was further confirmed in live cell experiments, where addition of 2 µM blebbistatin to cells resulted in prominent recruitment of GFP-supervillin to precursor podosomes (Suppl. Video 10).

Similarly, both myosin IIA and MLCK are required for the increase in cell polarization observed after modest overexpression of GFP-supervillin in macrophages (Figs. 1N; 8T). Knockdown of supervillin, myosin IIA, MLCK, a combined knockdown of myosin IIA and MLCK, or blebbistatin treatment had no effect on control cells treated with either luciferase siRNA or DMSO (Fig. 8T). By contrast, all of these treatments significantly reduced the extent of cell polarization induced by overexpression of GFP-supervillin (Fig. 8T). These data suggest that supervillin-induced cell polarization is largely dependent on MLCK-mediated myosin IIA contractility and is probably related to the selective recruitment of supervillin to successor podosomes.
DISCUSSION

We here identify supervillin as the first protein that shows a differential localization to podosome subpopulations in primary macrophages. Supervillin localizes primarily as a cap-like structure above the actin-rich podosome cores, a location that is consistent with the presence of three F-actin binding sites within the protein (Chen et al., 2003). This localization also is consistent with the co-localization of cortactin and supervillin at the cytoplasmic apexes of Src-induced invadosomes (Crowley et al., 2009) and apparently overlaps with the podosomal localization of the formin FMNL1 (Mersich et al., 2010). These results point to a more complex inner architecture of podosomes than previously appreciated (Gimona, 2008; Linder and Aepfelbacher, 2003).

Supervillin promotes podosome dissolution by increasing the local activation of myosin II. GFP-supervillin reduces podosome numbers and its recruitment to podosomes coincides with the gradual disappearance of their actin-rich cores. Deletion of the myosin II- and L-MLCK-binding N-terminal sequence (SV 1-174) eliminates the reduction in podosome numbers and abolishes the differential localization of supervillin to successor podosomes. Conversely, the focal adhesion-targeting sequence in supervillin (SV 343-571) (Takizawa et al., 2006) is not necessary for the reduction of podosome numbers. Any supervillin construct with a single F-actin binding site localizes to podosomes, suggesting that while F-actin binding can mediate podosome targeting, SV 1-174 is essential for promoting the differential localization to successors and for podosome dissolution. Moreover, siRNA-mediated knockdown of supervillin, myosin II, or both together led to a ~2-fold increase in podosome lifetime, indicating function in a common pathway. Taken together, these data argue that supervillin-mediated myosin IIA activation is important for podosome turnover.

Our results confirm and extend earlier findings that supervillin induces myosin II hypercontractility (Takizawa et al., 2007; Takizawa et al., 2006), by showing for the first time that myosin II ATPase activity is required for the interaction with supervillin. (Note: myosin IIA is the predominant myosin II isoform in monocytic cells, such as macrophages (Maupin et al., 1994)). Myosin IIA and L-MLCK both co-immunoprecipitate with the supervillin N-terminus (SV 1-174), and the association with myosin IIA is blocked by the myosin II ATPase inhibitor blebbistatin. Moreover, pMLC levels at podosomes are reduced by the knockdown of supervillin, myosin IIA or MLCK. These observations are in line with the identification of supervillin as part of the blebbistatin-sensitive myosin II interactome at focal adhesions (Kuo et al., 2011).
Our data clarify the role of myosin II in podosome regulation. Myosin II has been localized to the ring structure of podosomes in both osteoclasts (Krits et al., 2002) and dendritic cells (van Helden et al., 2008). Although the exact influence of myosin activity on podosome regulation is under debate (Burgstaller and Gimona, 2004; Clark et al., 2006; Collin et al., 2008; Kopp et al., 2006), a possibly unifying concept is that a low, basal myosin II activity supports the formation and maintenance of podosomes (Burgstaller and Gimona, 2004), whereas sudden increases in myosin II activity trigger podosome dissolution (van Helden et al., 2008). As reported previously (Burgstaller and Gimona, 2004), we find that myosin IIA localizes around and between macrophage podosomes. We also show that these myosin IIA-positive structures co-stain for pMLC, confirming that these myosin cables are indeed contractile. GFP-myosin IIA is enriched only at the most rearward, mRFP-supervillin-positive, successor podosomes, and myosin IIA enrichment immediately precedes podosome dissolution. By contrast, despite MLCK regulation of supervillin localization to successor podosomes, GFP-tagged L-MLCK itself is found at both successor and precursor podosomes, and no changes in GFP-L-MLCK signals were discernible prior to dissolution of podosomes from either subpopulation. Overexpression of GFP-supervillin increases the recruitment of myosin IIA and pMLC at and between podosomes, an effect that is even more prominent after expression of GFP-SV 1-830. These results are in good keeping with the report that myosin II activation results in rear-localized disassembly of actin networks (Wilson et al., 2010) and suggest a sequence of events at podosomes during their dissolution.

We propose the following model for coordinate regulation of podosome turnover by supervillin and L-MLCK-mediated activation of myosin II (Fig. 9): Precursor podosomes form at the leading edge through supervillin-independent F-actin nucleation (Linder, 2007). L-MLCK, which is a major MLC regulatory kinase in macrophages (Suppl. Fig. 8), localizes to all podosomes, probably because of its interactions with F-actin (Hatch et al., 2001; Yang et al., 2006). Myosin IIA becomes activated at low levels around or at successor podosomes, probably though not necessarily by MLCK. Supervillin binding to the activated myosin stabilizes the contractile filaments and helps connect them to L-MLCK and F-actin at successor podosomes. The cross-bridging of L-MLCK and myosin IIA by supervillin (Takizawa et al., 2007) increases local MLCK-mediated phosphorylation of MLC, which promotes more supervillin binding to myosin IIA heavy chain in a positive feedback loop that eventually triggers podosome dissolution.

Myosin II activation at the cell posterior also should be amplified by supervillin, which would explain the observed supervillin- and myosin II-dependent increase in cell
polarization. Increased actomyosin contractility within the cell can break the symmetry of round, unoriented cells, leading to their spontaneous polarization and enhanced migration, even in the absence of a stimulatory gradient (Cramer, 2010). Thus, the effect of supervillin on cell polarity is not necessarily coupled to its effects on podosome dynamics, but would be consistent with a speculative role for podosome function in the regulation of directional migration of macrophages (Linder et al., 2011).

Our model also is consistent with observations that branched filament disassembly is triggered by increases in myosin II activation in both cellular and in vitro systems (Wilson et al., 2010; L. Blanchoin, personal communication). Myosin contractility promotes the progressive alignment of actin filaments into arrays that facilitate myosin-based filament sliding. Incorrectly aligned filaments are lost from the coalescing structure and eventually disassemble. This process is further enhanced by the presence of F-actin bundling proteins. Therefore, supervillin-mediated actin bundling (Chen et al., 2003) may contribute to podosome dissolution. Indeed, myosin II itself is an important F-actin bundling and stabilizing protein (Choi et al., 2008; Xu et al., 2001).

Other mechanisms for supervillin-mediated podosome dissolution also may be involved. For instance, supervillin binding to cortactin (Crowley et al., 2009) could inhibit the conformational change in cortactin required for cortactin stimulation of Arp2/3-mediated actin polymerization (Evans et al., 2011). Simultaneous binding of the supervillin N-terminus to L-MLCK and cortactin might potentiate the association of L-MLCK with cortactin, an interaction that abolishes cortactin-mediated actin nucleation (Dudek et al., 2002). Supervillin also could indirectly inhibit cortactin-mediated actin polymerization at podosome cores by promoting the local formation of contractile assemblies of actin filaments containing tropomyosin, which inhibits Arp2/3-mediated actin nucleation from existing actin filaments (Blanchoin et al., 2001; Higgs et al., 1999; Machesky et al., 1999). Supervillin binding and bundling of actin filaments (Chen et al., 2003) might also directly inhibit their ability to bind activated Arp2/3.

The relationship between supervillin levels and podosome numbers is multi-faceted and dependent on cell-specific factors that may limit supervillin function at different points in a cyclical process. For instance, overexpression of EGFP-supervillin in cultured cells increases the number of invadosomes/cell (Crowley et al., 2009), in contrast to the decrease in podosome numbers observed here. Nevertheless, the decreased numbers of podosomes/cell observed after supervillin knockdown in macrophages are consistent with a role for supervillin in podosome formation or upkeep that is outweighed in macrophages by
podosome dissolution due to localized myosin II activation. A role in podosome formation could include supervillin-mediated, actin-dependent rapid recycling (Fang et al., 2010) of podosome components from dissolving podosomes to newly forming ones. Rapid recycling of podosome components could also facilitate the dynamic re-assembly of cytoskeletal and membrane components of podosomes.

The absence of a major effect on podosome numbers by GFP-SV 1-830 hints further at the possibility of a cyclical process, in which the supervillin C-terminus plays a role in controlling podosome dynamics. Calponin is one podosome regulatory factor that interacts with the supervillin C-terminus (Fig. 4A) (Gangopadhyay et al., 2004; Gimona et al., 2003). Another likely player is Tks5 (Fig. 4A), which interacts with the supervillin C-terminus and promotes podosome formation and matrix degradation (Crimaldi et al., 2009; Seals et al., 2005; Smith et al., 2010).

Because knockdown of supervillin and myosin IIA showed additive effects on matrix degradation, we suggest that supervillin and myosin IIA function in complementary pathways to degrade matrix. Our results are consistent with reports showing that siRNA-induced knockdown of supervillin decreases the number of invadopodia-induced holes in matrix degradation by MDA-MB-231 cells (Crowley et al., 2009) and that biochemical inhibition of myosin II reduces matrix degradation by invadopodia (Alexander et al., 2008). For instance, supervillin-mediated recycling of podosome components may be independent of myosin II function, whereas multiple pathways, separately including supervillin and gelsolin, could provide input to myosin II-mediated mechanosensing and/or matrix metalloprotease secretion (Alexander et al., 2008; Arora et al., 2011; Myers et al., 2011).

Our data also show functional redundancy between supervillin and gelsolin in the regulation of podosomal matrix degradation, but not in the regulation of podosome numbers. Gelsolin and supervillin are homologs that share amino acids required for the formation of five gelsolin-like folding repeats and residues in surface loops that are required for polyphosphoinositide binding by gelsolin (Janmey et al., 1992; Pestonjamasp et al., 1997; Smith et al., 2010). Gelsolin is a podosome regulator (Chellaiah et al., 2001) that localizes to all podosomes and binds F-actin but not myosin II. Functional redundancy between supervillin and gelsolin is consistent with gelsolin-independent formation of podosomes in dendritic cells (Hammarfjord et al., 2011) and with the observation that supervillin and gelsolin synergize to promote matrix invasion by breast cancer cells (Crowley et al., 2009). These results suggest commonalities in downstream functions although the large structural differences between supervillin and gelsolin imply different signalling mechanisms.
In conclusion, we identify supervillin as a critical regulator of podosome turnover, podosomal matrix degradation and cell polarization in primary human macrophages. We further show that supervillin-mediated coupling of MLCK-dependent myosin IIA contractility to podosomes is required for the regulation of podosome turnover. Moreover, our results reveal that podosome subpopulations in macrophages differ in their molecular makeup, and that their composition alters during their life cycle. The identification of supervillin as the first differentially localized component, its localization to a cap structure on top of the podosome core, and the supervillin-dependent recruitment of contractile myosin to podosomes thus shed new light on the increasingly apparent intricacies of podosome composition, architecture and turnover.
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FIGURE LEGENDS

Fig. 1. Supervillin is expressed in primary human macrophages and localizes to a substructure of successor podosomes. (A-F) Confocal immunofluorescence micrographs of 7d cultured primary human macrophages, in a quiescent (A-C) or polarized (D-F) state, that express GFP-supervillin (green; A,D), and are stained for F-actin (red; B,E); merged images shown in (C,F). Images in (G-I) show enlarged areas indicated by white box in (F). Dashed white lines in (D-F) indicate the leading edge of this polarized cell. Note absence of GFP-supervillin from precursors (cell periphery of migratory cell or leading edge of polarized cell). (J) Plots of fluorescence intensity for a single podosome; GFP-supervillin (green), F-actin (red). (K-M) 3D reconstruction of z-stacks of a single podosome from a macrophage expressing GFP-supervillin. Note cap-like distribution of GFP-supervillin (green) on top of the F-actin–rich podosome core (red), surrounded by a ring of vinculin (blue; see also Suppl. Video 1). (N) Cell polarization in cells expressing GFP or GFP-supervillin. For each value, 3x30 cells were evaluated; cells showing distinct leading and trailing edges (D-F), were scored as “polarized”. Polarization for cells expressing GFP: 22.2% ± 1.4%, polarization for cells expressing GFP-supervillin: 61.1± 2.8%, with P < 0.01 according to Student’s t-test. (O) Intensity of podosomal GFP-supervillin at 5 different locations between the cell interior and cell edge; locations of successor and precursor podosomes are indicated. Intensities were measured in 13 cells from 3 different donors. (P) Western blot of macrophage lysate stained with supervillin-specific H340 antibody. Molecular weights in kDa are indicated. Supervillin (SV) is indicated by an arrowhead. Left lane: lysate from macrophages treated with luciferase-specific siRNA as control; right lane: lysate from macrophages treated with supervillin-specific siRNA to demonstrate antibody specificity; β–actin loading control (Suppl. Fig. 8A). (Q) RT-PCR from macrophage mRNA using supervillin- or actin- (as a control) specific primers. Bands of the expected sizes (521 bp for supervillin, 445 bp for actin) are detected on an agarose gel stained with ethidium bromide. Molecular sizes in base pairs (bp) are indicated. (R-T) Confocal immunofluorescence micrographs of 7d cultured primary human macrophages, stained for supervillin (green) with specific antibody (R) and for F-actin (red) with Alexa Fluor 568 labeled phalloidin (S); merge in (T). Bars: 10 µm in (A-F, R-T) and 0.2 µm in (K-M).

Fig. 2. Dissolving podosomes acquire GFP-supervillin. (A-M) Still images from confocal time-lapse video of a 7d cultured macrophage expressing mRFP-β-actin (red) and GFP-
supervillin (green) for 18 h (Suppl. Video 2). (A) Note absence of GFP-supervillin from large podosome precursors at the leading edge (lower right). White box indicates area shown in (B-M), with merged images (B-E) containing the mRFP-β-actin signals (F-I) in red and the GFP-supervillin signals (J-M) in green. White arrows indicate a dissolving podosome, where disappearance of the mRFP-β-actin signal is coupled with enrichment of GFP-supervillin. Time since start of the experiment is indicated in seconds. (N-Y) Still images from a confocal time-lapse video of a 7d cultured macrophage expressing mRFP-Lifeact (red) and GFP-supervillin (green) for 18 h (Suppl. Video 3). White box indicates area enlarged in (N-Y), with merged images (N-Q) containing the mRFP-Lifeact signals (R-U) in red and the GFP-supervillin signals (V-Y) in green. Note the appearance of supervillin at precursors concomitant with the dissolution of the F-actin signal, as the cell begins to withdraw. Time since start of the experiment is indicated in seconds. Bars: 5 µm (B-M) and 10 µm (N-Y).

Fig. 3. Podosome numbers in macrophages expressing the designated supervillin constructs. (A) Supervillin contains a myosin IIA- and L-MLCK binding domain (red; aa 1-174), three F-actin binding regions (white, yellow, navy; aa 174-343, 343-571, 571-830) that partially overlap with cortactin-binding sites (aa 1-340, 571-830), a central region with a strong nuclear localization signal (NLS, white: aa 830-1010) and a C-terminal region with similarities to gelsolin and villin (light blue). The second F-actin binding region binds TRIP-6 while more C-terminal regions interact with the podosome protein Tks5, kinesins, calponin and cell cycle regulatory proteins (Smith et al., 2010). The supervillin N- and C-termini are indicated; numbers indicate amino acid residues at the start or end of each GFP fusion construct. Reduction of podosome numbers upon overexpression of respective constructs is indicated on the right (level of reduction indicated by “+++”, “++” and “+”). (B-G) Podosome numbers in macrophages expressing supervillin constructs (n = 3×30). GFP-supervillin (C) and SV Δ343-570 (F) greatly decrease podosome numbers, an ability lost after deletion of the myosin II-activating domain in SV 171-1792 (D); (G) the isolated SV 174-343 region also reduces podosome numbers (G), probably by acting as a competitive binder for full-length supervillin (see Suppl. Fig. 7). *, P < 0.05; **, P < 0.01. For specific values, see Suppl. Table 1. Data for the other constructs see Suppl. Fig. 4.

Fig. 4. Subcellular localization of supervillin deletion mutants. Confocal immunofluorescence micrographs of 7d cultured primary human macrophages expressing GFP-tagged supervillin constructs shown in Fig. 3A: (A-C) SV 1-830, (D-F) SV Δ343-570,
(G-I) SV 171-1792, or (J-L) SV 1-174. GFP signals in green (A,D,G,J); F-actin in red (B,E,H,K); merges in (C,F,I,L). Dashed white lines indicate leading edges of the cells. Bar: 10 µm. Note the presence of the SV 171-1792 construct at precursors at the leading edge (I).

**Fig. 5. Supervillin and myosin IIA regulate podosome lifetime and matrix degradation.** (A,B) Lifetimes of successor (A) or precursor (B) podosomes in macrophages expressing Lifeact-GFP after transfection with indicated siRNAs (control: luciferase siRNA). Each value represents an average lifetime of at least 10 podosomes in a single cell (n=9). (Note: only those precursors that formed and dissolved within the experimental period were evaluated) All values are significantly different from respective controls with at least $P < 0.001$, as determined by one-way ANOVA. (C) Summary of matrix degradation under cells treated with designated siRNAs. Degree of matrix degradation was analyzed by fluorescence measurements of 3x30 cells. Complete absence of labeled matrix beneath cells was set as 100% degradation. Cells were scored into groups according to matrix degradation (0-25%; 26-100%). *, $P < 0.05$; **, $< 0.01$. as determined by one-way ANOVA. Specific values for A and B see Suppl. Table 1. (D-J) Confocal laser scanning micrographs of macrophages transfected with (D) siRNA against luciferase as a control or with siRNA specific for (E) supervillin, (F) myosin IIA, (G) gelsolin or (H-J) combinations of specific siRNAs, as indicated. Cells were seeded on rhodamine-labeled gelatin matrix (red). Matrix degradation is visible as dark areas; insets show F-actin staining by Alexa Fluor 647-labeled phalloidin (white). Bars: 10 µm.

**Fig. 6. Myosin IIA cables connect supervillin-positive podosomes.** Co-localization of tagged supervillin with (A-H) endogenous and (I-O) co-expressed GFP-myosin IIA in (A-D) unpolarized and (E-H) polarized macrophages. (A-H) Confocal micrographs of macrophages expressing GFP-supervillin (A,E) and stained for myosin IIA (B,F), and for F-actin (C,G); merged images shown in (D,H). White bars: 10 µm. (I-O) GFP-myosin IIA overlaps with mRFP-supervillin at dissolving successor podosomes. Still images from confocal time-lapse videos of 7d cultured macrophages expressing GFP-myosin IIA (green) and mRFP-supervillin (red). Cell showing moderate overexpression in (I-L; Suppl. Video 4); cell with more pronounced overexpression (N,O; Suppl. Videos 5,6). Dashed white lines indicate position of the leading edge in (I-L, N) and total cell circumference in (M). Solid white arrow indicates current position of the trailing edge in (I,L), dashed white arrow indicates its prior position (L). Enrichment of GFP-myosin IIA at podosomes at the rear of the podosome field often
precedes their disappearance (white circles). (M) Net movement of the mRFP-supervillin-marked podosome field between time point 0 sec (blue line) and time point 648 sec (red line). As rearward podosomes dissolve, more forward-positioned podosomes acquire mRFP-supervillin. (N,O) GFP-myosin IIA-positive cables contact mRFP-supervillin-rich podosomes at the rear of the podosome field. (O) Enlargement of the area indicated by the white box in (N). Note that a network of GFP-myosin IIA connects mRFP-supervillin-positive podosomes. White bars: 10 µm. (P,Q) Macrophage supervillin interacts with myosin IIA and MLCK. Macrophage lysates were immunoprecipitated with anti-GFP antibody coupled to magnetic beads. (P) Western blots of (left) lysates from macrophages expressing GFP-SV 1-174 or GFP as a control and (right) anti-GFP immunoprecipitates after staining with anti-GFP antibody (left), anti-myosin IIA antibody (upper right), or anti-MLCK antibody (lower right). Left lanes: cells expressing GFP-SV 1-174, right lanes: GFP control. (Q) Western blots of (upper panels) lysates and (lower panels) anti-GFP immunoprecipitates from macrophages expressing GFP-SV 1-174 (left) or GFP-SV 1-830 (right), treated with either 10 µM blebbistatin or 0.035% DMSO prior to lysis. Blots were developed with anti-GFP antibody (upper blots) or anti-myosin IIA antibody (lower blots). (P,Q) Molecular weights in kDa are indicated to the left of each panel.

**Fig. 7.** The supervillin N-terminal 830 residues induce myosin light chain phosphorylation (pMLC) and myosin condensation at podosomes. (A-F) Confocal micrographs of macrophages transfected with supervillin- or luciferase-specific siRNA, or expressing GFP, GFP-supervillin, GFP-SV 1-830, or GFP-SV 1-174, as indicated, and stained for pMLC. Insets in red show F-actin staining; insets in green in (C-F) show GFP signal. (G) Enrichment of pMLC at podosomes in cells treated with the indicated siRNA or expressing the indicated supervillin constructs. **, P < 0.01, as determined by Mann-Whitney test (n=3x15 for overexpressing cells, 3x30 for siRNA-treated cells). (H-M) 3D reconstruction of optical z-stacks of single podosomes from macrophages expressing GFP-SV 1-830 (H-J) or GFP-supervillin (K-M) co-stained for myosin IIA (white) and F-actin (red). (Also see Suppl. Videos 7 and 8.) Note cap-like localizations of supervillin constructs above F-actin cores and the more compact localization of myosin IIA at the GFP-SV 1-830 decorated podosome. Bar: 0.15 µm.

**Fig. 8.** Supervillin localization and supervillin-induced cell polarization require myosin IIA. (A-R) Confocal micrographs of macrophages expressing GFP-supervillin (green) after
transfection with the indicated siRNA (A-L) or treatment with 0.007% DMSO (M-O) or 2 µM blebbistatin (P-R) and staining for F-actin to visualize podosome cores (red); merged images (C,F,I,L,O,R). Bars: 10 µm. (S) Differential localization of GFP-supervillin at podosome subpopulations in siRNA- or drug-treated cells. Cells were scored into groups: localization of GFP-supervillin at successors only (black bars), preferentially at successors (grey bars), or at both precursors and successors (white bars). *, $P < 0.05$; **, $P < 0.01$, as determined by Student’s $t$-test. (T) Polarity of control cells (unlabeled) or cells expressing GFP-supervillin (brackets) after treatment with specific siRNA or drugs, as indicated. Cell polarization was judged by the presence of leading and trailing edges, with accompanying recruitment of podosomes to the leading edge, as opposed to unpolarized, radially symmetric cells showing podosomes along the entire cell periphery. *, $P < 0.05$; **, $P < 0.01$, as determined by unpaired Student’s $t$-test. For each value in S and T, 3x30 cells were evaluated. For specific values for S and T, see Suppl. Table 1.

**Fig. 9. Model of supervillin and myosin IIA-regulated turnover of podosomes.** *Upper image:* polarized macrophage showing larger precursors at the leading edge and smaller successors at the trailing edge. L-MLCK is present at both podosome subpopulations, whereas only successors contain supervillin and myosin IIA and are connected to actomyosin cables. *Middle image:* as the cell changes polarity, enhanced local contractility leads to higher levels of supervillin and contractile myosin IIA at successors, while precursors lying in the contractile zone acquire both proteins. Enhanced local contractility also can increase cell polarity in a positive feedback loop. *Lower image:* podosomes that have acquired higher levels of supervillin and contractile myosin IIA at the new trailing edge are dissolving, while precursors are forming at the new leading edge.
SUPPLEMENTARY MATERIAL

Suppl. Figure 1. GFP-supervillin localizes preferentially to successor podosomes. Confocal immunofluorescence micrographs of a 7d cultured primary human macrophage expressing GFP-supervillin (A, green) and stained with anti-vinculin antibody (B, red), with merged images shown in (C). (D-F) An enlargement of the area denoted by the white box in (C). (G) Plots of fluorescence intensity for a single podosome. Note that maximal fluorescence intensities for vinculin do not coincide with those for GFP-supervillin. White bar indicates 10 µm.

Suppl. Figure 2. Gelsolin shows no differential localization to podosome subpopulations. Confocal immunofluorescence micrographs of a 7d cultured primary human macrophage expressing GFP-supervillin (A, green), and stained for gelsolin using specific primary antibody (B, red) and for F-actin using Alexa Fluor 647-labeled phalloidin (C, blue). (D) Merged image of (A) and (B). (E) Merged image of (A), (B) and (C). Note that gelsolin is present at all F-actin-stained podosomes in the cell (magenta). Bar, 10 µm.

Suppl. Figure 3. Endogenous supervillin at successor and precursor podosomes. (A-I) Confocal immunofluorescence micrographs of 7d cultured primary human macrophages, stained for endogenous supervillin (A,D,G; green) and F-actin (B,E,H; red), with merged images shown in (C,F,I). Randomly chosen successor (white circles) and precursor podosomes (magenta circles) were analyzed for their respective supervillin content by measuring fluorescence intensities. Bars, 10 µm. (J) Analysis of supervillin-based fluorescence intensities in successor and precursor podosomes. For each value, 40 successor or 40 precursor podosomes were analyzed (4 cells from 4 different donors). For specific values, see Suppl. Table 1. *, $P < 0.05$, as determined by Mann-Whitney test.

Suppl. Figure 4. Podosome numbers in macrophages transformed with various supervillin constructs. Macrophages were scored in groups according to podosome numbers. For each value and time, 30 randomly chosen cells from 3 independent experiments were evaluated. Values are given as mean percentage ± SEM of total cell counts (see also Suppl. Table 1 and Figure 4). *, $P < 0.05$; **, $P < 0.01$. 
Suppl. Figure 5. Overexpression of GFP-SV 1-174 induces the formation of myosin IIA-rich cables. Confocal immunofluorescence micrographs of a 7d cultured primary human macrophage expressing GFP-SV 1-174 (A) and stained for myosin IIA (B); merge shown in (C). Bar, 10 µm.

Suppl. Figure 6. Subcellular localization of supervillin deletion mutants. Confocal immunofluorescence micrographs of 7d cultured primary human macrophages expressing supervillin constructs: a construct comprising the first F-actin binding region (SV 174-343, A-C), a construct comprising the second F-actin binding region (SV 343-570; D-F), a construct comprising the third F-actin binding region (SV 571-830; G-I), a C-terminal construct (SV 830-1072; J-L), or a C-terminal construct lacking the nuclear localization signal (SV 1010-1072; M-O). GFP signals in green (A,D,G,J,M), F-actin staining in red (B,E,H,K,N), merge in (C,F,I,L,O). Bars, 10 µm. Note the presence at both precursor and successor podosomes of all constructs containing an actin-binding region.

Suppl. Figure 7. The SV 174-343 region competes with full-length supervillin for binding to podosomes. Confocal micrographs of macrophages expressing mRFP-supervillin (B,F), together with GFP-SV 174-343 (C) or GFP as control (G), and stained for F-actin (A,E); merges shown in (D,H). Note the retention of mRFP-supervillin on actin cables at the cell center (B) and its loss from podosomes (F) upon overexpression of GFP-SV 174-343. Bars: 10 µm.

Suppl. Figure 8. RNAi-induced knockdown of supervillin, myosin IIA and L-MLCK. Western blots of lysates from primary human macrophages (A left, B, C, D, E) or U2OS cells (A right), treated with the indicated siRNA and developed with antibodies against (A, B) supervillin (H340 or HSV715 antibody, as indicated); (C) myosin IIA; (D) MLCK; or (E) phosphorylated myosin light chain (pMLC). Lines in A, right indicate that lanes were not directly adjacent on original blots. Molecular weights of markers in kDa are indicated.

Suppl. Figure 9. Evaluation of podosome numbers following knockdown of supervillin, myosin IIA or gelsolin. Podosome numbers in 7d old macrophages, transfected with luciferase siRNA as control, or with supervillin-, myosin IIA- or gelsolin-specific siRNA or a combination thereof, as indicated. (A) Untransfected cells were scored in groups according to podosome numbers. For each value, 3x30 cells from 3 different donors were evaluated. For
specific values, see Suppl. Table 1. (B, C) Macrophages expressing GFP-Lifeact were transfected with specific siRNAs, as indicated, and scored for (B) the average number of podosomes/cell and (C) the average change in the number of podosomes/cell at the indicated time (n=9 in all cases). For specific values, see Suppl. Table 1.

**Suppl. Figure 10. L-MLCK-GFP localizes to all podosomes.** Confocal micrographs of polarized (A-D) and unpolarized (E-H) macrophages expressing GFP-L-MLCK (A,E) and mRFP-supervillin (B,F) counter-stained for F-actin with Alexa Fluor 647-coupled phalloidin (C,G); merged images (D,H). Note that GFP-L-MLCK is present at both precursor and successor podosomes, whereas mRFP-supervillin localizes preferentially to successor podosomes.
Video 1

**GFP-supervillin localizes at a cap structure on podosome cores.** 3D reconstruction showing a single podosome of a cell expressing GFP-supervillin (green), stained for F-actin (red) and vinculin (blue). Cell was imaged using a Leica SP-2 confocal microscope. Z stacks were taken from the bottom to the top of the cell with a step size of 0.1µm. Image processing was done using Volocity software (Improvision). Cell was cropped to the level of a single podosome and visualized in 3-D using the ‘3-D opacity’ option. Note the cap-like decoration of GFP-supervillin at the F-actin rich podosome core and the typical ring-like decoration of vinculin. The 3-D image was tilted along various axes and rendered into a movie. Frame rate = 15 frames/sec. For still images see Fig. 1K-M.

Video 2

**Dissolving podosomes acquire GFP-supervillin.** Confocal time-lapse video of a 7d cultured primary human macrophage expressing GFP-supervillin (green, left channel), m-RFP actin (red, middle channel), and merge (right channel). Region of interest from cell, as indicated in Fig.2A. Note also that disappearance of the mRFP-signal at podosomes is coupled with enrichment of GFP-supervillin. Exposure time: 500 ms, images were taken with a delay of 10 s between frames and are played back at 5 frames/s. Total elapsed time during experiment: 965.5 s, image width: 85 µm. For still images, see Fig. 2B-M.

Video 3

**Dissolving podosomes acquire GFP-supervillin.** Confocal time-lapse video of a 7d cultured primary human macrophage expressing GFP-supervillin (green, left channel), Lifeact-RFP (red, middle channel), and merge (right channel). Note that disappearance of the RFP-signal at podosomes is coupled with enrichment of GFP-supervillin. Exposure time: 569 ms, images were taken with a delay of 30 s between frames and are played back at 2 frames/s. Total elapsed time during experiment: 600 s, image width: 35 µm. For still images, see Fig. 2N-Y.

Video 4

**GFP-myosin IIA contacts dissolving podosomes.** Confocal time-lapse video of a 7d cultured primary human macrophage expressing GFP-myosin IIA (green) and mRFP-supervillin (red). Note formation of GFP-myosin IIA-enriched trailing edge and absence of mRFP-supervillin from podosomes at the leading edge (lower right). Contact of GFP-myosin IIA with mRFP-positive podosomes at the rear of the podosome field precedes their
disappearance, while podosomes at the forward side of the field acquire mRFP-supervillin. Exposure time: 1 s for 488 nm channel, 3 s for 568 nm channel, with 2x binning; images were taken with a delay of 10 s between frames and are played back at 10 frames/s. Total elapsed time during experiment: 788.1 s, image width: 73.6 µm. For still images, see Fig. 6I-L.

**Video 5**

**Supervillin-containing podosomes are connected by contractile GFP-myosin IIA-positive cables.** Confocal time-lapse video of a 7d cultured primary human macrophage expressing GFP-myosin IIA (green) and mRFP-supervillin (red). Podosomes at the rear of the podosome field (right) are connected and pulled upon by GFP-myosin-IIA-positive cables. Exposure time: 1 s for 488 nm channel, 3 s for 568 nm channel, with 2x binning; images were taken with a delay of 10 s between frames and are played back at 10 frames/s. Total elapsed time during experiment: 625.8 s, image width: 50.1 µm. For still image, see Fig. 6N.

**Video 6**

**Supervillin-containing podosomes are connected by contractile GFP-myosin IIA-positive cables.** Confocal time-lapse video of a 7d cultured primary human macrophage expressing GFP-myosin IIA (green, left channel) and mRFP-supervillin (red, middle channel), and merge (right channel). Region of interest from Suppl. Video 5, as indicated in Fig. 6N. Podosomes (from the rear of the podosome field in video 4) are connected and pulled upon by GFP-myosin-IIA-positive cables. Exposure time: 1 s for 488 nm channel, 3 s for 568 nm channel, with 2x binning; images were taken with a delay of 10 s between frames and are played back at 10 frames/s. Total elapsed time during experiment: 625.8 s, image width: 85 µm. For still image, see Fig. 6O.

**Video 7**

**GFP-L-MLCK localises to successor and precursor podosomes.** Confocal time-lapse video of a 7d cultured primary human macrophage expressing GFP-L-MLCK (green; left channel) and mRFP-supervillin (red; middle channel), and merge (right channel). GFP-L-MLCK localizes to both successor and precursor podosomes, whereas mRFP-supervillin is observed only at successor podosomes. Exposure time: 841 ms for 488 nm channel, 500 ms for 568 nm channel, with 1x binning; images were taken with a delay of 15 s between frames and are played back at 10 frames/s. Total elapsed time during experiment: 1837s, image width: 90 µm.
Video 8
Cells expressing GFP SV 1-830 show formation of a compact myosin IIA ring around the podosome core. 3D reconstruction showing a single podosome in a macrophage expressing GFP-SV 1-830 (green) counter-stained for F-actin (red) and myosin IIA (white). Cell was imaged using a Leica SP-2 confocal microscope. Z stacks were taken from the bottom to the top of the cell with a step size of 0.1µm. Image processing was done using Volocity software (Improvision). Cells were cropped to the level of a single podosome and visualized in 3-D using the ‘3-D opacity’ option. Note the tight ring-like decoration of myosin IIA around the podosome core. The 3-D image was tilted along various axes and rendered into a movie. Frame rate: 15 frames/sec. For still images see Fig. 7H-J.

Video 9
Cells expressing GFP-supervillin show a more dispersed localization of myosin IIA around the podosome core. 3D reconstruction showing a single podosome in a macrophage expressing GFP-supervillin (green) counter-stained for F-actin (red) and myosin IIA (white). Cells were imaged using a Leica SP-2 confocal microscope. Z stacks were taken from the bottom to the top of the cell with a step size of 0.1 µm. Image processing was done using Volocity software (Improvision). Cells were cropped to the level of a single podosome and visualized in 3-D using the ‘3-D opacity’ option. Note the dispersed ring-like decoration of myosin IIA around the podosome. The 3-D image was tilted along various axes and rendered into a movie. Frame rate: 15 frames/sec. For still images see Fig. 7K-M.

Video 10
Precursor podosomes acquire GFP-supervillin on addition of blebbistatin. Confocal time-lapse video of a 7d cultured primary human macrophage expressing GFP-supervillin (green, left channel), Lifeact-RFP (red, middle channel), and merge (right channel). 2 µM blebbistatin was added 11 min after the start of the experiment Exposure time: 273 ms, images were taken with a delay of 60 s between frames and are played back at 3 frames/s. Total elapsed time during experiment: 94 .24 minutes, image width: 65 µm.
REFERENCES


Figure 1

GFP-supervillin  |  F-actin  |  merge

A  |  B  |  C

D  |  E  |  F

G  |  H  |  I

J

K  |  L  |  M

N

O  |  P  |  Q

R  |  S  |  T

successors  |  precursors

intensity of GFP-supervillin (a.u.)  |  IgG HCl

vinculin

SV control  bp

GFP  |  GFP-supervillin

Supervillin  |  F-actin  |  merge

supervillin  |  F-actin  |  merge

**
Figure 2

A

GFP-supervillin, mRFP-β-actin

B

193.0 s

C

470.7 s

D

682.5 s

E

823.7 s

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Lifeact-RFP

merge

GFP-supervillin

mRFP-β-actin

510s000s 150s 420s

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Figure 3

B GFP

C GFP-SV

D GFP-SV 171-1792

E SV 1-174-GFP

F GFP-SV Δ343-570

G GFP-SV 174-343
Figure 4

GFP-signal | F-actin | merge

A. GFP-SV 1-830

D. GFP-SV Δ343-570

G. GFP-SV 171-1792

J. SV 1-174-GFP

H. GFP-SV 171-1792

K. SV 1-174-GFP

C. merge

F. merge
Figure 5

A) successors

B) precursors

C) degree of matrix degradation

siRNA treatment

successors

precursors

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Figure 6

**P**

- **GFP-SV 1-174**
  - **anti-GFP**: kDa 55, 72, 42
  - **anti-Myo IIA**: kDa 170
  - **anti-MLCK**: kDa 170

- **GFP**

**Q**

- **GFP-SV 1-174**
  - **anti-GFP**: kDa 55, 130
  - **anti-Myo IIA**: kDa 170

- **GFP-SV 1-830**
  - **anti-GFP**: kDa 170
  - **anti-Myo IIA**: kDa 170
Figure 7

[Images of immunofluorescence staining showing effects of luciferase siRNA, SV siRNA, and GFP on p-MLC and F-actin localization in podosomes]

GFP-SV 1-830

**Figure 7 continued**

(area of pMLC/area of podosome (%)

[Graph showing area of pMLC in relation to area of podosome across different treatments]

GFP-SV 1-174-GFP

[Images of additional fluorescent markers showing GFP-supervillin 1-830 and Myo IIA]
Figure 8

GFP-supervillin  F-actin  merge

siRNA treatment  drug treatment

S  GFP-supervillin Localisation

T  Cell Polarisation

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Figure 9

*Change in cell polarity*

**Trailing edge**
- **Successors**
- **Precursors**

**Leading edge**
- **Formation of new podosomes**

**Cell rear**
- Increased contractility
- Disappearance of dissolving podosomes

**Dissolving successors**
- Increased recruitment of myosin & supervillin

**Dissolving precursors**
- Recruitment of myosin & supervillin

**Establishment of new cell polarity**