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► **To cite this version:**

K. Jerabkova-Roda, A. Mousson, M. Peralta, R. Karali, H. Justiniano, et al.. Peripheral positioning of lysosomes supports melanoma aggressiveness. 2023. hal-04308597

**HAL Id: hal-04308597**

**<https://hal.science/hal-04308597>**

Preprint submitted on 27 Nov 2023

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# Peripheral positioning of lysosomes supports melanoma aggressiveness

K. Jerabkova-Roda<sup>1-5\*</sup>, A. Mousson<sup>2,6</sup>, M. Peralta<sup>1-4</sup>, R. Karali<sup>2,6</sup>, H. Justiniano<sup>2,6</sup>, L.M. Lisii<sup>2,6</sup>, P. Carl<sup>2,6</sup>, N. Asokan<sup>1-4</sup>, I. Busnelli<sup>1-4</sup>, A. Larnicol<sup>1-4</sup>, O. Lefebvre<sup>1-4</sup>, H. Lachuer<sup>5,†</sup>, A. Pichot<sup>2,3,8</sup>, T. Stemmelen<sup>2,3,8</sup>, A. Molitor<sup>2,3,8,9</sup>, A. Hirschler<sup>10</sup>, F. Delalande<sup>10</sup>, E. Sick<sup>2,6</sup>, R. Carapito<sup>2,3,8,9</sup>, C. Carapito<sup>10</sup>, V. Hyenne<sup>1-4,11\*</sup>, K. Schauer<sup>5,7\*†</sup>, P. Rondé<sup>2,6\*†</sup>, and J.G. Goetz<sup>1-4\*†</sup>

<sup>1</sup>Tumor Biomechanics, INSERM UMR\_S 1109, Strasbourg, France

<sup>2</sup>Université de Strasbourg, Strasbourg, France

<sup>3</sup>Fédération de Médecine Translationnelle de Strasbourg (FMST), Strasbourg, France

<sup>4</sup>Équipe labellisée Ligue Contre le Cancer

<sup>5</sup>Institut Curie, PSL, CNRS, UMR144, Paris, France

<sup>6</sup>CNRS UMR7021, Faculté de Pharmacie, Illkirch, France

<sup>7</sup>Institut Gustave Roussy, INSERM UMR1279, Université Paris-Saclay, Villejuif, France

<sup>8</sup>Plateforme GENOMAX, Institut thématique interdisciplinaire (ITI) de Médecine de Précision de Strasbourg Transplantex NG, Fédération Hospitalo-Universitaire OMICARE

<sup>9</sup>Service d'Immunologie Biologique, Plateau Technique de Biologie, Pôle de Biologie, Nouvel Hôpital Civil, Hôpitaux Universitaires de Strasbourg, 1 Place de l'Hôpital, 67091, Strasbourg, France

<sup>10</sup>CNRS, Laboratoire de Spectrométrie de Masse Bio-Organique (LSMBO), IPHC, UMR 7178, Université de Strasbourg, Strasbourg 67087, France

<sup>11</sup>CNRS SNC5055, Strasbourg, France

<sup>†</sup>Current address : Université de Paris, CNRS, Institut Jacques Monod, 75013 Paris, France

Equal contribution

\* Corresponding authors

1 **Emerging evidences suggest that both function and position of** 37  
2 **organelles are pivotal for tumor cell dissemination. Among** 38  
3 **them, lysosomes stand out as they integrate metabolic sens-** 39  
4 **ing with gene regulation and secretion of proteases. Yet, how** 40  
5 **the function of lysosomes is linked to their position and how** 41  
6 **this controls metastatic progression remains elusive. Here,** 42  
7 **we analyzed lysosome subcellular distribution in micropat-** 43  
8 **terned patient-derived melanoma cells and found that lysosome** 44  
9 **spreading scales with their aggressiveness. Peripheral lyso-** 45  
10 **somes promote invadopodia-based matrix degradation and in-** 46  
11 **vasion of melanoma cells which is directly linked to their lyso-** 47  
12 **somal and cell transcriptional programs. When controlling lyso-** 48  
13 **somal positioning using chemo-genetical heterodimerization in** 49  
14 **melanoma cells, we demonstrated that perinuclear clustering** 50  
15 **impairs lysosomal secretion, matrix degradation and invasion.** 51  
16 **Impairing lysosomal spreading in a zebrafish metastasis model** 52  
17 **significantly reduces invasive outgrowth. Our study provides a** 53  
18 **mechanistic demonstration that lysosomal positioning controls** 54  
19 **cell invasion, illustrating the importance of organelle adaptation** 55  
20 **in carcinogenesis.** 56

21 **Keywords:** lysosome, invasion, metastasis

22 **Correspondence:** [katerina.jerabkova@inserm.fr](mailto:katerina.jerabkova@inserm.fr) [kris-philippe.ronde@unistra.fr](mailto:kris-philippe.ronde@unistra.fr)  
23 [tine.schauer@gustaveroussy.fr](mailto:tine.schauer@gustaveroussy.fr)  
24 [jacky.goetz@inserm.fr](mailto:jacky.goetz@inserm.fr)

## 25 Main

26 Metastases are responsible for the majority of cancer-related 62  
27 deaths (Dillekås et al., 2019). Melanoma shows strong 63  
28 negative correlation between cancer stage and 5-year pa- 64  
29 tient survival, making it an ideal model to study phenotypic 65  
30 changes leading to cancer cell invasion, adaptation and sur- 66  
31 vival. Melanoma progression consists of multiple sequen- 67  
32 tial events. First, melanocytes are transformed and grow in 68  
33 the epidermis during radial growth phase (RGP), forming a 69  
34 premalignant lesion. Changes in their transcription program 70  
35 lead to expression of matrix-degrading enzymes and to inva- 71  
36 sion through the dermis during vertical growth phase (VGP) 72

followed by cancer dissemination through vascular and lym-  
phatic routes, progressing into metastatic stages (Brauer et  
al., 2011). To colonize secondary organs during metasta-  
sis, melanoma cells sense their microenvironment and react  
by locally degrading and remodeling the extracellular matrix  
(ECM). Invading melanoma cells frequently form dynamic  
actin-rich protrusive structures with high proteolytic activity,  
called invadopodia, that mediate ECM degradation (Augoff  
et al., 2020). Invadopodia formation involves the local dock-  
ing of endosomal compartments, such as multivesicular bod-  
ies, and their fusion with the plasma membrane (Hoshino et  
al., 2013). This suggests that melanoma metastasis requires  
specific invasion programs for an efficient and targeted deliv-  
ery of ECM-degrading enzymes to highly-specialized struc-  
tures. Yet how this is orchestrated in invasive cells remains  
unclear. Over the past decade, several reports indicated that  
lysosomes constitute novel regulators of invasion by allowing  
cells to sense their microenvironment and trigger adapted re-  
sponses, notably through the exocytic release of their content  
(Ballabio and Bonifacino, 2020). For instance, lysosomal ex-  
ocytosis drives the formation of invasive protrusions result-  
ing in basement membrane breaching in *C. elegans* (Naegeli  
et al., 2017). In addition, secretion of lysosomal cathepsin  
B promotes cancer cell invasion and metastasis (Bian et al.,  
2016). Besides, lysosome secretion contributes to the repair  
of plasma membrane damages occurring during cell migra-  
tion and results in better cell survival under mechanical stress  
(Corrotte and Castro-Gomes, 2019). Importantly, lysosomal  
activity is regulated by their subcellular location (Johnson  
et al., 2016; Korolchuk et al., 2011). Peripheral lysosomes  
are prone to exocytosis and drive growth factor signaling (Jia  
and Bonifacino, 2019), while perinuclear lysosomes have a  
decreased pH and higher proteolytic activity (Johnson et al.,  
2016). Lysosome distribution is in turn directly impacted by  
the cellular microenvironment (Steffan et al., 2009). Lyso-  
somes are transported to the plasma membrane via kinesins

(anterograde transport) in response to growth factors and nutrients presence, conversely, during starvation and in alkaline environment, lysosomes are transported to the perinuclear region (retrograde transport) in a dynein-dependent manner (Ballabio and Bonifacino, 2020). While the molecular mechanisms driving lysosomal positioning have been partially elucidated, it remains unclear whether it can control invasion programs of melanoma cells. A parallel study by Marwaha and colleagues (Marwaha et al., 2023) demonstrates that peripheral lysosomes control the emergence of leader cells during epithelial collective migration events, suggesting that lysosomal positioning is likely a universal mechanism controlling cell invasion within tissues. Here, we investigated how lysosome dynamics contribute to cancer aggressiveness and metastatic progression. We reveal a phenotypic switch concerning lysosome positioning in aggressive melanoma that is supported by distinct transcriptional programs and controls migration and invasion. Our study illustrates the importance of organelle adaptation in carcinogenesis by providing mechanistic evidences that lysosomal positioning controls secretory pathways of malignant transformation.

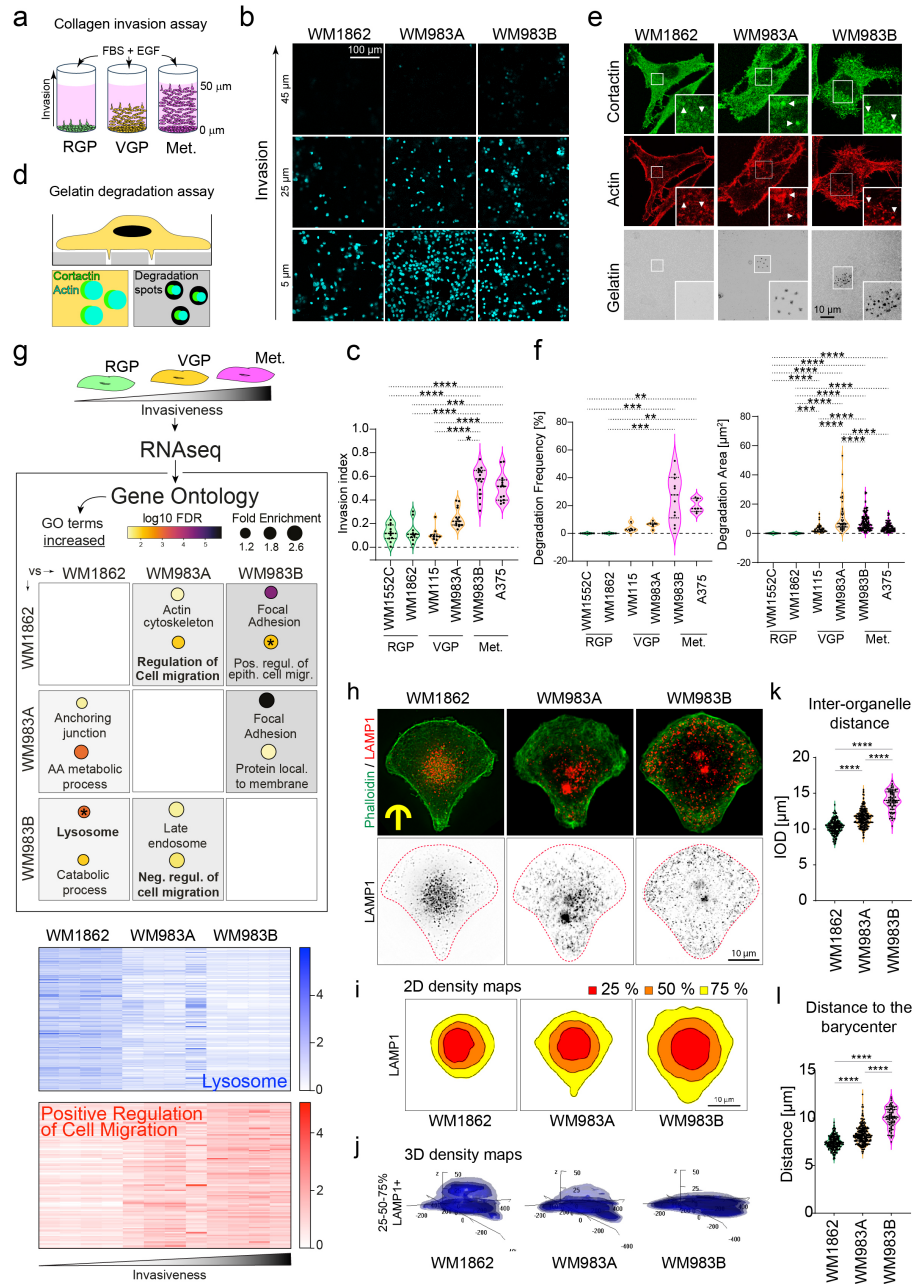
### **Melanoma invasiveness scales with lysosome spreading**

Cells progressing through the metastatic cascade display tremendous phenotypic plasticity in the benefit of increased invasion and ECM degradation potential. Transition of melanoma cells from RGP to VGP involves a series of invasion-promoting programs, particularly the concerted expression of matrix-degrading enzymes (Braeuer et al., 2011) and the maturation of invadopodia (Weidmann et al., 2016). In order to investigate which organelles control such invasion-promoting programs, we first characterized the invasive properties of a collection of patient-derived melanoma cells from different stages (RGP: WM1552c, WM1862, VGP: WM115, WM983A and metastatic: WM983B, A375 cells). Using collagen invasion assay (Fig 1a), we identified three patient-derived cell lines with low, medium and high invasion potential (Fig 1b,c), which correlated with their cancer progression state (RGP, VGP, metastatic). Using gelatin degradation assay (Fig 1d), we further showed that cells with high invasion index displayed significantly increased gelatin degradation frequency and area (Fig 1e,f) confirming previous observations (Mousson et al., 2021). Notably, gelatin degradation areas were mainly located at invadopodia identified by the invadopodia markers, actin and cortactin (Fig 1e). Because ECM degradation is mostly mediated by enzymes, such as metalloproteases (MMPs) or cathepsins, contained in lysosomes, and released by exocytosis at invadopodia (Jacob and Prekeris, 2015), we tested whether lysosomal marker LAMP1 co-localizes with active invadopodia. We found that LAMP1 colocalized with actin and cortactin in a subset of degradation areas (Fig S1 a-d) suggesting at least transient presence of lysosomes at podosomes as reported previously in v-*Src* transformed fibroblasts (Tu et al., 2008). We accessed transcriptional programs of the three representative patient-derived cell lines with graded invasion index (WM1862, WM983A, WM983B) using RNAseq and

Gene Ontology analysis. This showed an overrepresentation of metabolism, actin cytoskeleton and cell migration pathways associated with a concomitant reduction of transcripts linked to the lysosomal pathway (Fig.1g and Table 1). We thus hypothesized that increased invasion occurs through a phenotypic switch from a lysosomal signature characteristic of RGP cells (WM1862) to a migratory signature found in metastatic cells. We next investigated the sub-cellular localization of LAMP1, a marker of late endosomes and lysosomes (referred to as lysosomes hereafter) in these cell lines. Micropatterning allows for high-throughput study of cells with reproducible shapes (Fig 1h) and facilitates the comparison and quantitative positional analysis of lysosomes in 2D (Figure 1i) or in full cell volume (Figure 1j). While RGP cells had mostly perinuclear lysosomes, metastatic cells showed significant dispersion of LAMP1-compartments towards the cell periphery (Fig 1h-j) characterized by a significant increase in the mean inter-organelle distance and the mean distance to the barycenter (Figure 1k,l). Moreover, LAMP1-compartments were smaller and more numerous in metastatic cells (Fig S1e,f), revealing that the observed transcriptional changes in melanoma cells correlate with changes in LAMP1 distribution. Our results identify and characterize a remarkable cellular phenotype of scattered lysosomes associated with aggressive malignancy in melanoma. Since peripheral lysosome positioning has been reported earlier in other cancer types (breast cancer (Ping-Hsiu Wu et al., 2020) and bladder cancer (Mathur et al., 2023)), it is tempting to speculate that peripheral lysosome repositioning could represent a universal feature of cancer progression. Indeed, our transcriptomics data demonstrate that genes known to promote perinuclear localization of lysosomes, such as RILP (Pu et al., 2016) or RNF167 (Nair et al., 2020), show decreased expression, and conversely, genes linked to anterograde transport, such as KIF1B and KIF5B (Moamer et al., 2019), are overexpressed in metastatic cells. Interestingly, lysosomal genes previously associated with cancer progression such as LAMP1 (Machado et al., 2015; Wang et al., 2017), lysosomal Ca<sup>2+</sup> channel MCOLN1 (Medina et al., 2011) and several MMPs (Hua et al., 2011) (MMP2, MMP15, MMP16, MMP17) are upregulated in metastatic cells, besides an overall loss of lysosomal signature in these cells. Yet, lysosomal dispersion could be uncoupled from lysosome biogenesis as some studies report its induction in highly metastatic breast cancer cells (Glunde et al., 2003) or during pancreatic ductal adenocarcinoma progression (Perera et al., 2015). Further work is therefore needed to identify what are the molecular programs controlling the positioning and the biogenesis of lysosomes in invasive melanomas cells and what triggers their activation.

### **Forcing lysosome perinuclear clustering in melanoma cells**

We next engineered melanoma cells employing a chemo-genetic strategy based on the heterodimerization of the FKBP-FRB domains by Rapalog or derivatives (Kapitein et al., 2010) for lysosome positioning control. We chose WM983A and WM983B cell lines, derived from the primary tumor and metastatic site of the same patient, respectively.



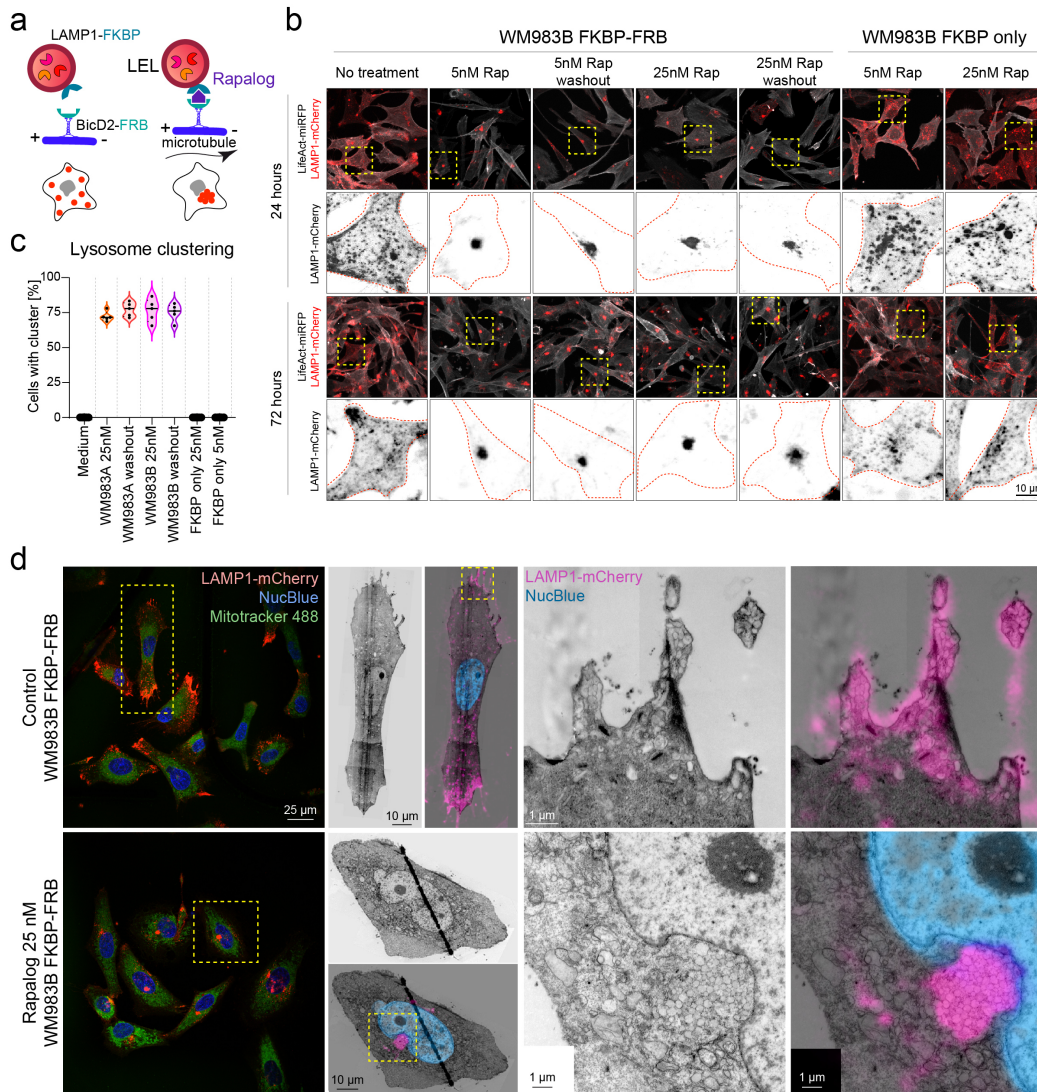
**Fig. 1.** Melanoma invasiveness correlates with lysosome spreading a-c) Invasive potential of human melanoma cells (WM1552c, WM1862, WM115, WM983A, WM983B, A375) was analyzed using collagen invasion assay. b) Cells were seeded into a collagen plug, cultured for 24 hours and their spreading at distance of the seeding position was analyzed by confocal imaging. c) For each cell line, the invasion index equals the number of nuclei above 10 μm distance divided by the total number of cells. Invasion index (Mean ± SD) = 0.1213 ± 0.070, 0.1334 ± 0.089, 0.2500 ± 0.080, 0.1036 ± 0.060, 0.5562 ± 0.125, 0.5070 ± 0.117, respectively, in triplicate, one dot represents 1 field of view. Kruskal-Wallis with Dunn's multiple comparison post-hoc test. d-f) Degradation capacity of the melanoma cell lines was assessed using gelatin degradation assay allowing to visualize actin, cortactin and degradation spots. e) Cells were seeded on FITC-gelatin for 24 hours, fixed and stained with Cortactin antibody and Phalloidin to label invadopodia and imaged using confocal microscopy. f) Degradation frequency (DF) was calculated as a percentage of cells displaying gelatin degradation activity. DF (Mean ± SD) = 0.00, 0.00, 6.19 ± 2.26, 4.00 ± 2.94, 26.08 ± 16.52, 20.08 ± 4.79, respectively, in triplicate one dot represents 1 field of view. For cells displaying degradation activity, the degradation area (DA, total area per cell) was quantified. DA (Mean ± SD) = 0.00, 0.00, 11.13 ± 11.00, 3.19 ± 2.76, 7.48 ± 5.59, 3.99 ± 2.97, respectively, in triplicate one dot represents 1 cell. Kruskal-Wallis with Dunn's multiple comparison post-hoc test. g) Transcriptomics analysis of WM1862, WM983A and WM983B cell lines was performed in quadruplicate, genes showing statistically significant differential expression (pAdj<0,01) were analyzed using gene ontology (GO). Selected GO terms of differentially regulated pathways are listed showing their fold enrichment and log10 FDR in paired comparisons (left panel) and heatmaps are shown for the two main pathways identified (right panel). h-j) WM1862, WM983A, WM983B cells were analyzed using micropatterning. Cells were seeded on 36μm crossbow micropatterns, let spread for 4 hours, fixed and stained with LAMP1 antibody and cell spreading was controlled by actin staining (Phalloidin). 2D (i) and 3D (j) density maps of LAMP1 staining were calculated using R software, displaying the smallest area that can be occupied by 25, 50 and 75per cent of all compartments. k) Inter-organelle distance (IOD) represents average of distances between all lysosomes. IOD (Mean ± SD) = 10.30 ± 0.94, 11.56 ± 1.15, 13.99 ± 1.40, respectively. One dot represents 1 cell. l) Distance to barycenter (DB) represents the distance from each lysosome to the center of mass. DB (Mean ± SD) = 7.40 ± 0.69, 8.21 ± 0.93, 10.03 ± 1.07, respectively. One dot represents 1 cell. Kruskal-Wallis with Dunn's multiple comparison post-hoc test. (i,j,k,l) WM1862, n= 182 cells, WM983A, n= 231 cells, WM983B, n= 82 cells, in triplicate. \*p<0,05; \*\* p<0,01; \*\*\* p<0,001; \*\*\*\* p<0,0001.

We stably expressed FKBP domain fused to LAMP1 and the FRB domain fused to dynein adaptor BicD2. Rapalog treatment forced binding of BicD2 and recruitment of Dynein to LAMP1 for the movement towards the minus end of microtubules, and thus perinuclear clustering of the LAMP1 compartment around the microtubule organizing center (Fig. 3a, S2d). Clustering was fast, dose-dependent (Figure 2a,b) and persistent in time (Figure 2b, c, Figure S2c), as previously described (Kapitein et al., 2010), allowing precise control of lysosomal positioning in vitro and in vivo investigations. Engineered control cells expressing only single domain FKBP (FKBP only) did not display lysosome clustering upon Rapalog treatment (Fig 2b, c, S2A). Correlative light and electron microscopy (CLEM) of control- or Rapalog-treated WM983B-LAMP1-mCherry cells showed colocalization between LAMP1-mCherry and vesicular compartments, which clustered as expected in the perinuclear region upon Rapalog treatment (Fig 2d). Notably, Rapalog had no effect on the colocalization between LAMP1-mCherry and the BODIPY-Pepstatin A (Fig S2e), which is delivered to lysosomes via endocytic pathway (Chen et al., 2000) and binds to the active site of cathepsin D under acid condition. Thus, clustering by Rapalog did not disrupt cargo delivery and lysosomal catabolic activity in melanoma cells, indicating that we could control lysosomal positioning in relevant patient-derived cell lines, without interfering with basic lysosomal functions. While our study exploited a chemo-genetic model of forced lysosome clustering, the study by (Marwaha et al., 2023) built on the induction of lysosomal spreading demonstrating that fine control or organelle positioning is now at reach. Both approaches require cell engineering and would benefit from the complementary use of small molecules that regulate lysosome positioning, particular for clinical applications. We previously identified PI3K inhibitors as potent lysosome clustering agents in bladder cancer (Mathur et al., 2023). Indeed, lysosome positioning, and more general organelle topology (Wang et al., 2023), were used as a readout in a screen for novel therapeutic drugs and targets (Circu et al., 2016). This opens an exciting area of research leading to a wider drug discovery approach centered on organelle positioning.

**Peripheral lysosomes promote secretion and matrix degradation** Lysosome exocytosis of different proteases and subsequent extracellular matrix degradation promotes invasion to adjacent tissues (Monteiro et al., 2013; Naegeli et al., 2017). We thus first investigated how altering positioning of the LAMP1 compartment impacts the cell secretome. We analyzed the concentrated cell supernatant of WM983B cells in the presence and absence of Rapalog treatment (Fig 3a) by mass spectrometry and identified a significant decrease of several lysosome-associated proteins upon clustering (Fig 3a), including Cathepsins. These enzymes contribute to ECM degradation (Vidak et al., 2019) and have been linked with metastatic progression, for instance in the case of Cathepsin S or B in gastric and colorectal cancers respectively (Bian et al., 2016; da Costa et al., 2020). To confirm that clustering inhibits lysosome secretion, we im-

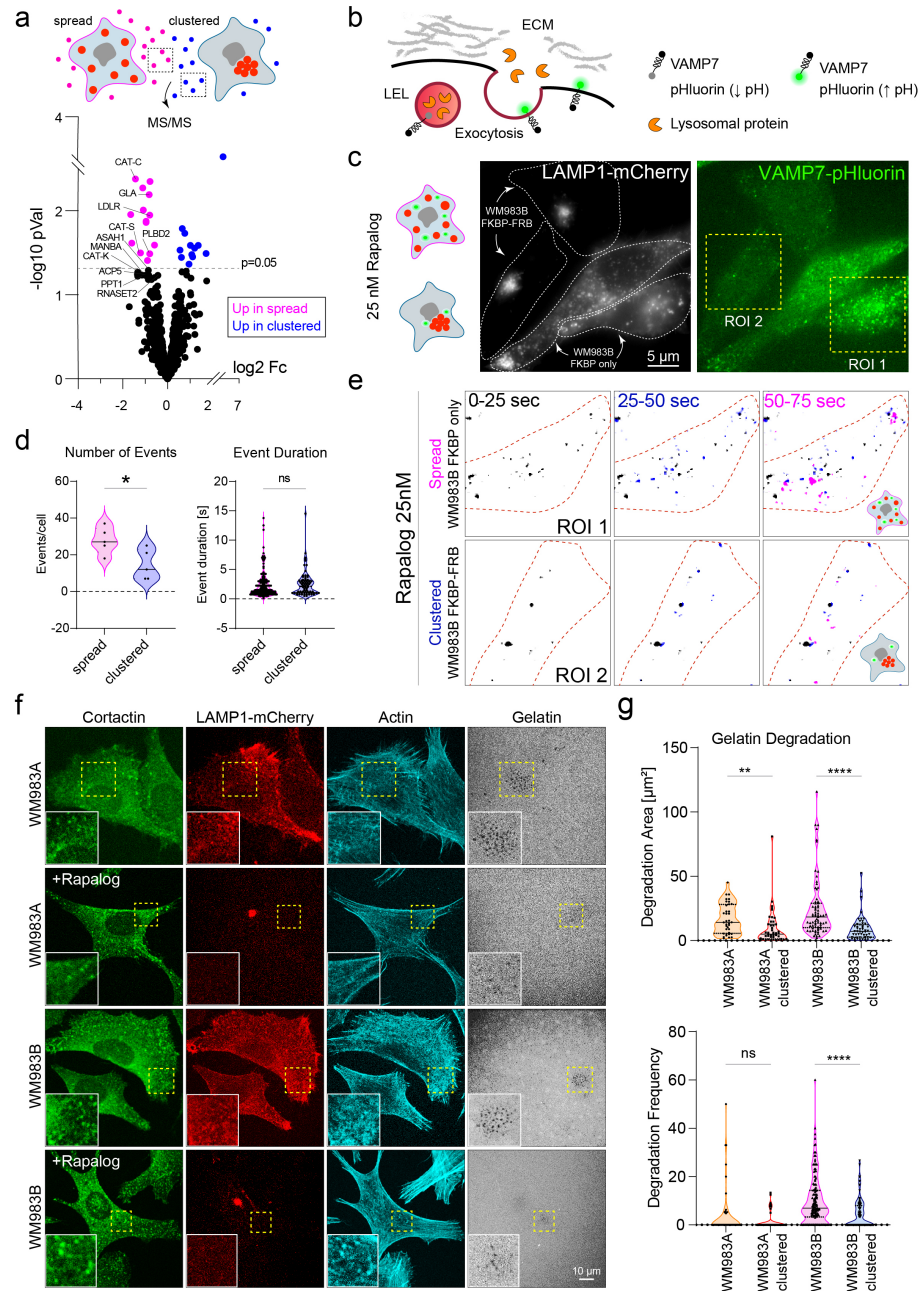
aged VAMP7-pHluorin, a v-SNARE involved in the fusion of lysosome with plasma membrane (Chaîneau et al., 2009) using TIRF microscopy (Fig 3b). We mixed cells expressing either FKBP only or FKBP-FRB and treated them with 25nM Rapalog (Fig 3c). Cells with clustered LAMP1 compartment showed significantly reduced numbers of VAMP7 secretory events (Fig 3d left, Fig 3e), with no impact on the duration of the secretion process (Fig 3d right). Together, these experiments demonstrated that perinuclear localization of lysosomes strongly decreased lysosome secretion in melanoma cells. These results are in line with a previous observation of peripheral lysosomes promoting their fusion with plasma membrane and thus exocytosis in a model of lysosomal storage disease (Medina et al., 2011). Next, we investigated whether lysosome clustering impairs invadopodia-based ECM degradation (Fig 3f). In both cell lines (WM983A, WM983B), we observed a significant decrease in the degradation area (Fig 3g top) upon LAMP1-clustering. While degradation frequency in VGP WM983A cells remained unaltered, metastatic WM983B cells showed a significant decrease (Fig 3g bottom). These results further confirmed that peripheral lysosome positioning promotes lysosome exocytosis and ECM degradation. Forcing perinuclear lysosome localization rescues this phenotype, suggesting that that lysosome position and subsequent secretion promotes ECM remodeling, feature often seen in aggressive cancers (Winkler et al., 2020). More globally, cancer cell secretion is likely to further shape pro-metastatic features of the tumor microenvironment, and favor the emergence of, for example, cancer associated fibroblasts, whose ECM remodeling expertise is pivotal during tumor progression (Kalluri, 2016; Sahai et al., 2020) Our results further suggest that the formation of functional invadopodia requires local lysosome exocytosis. Similarly, targeted secretion of CD63-positive multi-vesicular bodies promotes invadopodia formation. Indeed, other secretory organelles are relocalized to the cell periphery and protrusive structures, and favor, for example, the secretion of pro-tumorigenic extracellular vesicles (Ghoroghi et al., 2021) as it has been shown for CD63+ endo/lysosomes (Hoshino et al., 2013). Whether distinct types of late endosomes act in concert to favor invadopodia-mediated ECM degradation, or whether it involves hybrid late endo-lysosome compartments remains to be determined.

**Forcing lysosomal clustering impairs invasion potential of melanoma cells** Building on the stability of Rapalog-mediated lysosomal clustering in patient-derived cells and the demonstration that it impairs ECM degradation, we sought to monitor if lysosome position impacted the cancer cell dissemination in vivo. To do so, we intravenously injected melanoma cells with different clustering status in two days post-fertilization (dpf) zebrafish embryos (Follain et al., 2018) to probe lysosomal clustering while assessing the metastatic and invasive potential of melanoma cells over time (Fig 4a). Rapalog treatment (5nM) had no effect on cell proliferation or cell viability (Fig S3a, b) during the 3-days time course. Lysosome clustering was stable in vivo and visible in round circulating tumor cells that had just performed



**Fig. 2.** Induced lysosome clustering in melanoma cells a) Lysosomes in WM983A or WM983B cells stably expressing LAMP1-mCherry-FKBP and BicD2-FRB can be clustered using the compound Rapalog (Rap) which induces FKBP-FRB rapid heterodimerization. b) Cells were seeded in glass-bottom dishes, treated with 5nM or 25nM Rapalog and imaged at 24 hours and 72 hours timepoints. Washout condition – cells were treated for 1 hour with Rapalog, washed 3x in PBS and cultured in normal growth medium. WM983B FKBP-FRB = cells expressing both heterodimerizing domains, lysosomes can cluster. WM983B FKBP only = cells expressing only one heterodimerizing domain, clustering is not achieved. Yellow square delineates the zoomed region shown below; red dotted line shows the cell contour. c) Percentage of cells displaying clustered lysosomes was quantified for each condition at 72 hours timepoint. Percentage = 0.0, 73.3 ± 3.6, 78.5 ± 5.1, 77.7 ± 8.3, 76.4 ± 6.2, 0.0, 0.0 respectively. Data come from five randomly selected fields of view per condition. One dot represents 1 field of view. Kruskal-Wallis with Dunn's multiple comparison post-hoc test, adjusted p-value: WM983A Rap vs. washout,  $p > 0.9999$ , WM983B Rap vs. washout,  $p > 0.9999$ . d) Correlative light and electron microscopy. WM983B cells expressing LAMP1-mCherry-FKBP and BicD2-FRB were cultured in control medium, or in medium supplemented with 25 nM Rapalog for one hour and imaged by spinning disc microscope. Samples were processed for electron microscopy and imaged by TEM. Representative images with high magnification of spread or clustered lysosomes and overlay of fluorescent and electron microscopy images (LAMP1 appears in pink, nucleus in blue) are shown for each condition.

301 arrest in the vasculature after injection (Fig 4b). Melanoma 313  
 302 cells with spread lysosomes efficiently extravasated, and dis- 314  
 303 played high metastatic potential in vivo (Fig 4c, left). On 315  
 304 the contrary, when lysosomes were clustered in melanoma 316  
 305 cells before injection, their ability to invade from the pri- 317  
 306 mary tumor site was strongly impaired (Fig 4c, right) al- 318  
 307 though they had similarly completed intravascular arrest and 319  
 308 extravasation as quantified by their post-extravasation inva- 320  
 309 sion potential (Figure 4d, Figure S4a, b). Collectively, these 321  
 310 data show that lysosome positioning is an important driver 322  
 311 of cell aggressiveness, contributing to the ECM remodeling 323  
 312 and to cell invasiveness. As seen in our study, cells with 324  
 peripheral lysosomes have higher invasion potential in vitro  
 as well as in vivo, which can be rescued by promoting the  
 perinuclear lysosome clustering and thus reducing their ma-  
 lignancy, providing the first in vivo evidence for the role of  
 lysosome positioning in metastatic progression. Organelles  
 are dynamic, self-organized structures whose specific func-  
 tion is inevitably linked to their position and morphology  
 (Schauer et al. 2010), in space and time within cells (Bal-  
 labio and Bonifacino, 2020; van Bergeijk et al., 2016). In  
 this study, we provide the first mechanistic demonstration  
 that the position of lysosomes within cells tightly controls the  
 targeted secretion of matrix-degrading enzymes which subse-



**Fig. 3.** Lysosome clustering inhibits lysosome secretion and matrix degradation a) Differential quantitative mass spectrometry analysis of proteins secreted in the cell medium by WM983B cells expressing LAMP1-mCherry-FKBP and BicD2-FRB. Cells were cultured in serum-free medium for 24 hours in the absence or presence of 5nM Rapalog. Each dot represents a protein. Proteins known to be lysosome-associated are labeled with their name. Magenta = proteins upregulated in cells with spread lysosomes, blue = proteins upregulated in cells with clustered lysosomes ( $p < 0,05$ ). b-e) Lysosome secretion was assessed by TIRF microscopy using VAMP7-pHluorin probe, which is quenched in lysosome acidic environment and has bright fluorescence once exposed to the alkaline pH of the extracellular space. c) WM983B-LAMP1-mCherry cells expressing single heterodimerizing domain (FKBP only) and both the domains (FKBP-FRB) were co-cultured, treated with 25nM Rapalog for 1 hour and analyzed by TIRF microscopy. d) Number of secretion events (left graph, one dot represents one cell) and event duration (right graph, one dot represents one event) were manually counted in five different cells of each type. Events per cell, spread =  $27.80 \pm 7.19$ , clustered =  $14.40 \pm 8.23$ , p value = 0.0397, Mann-Whitney test. Event duration, spread =  $2.46 \pm 2.36$ , clustered =  $2.56 \pm 2.20$  seconds, p value = 0.3518, Mann-Whitney test. e) TIRF movie was divided into three time-segments (black: 0-25 seconds, blue: 25-50 seconds, magenta: 50-75 seconds) and displayed as maximum projection showing the number of events per each time-segment. Two representative examples from panel c are shown, ROI 1 = spread lysosomes, ROI 2 = clustered lysosomes. f-g) WM983A or WM983B cells expressing LAMP1-mCherry-FKBP and BicD2-FRB were cultured on FITC-gelatin in presence or absence of 25nM Rapalog for 24 hours, fixed and stained with cortactin antibody and Phalloidin and imaged by confocal microscopy. g) Degradation area (DA) per cell and degradation frequency (DF) were quantified from the immunofluorescence images. DA (Mean  $\pm$  SD) =  $16.94 \pm 11.71$  n= 36 cells,  $9.38 \pm 13.47$  n= 45 cells,  $24.75 \pm 23.29$  n= 73 cells,  $9.41 \pm 9.94$  n= 50 cells, respectively, in triplicate. DF (Mean  $\pm$  SD) =  $3.62 \pm 9.73$  n= 57 cells,  $1.49 \pm 3.44$  n= 58 cells,  $10.14 \pm 10.45$  n= 138 cells,  $4.43 \pm 6.04$  n= 102 cells, respectively, in triplicate, Kruskal-Wallis with Dunn's multiple comparison post-hoc test. One dot represents one cell. \* $p < 0,05$ ; \*\* $p < 0,01$ ; \*\*\* $p < 0,001$ ; \*\*\*\* $p < 0,0001$ .

quently promotes melanoma cell invasion and metastatic progression. This echoes a parallel study showing that relocalization of lysosomes to the cell periphery promotes the emergence of leader cells in collective epithelial cell migration (Marwaha et al., 2023). While more work is required to understand the switches that relocalize lysosomes, both studies demonstrate that peripheral lysosome positioning can impact cell migration, through different mechanisms, either through lysosomal secretion (our study) or through a Rac1-dependent control of actin polymerization and lamellipodium formation (Marwaha et al., 2023). In addition, lysosome repositioning could potentially alter tumor progression by mediating nutrient sensing (Korolchuk et al., 2011) or chemoresistance through the secretion of chemotherapeutics stored in lysosomes (Machado et al., 2015). Probing of spatial distribution and morphology of lysosomes in tumors could constitute a novel indicator of tumor progression, as it is the case for cell shape or additional morphometric analysis (Sero et al., 2015; Pei-Hsun Wu et al., 2020). Several other organelles, such as mitochondria, are intimately linked to cancer progression and probing simultaneously all organelles, as it can be done using whole-cell segmentation of high-resolution images (Heinrich et al., 2021), could document precisely which organelles, and their contacts, are repositioned during melanoma progression. Interestingly, deep learning-based approaches recently demonstrated the power of correlating breast cancer status with organelle topology, which out-performed morphology-based features, further highlighting the need to consider organelles positioning, and their interactome, as a new cancer rheostat that could be exploited for better diagnosis (Wang et al., 2023).

## Methods

**Antibodies** Anti-Cortactin (p80/85) clone 4F11 (ref. n°05-180-I) and Anti-LAMP1 (ref. n°L1418) are from Merck (Sigma-Aldrich). Alexa Fluor™ 488 Phalloidin (ref. n° A12379), Alexa Fluor™ 568 Phalloidin (ref. n°A12380), Alexa Fluor™ 647 Phalloidin (ref. n°A22287) and Alexa Fluor-conjugated secondary antibodies, are all from Thermo Fisher Scientific. Secondary antibodies include Alexa Fluor™ 405 conjugated Goat anti-Rabbit (ref. n°A-31556), Alexa Fluor™ 488 conjugated Goat anti-Rabbit (ref. n°A-11034), Alexa Fluor™ 555 conjugated Goat anti-Rabbit (ref. n°A21429) Alexa Fluor™ 647 conjugated Goat anti-Rabbit (ref. n°A-21244).

**Cell culture** Primary human melanoma cell lines were purchased from Rockland and cultured in MCDB153 (ref. n°M7403, Merck/Sigma-Aldrich) and Leibovitz's L-15 medium (ref. n°11415064, Thermo Fisher Scientific) in a 4 to 1 ratio, supplemented with 2 per cent foetal bovine serum, 1.68 mM CaCl<sub>2</sub> and 1 per cent penicillin/streptomycin.

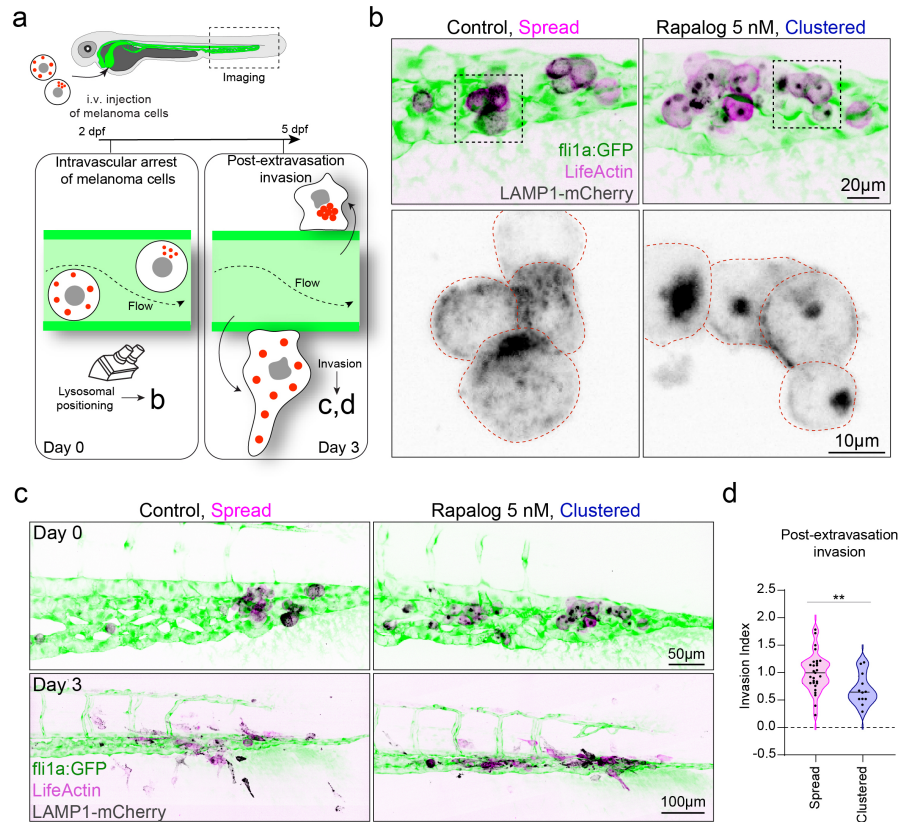
**Lentivirus transduction and plasmid transfection** Transduction: pLSFFV-LAMP1-mCherry-FKBP (FK506 binding protein), pLSFFV-BicD-FRB (FKBP-rapamycin binding domain) and pLSFFV-LifeActin-miRFP lentivirus were produced in HEK293T cells using JetPRIME® transfection reagent (Polyplus). WM983B cells were infected by

lentivirus in the presence of 5µg/mL polybrene (ref. n°TR-1003, Merck/Sigma-Aldrich) followed by antibiotic selection (puromycin 1µg/mL, blasticidin 5µg/mL, hygromycin 200µg/ml). Transfection: Cenexin-GFP plasmid was transfected using JetOPTIMUS® transfection reagent (Polyplus) in a live-video dish, 46h prior experiment.

**Invasion assay** The 3D collagen invasion assay was adapted from (Sadok et al., 2015). Briefly, melanoma cell lines at 3x10<sup>5</sup> cells/ml were labelled with Hoechst 33342 and suspended in 3mg/mL of serum free solution of neutralized Type I Bovine Collagen (PureCol® 5005-B, Advanced Biomatrix). Then, 600µL were distributed into black 24 well plates (ref. n°058062, Dutscher) coated with bovine serum albumin. The plates were centrifuged at 1500 rpm at 4°C for 5min and incubated at 37°C for 3 hours. Once collagen had polymerized, medium supplemented with 10 per cent foetal bovine serum, 100ng/mL EGF was added on top of the collagen. 24h after, cells were observed using a Leica TSC SPE confocal microscope (x20 HCX PI Apo 0.7 NA objective, Wetzlar, Germany) and z-stacks were acquired. Four fields per sample were imaged. Nuclear localization was quantified by IMARIS (interactive microscopy image analysis software) at each plane. The invasion index was calculated by reporting the number of cells above 10µM on the total number of cells by field.

**Matrix degradation assay and immunofluorescence** Glass coverslips were coated with fluorescent-labelled-FITC gelatine or Cy3 gelatine as described previously (Kolli-Bouhafs et al., 2014). Then, previously starved melanoma cells expressing or not LAMP1-mCherry-FKBP and BicD2-FRB were plated on fluorescent gelatine and incubated at 37°C for 6 to 24h in medium supplemented with 10 per cent foetal bovine serum with or without Rapalog. Cells were fixed using 4 per cent paraformaldehyde permeabilized using triton-X-100 at 0,1 per cent and incubated in 2 per cent of bovine serum albumin at room temperature. Cells were then labelled for 1h with Anti-Cortactin (1/250). After three washes with PBS, cells were incubated with either Alexa Fluor™ 405 conjugated Goat anti-Rabbit (1/1000), Alexa Fluor™ 488 conjugated Goat anti-Rabbit (1/1000) or Alexa Fluor™ 647 conjugated Goat anti-Rabbit (1/1000) and Alexa Fluor™ 568 Phalloidin (1/250) or Alexa Fluor™ 647 Phalloidin (1/250) for 1h, washed and mounted in ProLong™ Gold Antifade Mountant (ref. n°P10144, Thermo Fisher Scientific). Cells were imaged using Leica TSC SPE or SP8 confocal microscope (x63 HCX PI Apo 1.40 NA or x20 HCX PI Apo 0.7 NA objective, Wetzlar, Germany). Invadopodia were identified as actin and cortactin rich punctate structure. Areas of degradation were identified as "black holes" within the fluorescent gelatine. Invadopodia and areas of degradations were quantified using ImageJ software. Degradation areas measurements were based on cells displaying degradation activity, and the frequency of degradation was based on randomly selected cells. Maximum filter, background subtraction and gaussian blur filters were then applied to extract the gel degradation areas by thresholding. Then, M1 Manders coefficients between Lamp1 and inverted Gelatin inten-





**Fig. 4.** Lysosome clustering impairs cancer cell invasion in vivo a) Fli1a:GFP (green endothelium) zebrafish embryos (48 hpf) were injected intra-vascularly with WM983B cells having either spread or clustered lysosomes and imaged by confocal microscopy at day 0 and day 3 post injection to assess the post-extravasation invasion potential of cancer cells with different lysosomal positioning. b) WM983B cells stably expressing LAMP1-mCherry-FKBP, BicD2-FRB and LifeActin-miRFP were cultured in medium or in medium supplemented with 5nM Rapalog for 1 hour before injection. Representative confocal images at 3 hours post-injection are shown as maximum z projection for each condition, control = spread lysosomes, 5nM Rapalog = clustered lysosomes. c) Zebrafish embryos were imaged at day 0 (3 hours after cell injection) and at day 3 (72 hours post injection) using a spinning disc microscope. Representative images are shown as a maximum z projection. d) Post-extravasation invasion (PEI) potential was calculated as a proportion of cells (area) that migrated outside of the vasculature region to the total area of cells. PEI (Mean  $\pm$  SD), control =  $1.000 \pm 0.3491$  n = 29 embryos, clustered =  $0.696 \pm 0.285$  n = 12 embryos, in triplicate, p value = 0.0090, Mann-Whitney test. One dot represents one fish, normalized to control.

438 sity pictures for these selections were calculated using the 459  
439 colocalization finder plugin. 460

440 **Clustering dynamics, washout experiments proliferation** 462  
441 **assay** Mechanism of lysosome clustering: clustering was per- 463  
442 formed by heterodimerization between LAMP1-mCherry- 464  
443 FKBP (lysosomes) and BicD-FRB (dynein adaptor) by the 465  
444 use of Rapalog (A/C Heterodimerizer ref. n°635057, from  
445 Takara Bio Inc.). Cells were cultured in glass bottom dishes 466  
446 and imaged at different time points (24, 48 and 72 hours) 467  
447 using Olympus Spinning Disk (60X objective, N.A. 1.2). 468  
448 To establish time needed for lysosome clustering, WM983A 469  
449 and WM983B cells expressing both heterodimerizing do- 470  
450 mains (FKBP-FRB) were treated with 5nM or 25nM Ra- 471  
451 palog and followed in time. Time to appearance of lyso- 472  
452 some cluster was counted. Washout experiments: WM983B 473  
453 cells expressing either one (FKBP only) or two (FKBP-FRB) 474  
454 heterodimerizing domains were cultured in medium supple- 475  
455 mented with 5nM or 25nM Rapalog. To assess the stability of 476  
456 clustering, cells were treated for 1h with 5nM or 25nM Rapa- 477  
457 log, washed 3x in PBS and cultured in normal medium for the 478  
458 duration of the experiment. Cells cultured in normal growth 479

medium were used as a control. Proliferation assay: prolif-  
eration rates of WM983B cells treated with increasing con-  
centrations of Rapalog were analyzed using the Incucyte®  
Live-Cell Analysis System. Confluences were automatically  
calculated by the Incucyte® software based on bright field  
images, all values are normalized to time zero. Acquisition  
was performed for 96h.

**Micropatterning Immunofluorescence** Micropatterns were  
prepared using photo-lithography methods as previously de-  
scribed (Duong et al., 2012; Schauer et al., 2010). Briefly,  
cover slides were cleaned in EtOH, dried, cleaned with UV  
for 10 minutes, coated in PLL-PEG and exposed to UV 10  
minutes through a Photomask with 36µm crossbow micropat-  
terns. Coverslips were coated in Fibronectin (40µg/ml –  
ref. n°F1141 Merck/Sigma-Aldrich). WM1862, WM983A,  
WM983B cells were trypsinized, resuspended in 5ml culture  
medium and seeded an micropatterned coverslips. Cells were  
let to spread for 4 hours before fixing them in 4 per cent  
PFA. For micropatterning, cells were fixed in 4 per cent  
PFA, washed 3x in PBS, permeabilized in saponin 0.5 per cent /  
2 per cent BSA in PBS for 20 minutes, blocked 30 minutes

in 2 per cent BSA, stained with Anti-LAMP1 (ref. n°L1418) primary antibody for 1 hour, washed 3x in PBS, stained with Alexa Fluor™ 555 secondary Goat anti-Rabbit (1:500) and Alexa Fluor™ 488 Phalloidin (1:200) 45 minutes. Mounted in Fluoromount-GTM mounting medium (ref. n°00-4958-02, Thermo Fisher Scientific with DAPI. Z stacks were acquired for the full cell volume (0.2µm between layers) using an Olympus Spinning Disk (60X objective, N.A. 1.2). Image analysis and processing were performed using the Fiji (2.0.0) (Schindelin et al., 2012), Cell Profiler (4.2.1) (Stirling et al., 2021), Metamorph and R software.

**Live cell imaging** For live cell imaging, Lamp1-mCherry and Lifeact-miRFP expressing cells were plated on fluorescent gelatin-coated coverslips and mounted in a Ludin Chamber (Life Imaging Services). The cells were then placed at 37°C, 5 per cent CO<sub>2</sub> on an iMIC microscope equipped with a multi-LED Lumencor Spectra X. Images were acquired with an Olympus 60x TIRFM (1.45 NA) objective every 2.5min during 4h and a Hamamatsu Flash 4 V2+ camera (Iwata) piloted by the Live Acquisition software (Till Photonics). Expressing cells were initially located via both the mCherry and Lifeact-miRFP signals, and were subsequently followed via dual phase contrast/fluorescent signal together with the FITC-coupled gelatin substrate. 10 to 20 different fields were sequentially recorded during each experiment using a Marzhauser Motorized Stage piloted by the iMIC software. JeasyTFM software were then used for automatic selection and repositioning of the best focused images in all channels and time-points. Mean fluorescence intensity of actin, Lamp1 and the underlying substrate at each time points were calculated at invadopodia identified using gelatin degradation.

**TIRF microscopy** WM983B cells expressing FKBP only (spread) and WM983B cells expressing both FKBP and FRB (clustered) were mixed in an 1:1 ratio, seeded in fibronectin-coated low glass bottom µ-Dish 35mm (ref. n°80137, Ibidi) 48h prior to imaging, treated with 25nM Rapalog for 1h. Imaging was performed in culture medium using an inverted Leica DMI8 microscope (objective 100X HC PL APO 1.47 oil). Recording was done with an Evolve® 512 camera (for TIRF-HILO), at 512X512 pixels resolution, at an acquisition rate of 250ms between frames, for a total duration of 90 seconds, with AFC (Adaptive Focus Control). Exocytosis events were identified based on VAMP7-pHluorin signal, marking lysosome exocytosis (Lachuer et al., 2023). Secretion events were detected and counted manually.

**Experimental metastasis assay in zebrafish** All zebrafish (ZF) procedures were performed in accordance with French and European Union animal welfare guidelines and supervised by local ethics committee (Animal facility A6748233; APAFIS 2018092515234191). Tg(fli1a:eGFP) (Lawson and Weinstein, 2002) embryos were maintained in Danieau 0.3X medium (17.4mM NaCl, 0.2mM KCl, 0.1mM MgSO<sub>4</sub>, 0.2mM Ca(NO<sub>3</sub>)<sub>2</sub>) buffered with HEPES 0.15mM (pH=7.6), supplemented with 200µM of 1-phenyl-2-thiourea (PTU, ref. n°P7629, Merck/Sigma-Aldrich) to avoid pigmentation. Two days post-fertilization (2dpf) embryos were mounted in 0.8 per cent ultrapure low melting point agarose (Invitro-

gen) containing 0.17mg/ml tricaine (ethyl-3-aminobenzoate-methanesulfonate, ref. n°E10521, Merck/Sigma-Aldrich). WM983B cells of different lysosome clustering status (spread, clustered) were injected with a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company) and microforged glass capillaries (25 to 30µm inner diameter) filled with mineral oil (ref. n°M5904, Merck/Sigma-Aldrich). 13.8nL of cell suspension from confluent T25 flasks (50x10<sup>6</sup> cells per ml approx.) were injected in the duct of Cuvier under a M205 FA Fluorescence stereomicroscope (Leica), as previously described (Stoletov et al., 2010). Embryos were injected with WM983B cells or with WM983B cells treated with 5nM rapalog 1h prior to injection, and then kept in Danieau with PTU. Caudal plexus was recorded at day 0 (injection day) and 3dpi using the inverted spinning-disk Olympus IXplore Spin, 30x / 1.05 NA (silicone) objective. Z-stacks of the caudal plexus were acquired for each embryo (3µm or 5µm between layers, at day 0 and 3dpi respectively) with the following settings: 488nm laser at 2 per cent for 100ms / 561nm laser at 15 per cent for 300ms / 640nm laser at 15 per cent for 300ms). Detailed lysosome status (Fig.4b) was imaged at 3 hpi using a 60x/1.2 NA (water) objective.

**Correlative Light and Electron Microscopy (CLEM)** WM983B cells expressing LAMP1-mCherry-FKBP and BicD2-FRB were cultured in control medium, or in medium supplemented with 25nM Rapalog for one hour and imaged with an Olympus Spinning Disk (60X objective, N.A. 1.2). The samples were then chemically fixed right after the photonic acquisition with 0,05 per cent malachite green, 2.5 per cent glutaraldehyde in 0.1M sodium cacodylate buffer (NaCac), pH7.4 during 30min in an ice bath. Subsequently the samples were post-fixed in 1 per cent OsO<sub>4</sub> - 0.8 per cent K<sub>3</sub>[Fe(CN)<sub>6</sub>] - 0.1M NaCac buffer pH7.4 (under a fume hood) kept in an ice bath for 50 min, and then washed 2 times with ice-cold 0.1M NaCac. Then the samples were incubated in 1 per cent aqueous tannic acid solution for 25 min in an ice bath and finally washed 5 times with distilled water. Samples were then kept in 1 per cent uranyl acetate aqueous solution overnight at 4°C sheltered from the light. The samples were serially dehydrated with ethanol solutions (25 per cent, 50 per cent, 70 per cent 95 per cent and 100 per cent). Subsequently the samples were incubated in a serial resin-ethanol 100 per cent mix (1:3; 1:1; 3:1), ending with an incubation in 100 per cent Epon resin 3 times 1h at room temperature. The samples were allowed to polymerize in an oven at 60°C for 48h. The resin blocks were trimmed by ultramicrotomy, 90nm thin sections were collected and placed in copper/formvar slot grids. The transmission electron microscopy (TEM) data sets were acquired with a Hitachi 7500 TEM, with 80 kV beam voltage, and the 8-bit images were obtained with a Hamamatsu camera C4742-51-12NR. Correlative light and electron images were obtained/combined using Adobe Photoshop v.24.4.

**Image analysis** Organelles segmentation was performed using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices) and the ImageJ Modular Image Analysis (MIA) plugin. Segmentation on LAMP1

594 images were performed to get coordinates for individual 651  
595 LAMP1+ objects and their number. 3D Density maps and 652  
596 inter-organelle distance and distance to barycenter were ob- 653  
597 tained through the use of R software. Cell invasion in ze- 654  
598 brafish was performed by ImageJ Cell Profiler (Molecular 655  
599 Devices) software. Zebrafish images were divided into 6 re- 656  
600 gions – plexus and 5 regions outside (bins). Area of cells was 657  
601 quantified for each region using Cell Profiler and percentage 658  
602 of cells (from total) was calculated per each region and plot- 659  
603 ted in a graph. Invasion potential was calculated as area under 660  
604 the curve for all cells that extravasated and invaded outside of 661  
605 the vasculature region. 662

606 **Mass Spectrometry - Quantitative Proteomics** A 664  
607 PierceTM 660nm protein assay quantification (ref. n°22660, 665  
608 Thermo Fisher Scientific), 2µg of each protein extract 666  
609 were digested using the automated Single Pot Solid Phase 667  
610 enhanced Sample Preparation (SP3) protocol as described 668  
611 in (Hughes et al., 2019) on the Bravo AssayMAP plat- 669  
612 form (Agilent Technologies). Extracted peptides were 670  
613 cleaned-up using automated C18 solid phase extraction on 671  
614 the same platform and analysed by nanoLC-MS/MS on a 672  
615 nanoUPLC system (nanoAcquityUPLC, Waters) coupled 673  
616 to a quadrupole-Orbitrap mass spectrometer (Q-Exactive 674  
617 HF-X, Thermo Scientific). Chromatographic separation was 675  
618 conducted over a 60 minutes linear gradient from 2 to 40 per 676  
619 cent of solvent B (0.1 per cent formic acid in acetonitrile) 677  
620 at a flow rate of 350 nL/min. A Top 10 method was used 678  
621 with automatic switching between MS and MS/MS modes 679  
622 to acquire high resolution MS/MS spectra. To minimize 680  
623 carry-over, a solvent blank injection was performed after 681  
624 each sample. NanoLC-MS/MS data was interpreted to do 682  
625 label-free extracted ion chromatogram-based differential 683  
626 analysis. Searches were done using Mascot software (version 684  
627 2.5.1, MatrixScience) against a composite database including 685  
628 Homo Sapiens and Bos Taurus protein sequences, which 686  
629 were downloaded from UniProtKB-SwissProt (28-07-2021; 687  
630 26.031 sequences, Taxonomy ID: 9913 and 9606 respec- 688  
631 tively) to which common contaminants and decoy sequences 689  
632 were added. One trypsin missed cleavage was tolerated. 690  
633 Carbamidomethylation of cysteine residues was set as a fixed 691  
634 modification. Oxidation of methionine residues and acetyla- 692  
635 tion of proteins n-termini were set as variable modifications. 693  
636 Identification results were imported into the Proline software 694  
637 (version 2.2.0) (Bouyssie et al., 2020) and validated. The 695  
638 maximum false discovery rate was set at 1 per cent at peptide 696  
639 and protein levels with the use of a decoy strategy. Peptides 697  
640 abundances were extracted with cross assignment between 698  
641 all samples. Protein abundances were computed using the 699  
642 best ion of the unique peptide abundances normalized at the 700  
643 peptide level using the median. To be considered, proteins 701  
644 must be identified in at least three out of the four replicates in 702  
645 at least one condition. The imputation of the missing values 703  
646 and differential data analysis were performed using the 704  
647 open-source ProStaR software (version 1.30.7) (Wieczorek 705  
648 et al., 2017). Imputation of missing values was done using 706  
649 the approximation of the lower limit of quantification by 707  
650 the 2.5 per cent lower quantile of each replicate intensity 708

distribution (“det quantile”). A Limma moderated t-test was applied on the dataset to perform differential analysis. The adaptive Benjamini-Hochberg procedure was applied to adjust the p-values and False Discovery Rate. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2022) with the dataset identifier PXD042007.

**Transcriptomic analysis** RNA integrity was assessed by Bioanalyzer (total RNA Pico Kit, 2100 Instrument, Agilent Technologies, Paolo Alto, CA, USA). All samples had RNA integrity numbers above 9.5. Sequencing libraries were prepared using “NEBNext Ultra II Directional RNA Library Prep Kit for Illumina” combined with “NEB Ultra II polyA mRNA magnetic isolation” for mRNA enrichment (New England Biolabs, Ipswich, MA, USA). Libraries were pooled and sequenced (single-end, 100bp) on a NextSeq2000 according to the manufacturer’s instructions (Illumina Inc., San Diego, CA, USA). For each sample, quality control was carried out and assessed with the NGS Core Tool FastQC (Andrews S, 2010). Sequence reads (minimum 33 Million per sample) were mapped to Homo Sapiens genome version GRCh38 using STAR (Dobin et al., 2013) to obtain a BAM (Binary Alignment Map) file. An abundance matrix was generated based on read counts identified by Featurecounts (Liao et al., 2014) using default parameters. At last, differential expression analyses were performed using the DESeq2 (Love et al., 2014) package of the Bioconductor framework for RNASeq data (Gentleman et al., 2004). Up- and down-regulated genes were selected based on the adjusted p-value (< 0.05) and the fold-change (> 1.5). Functional enrichment analyses were performed using STRING v11 (Szklarczyk et al., 2019) and Gene Ontology (Carbon et al., 2021). Raw data (FASTQ files) are available at the EMBL-EBI ArrayExpress archive (Accession number E-MTAB-13165).

**Statistical analyses** Statistical analysis of the results was done using GraphPad Prism 9 (version 9.5.1 for Windows). Mann-Whitney (two groups) or Kruskal-Wallis (>2 groups) statistical tests were performed as specified in the figure legends, to the exception of quantitative proteomics and transcriptomic analysis which have dedicated statistical methodologies specified above. Illustrations of the statistical analyses are displayed in the figures as the mean +/- standard deviation (SD). p-Values smaller than 0.05 were considered as statistically significant. \*, p<0.05, \*\*, p<0.01, \*\*\*, p<0.001, \*\*\*\*, p<0.0001.

**Acknowledgements** We thank all members of J.G. Goetz’s and K. Schauer teams for discussions on this topic. J.G. Goetz is the coordinator of the NANOTUMOR Consortium, a program from ITMO Cancer of AVIESAN (Alliance Nationale pour les Sciences de la Vie et de la Santé, National Alliance for Life Sciences Health) within the framework of the Cancer Plan (France) that has mostly supported this work, including the teams of KS and PR. Work and people in the lab of J.G. are also supported by the INCa (Insti-

707 tut National Du Cancer, French National Cancer Institute), 761  
708 charities (La Ligue contre le Cancer, which also supports 762  
709 PR, and ARC (Association pour la Recherche contre le Can- 763  
710 cer), FRM (Fondation pour la Recherche Médicale)), the Na- 764  
711 tional Plan Cancer initiative, the Region Est, INSERM and 765  
712 the University of Strasbourg. Proteomics experiments were 766  
713 supported by the French Proteomic Infrastructure (ProFI 767  
714 FR2048, ANR-10-INBS-08-03). K. Jerabkova-Roda is sup- 768  
715 ported by a post-doctoral fellowship SPF202004011876 from 769  
716 FRM and the NANOTUMOR consortium. HL was sup- 770  
717 ported by ARC (Association pour la Recherche contre le 771  
718 Cancer) and FRM (Fondation pour la Recherche Médicale). 772  
719 We are also thankful to recent donators (Rohan Athlétisme 773  
720 Saverne) to support our work. The imaging was supported 774  
721 by CRBS imaging platform PIC-STRA with assistance from 775  
722 P. Kessler, by the Imaging Center of IGBMC with assistance 776  
723 from E. Grandgirard and E. Guiot and by the PIQ Platform 777  
724 with assistance from R. Vauchelles.. We acknowledge the 778  
725 Cell and Tissue Imaging Platform (PICT-IBiSA), member of 779  
726 the national infrastructure France-BioImaging supported by 780  
727 the French National Research Agency (ANR-10-INBS-04). 781  
728 We are grateful to T.Galli (IPNP, INSERM U1266, Paris) 782  
729 for sharing the VAMP7-pHLuorin constructs. We thank A- 783  
730 C. Reymann and R. Benoit for sharing their resources, D. 784  
731 Sampaio Goncalves for help with ImageJ macro writing and 785  
732 M. Durik for valuable feedback on the work. 786

733

734 **Author Contributions** KJR, VH, KS, PR and JGG con- 789  
735 ceived project and designed the experiments. KJR performed 790  
736 most of the experiments and analysis. AM, RK, HJ, LML, 791  
737 PC, ES and PR performed collagen gel invasion assays and 792  
738 invadopodia experiments. KJR, NA and MP performed zeb- 793  
739 rafish experiments. KJR and IB performed CLEM experi- 794  
740 ments. AL and OL performed molecular biology. KJR per- 795  
741 formed and analyzed (with help from HL) micropatterning 796  
742 experiments. AP, TS, AM and RC performed and analyzed 797  
743 RNAseq experiments. AH, FD and CC performed and ana- 798  
744 lyzed mass spectrometry experiments. VH, KS, PR and JGG 799  
745 provided funding. VH and JGG supervised the study. KS 800  
746 and PR co-supervised the study. KJR, VH and JGG wrote the 801  
747 manuscript, with input from KS and PR. All authors proof- 802  
748 read the manuscript. 803

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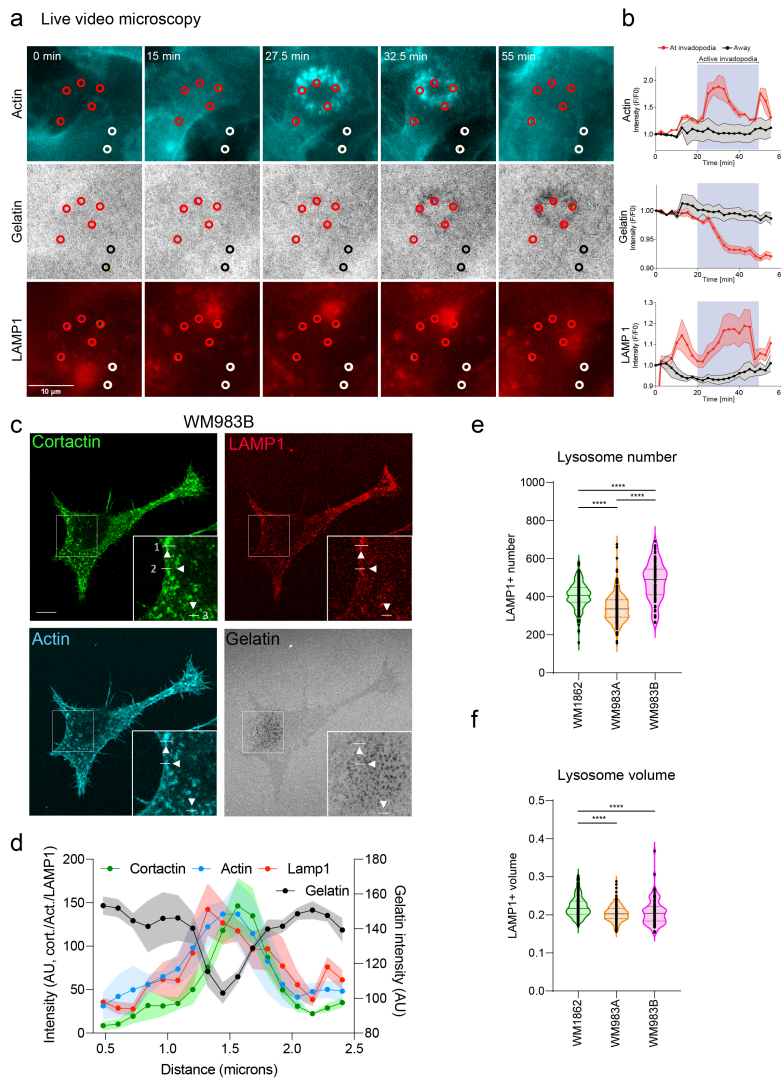
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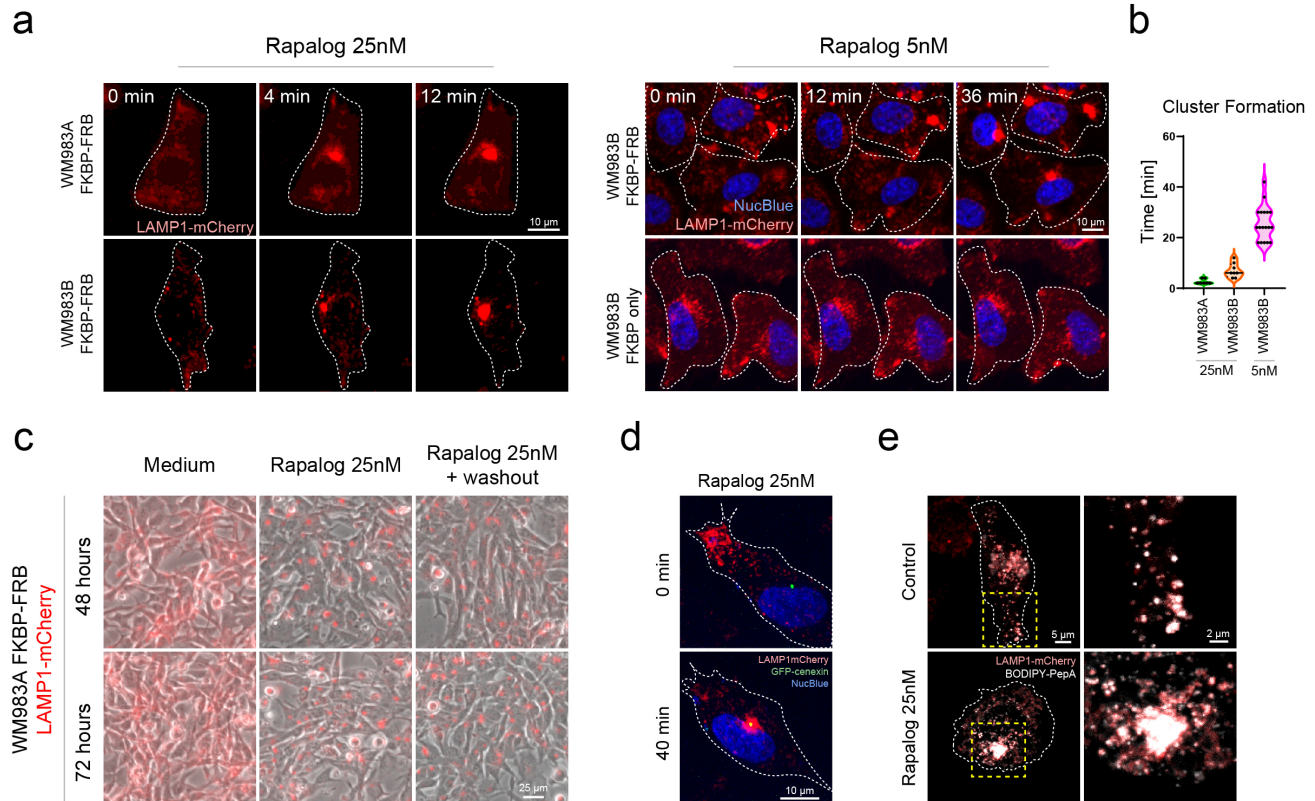
**Fig. S1.** Lysosomal marker LAMP1 colocalizes with active invadopodia a) WM983B cells stably expressing lysosome marker LAMP1-mCherry and LifeActin-miRFP were seeded on gelatin 24 hours prior to the experiment. Time-lapse movies were acquired during one hour and the signal intensity was quantified in five invadopodia-rich regions (red) and in two regions outside of active invadopodia (gray), blue rectangle marks the duration of active invadopodia. b) Intensity profiles over time are plotted for actin (invadopodia), gelatin (degradation) and LAMP1 (lysosomes). c) WM983B cells were seeded on gelatin 24 hours prior to the experiment, fixed and stained with cortactin, and LAMP1 antibody and with phalloidin (actin). d) The level of colocalization was quantified by line profiling in ImageJ. e-f) Cells were grown on crossbow micropatterns for 4 hours, fixed and stained with LAMP1 antibody. Images were segmented in Metamorph. R software was used to quantify total lysosome number per cell and the average volume of lysosome per cell. One dot represents 1 cell. Kruskal-Wallis with Dunn's multiple comparison post-hoc test. WM1862, n= 182 cells, WM983A, n= 231 cells, WM983B, n= 82 cells, in triplicate. e) Lysosome number (Mean  $\pm$  SD) = 405.9  $\pm$  68, 341.9  $\pm$  74, 479.7  $\pm$  99, respectively. f) Lysosome volume = 0.2211  $\pm$  0.023, 0.2035  $\pm$  0.021, 0.2071  $\pm$  0.034, respectively. \*p<0,05; \*\* p<0,01; \*\*\* p<0,001; \*\*\*\* p<0,0001.

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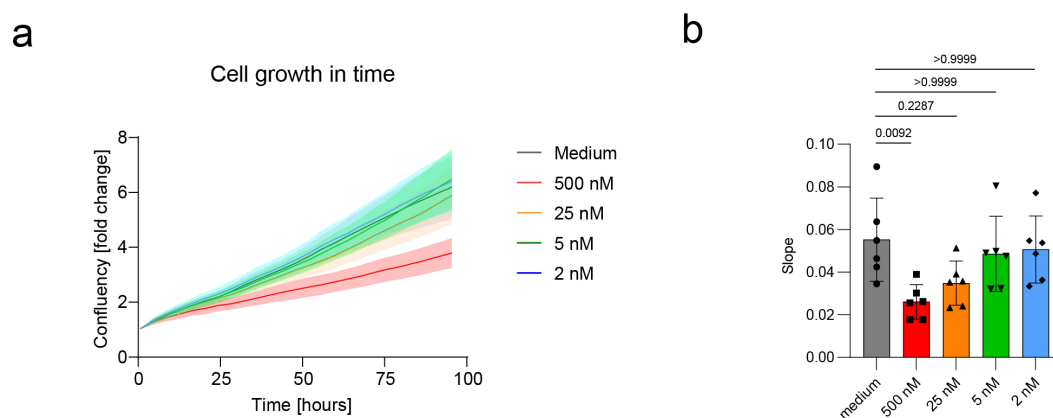
1076 **Supplementary material** Table 1: Lysosome genes differentially expressed (refers to Fig1g)

1077 Table 2: Positive Regulation of cell migration genes differentially expressed (refers to Fig1g)

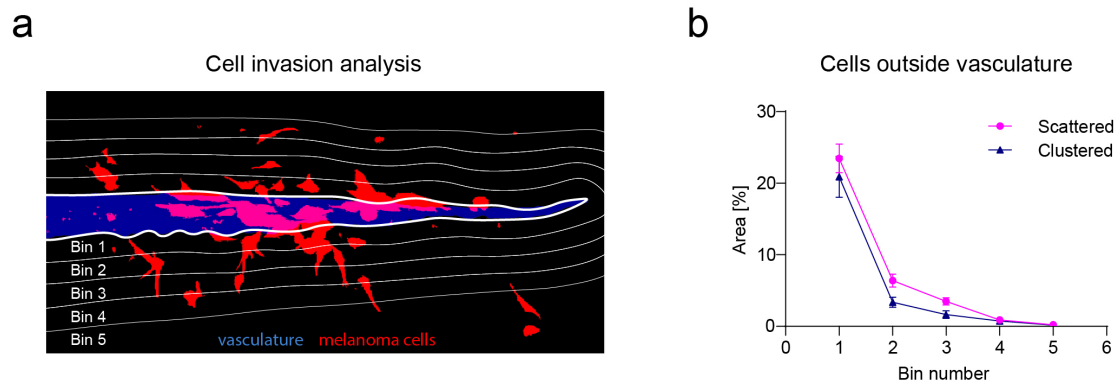




**Fig. S2.** Dynamics of Rapalog-induced lysosome clustering a) WM983A and WM983B cells expressing two heterodimerizing domains (FKBP-FRB) and WM983B cells expressing only one heterodimerizing domain (FKBP only) were seeded in glass bottom dishes and 1-hour time-lapse movies were acquired using spinning disc. After acquiring the first time-frame, cells were treated with 25 nM Rapalog (left) or with 5 nM Rapalog (right). b) Cluster formation was quantified as the time needed until a visible cluster appeared. Time [min] =  $2.6 \pm 1.0$ ,  $7.0 \pm 2.8$ ,  $25.7 \pm 6.8$ , respectively. One dot represents 1 cell. c) WM983A (FKBP-FRB) cells were treated with 25 nM Rapalog and imaged at 48 hours and 72 hours timepoints. Washout condition – cells were treated for 1 hour with Rapalog, washed 3x in PBS and cultured in normal growth medium for the duration of the experiment. d) WM983B (FKBP-FRB) cells were seeded in glass bottom dishes and 45 minutes time-lapse movies were acquired using spinning disc. Rapalog induces lysosome clustering around the microtubule organizing center, labelled by cenexin-GFP. e) WM983B (FKBP-FRB) cells expressing LAMP1-mCherry (red) were cultured in medium or in medium with 25 nM Rapalog for 1 hour and then treated with PepstatinA-BODIPY (white) probe for 45 minutes. Cells were imaged live using spinning disc.



**Fig. S3.** Low dose of Rapalog treatment does not inhibit cell proliferation a-b) Proliferation rate of WM983B cells treated with increasing concentrations of Rapalog was analyzed using incuCyte. a) Cell confluency was quantified for each time-point, all values were normalized to time 0 and the growth curves were plotted. Colored lines depict different culture conditions: medium (black), 2 nM (blue), 5 nM (green), 25 nM (orange), 500 nM Rapalog (red) and are representing average value of three experiments  $\pm$  SD. b) Slopes were calculated for the different growth conditions. Slope (Mean  $\pm$  SD) =  $0.0552 \pm 0.02$ ,  $0.0261 \pm 0.01$ ,  $0.0348 \pm 0.01$ ,  $0.0486 \pm 0.02$ ,  $0.0507 \pm 0.02$ , respectively. Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, respective adjusted p-values are shown in the graph. One dot represents one experiment (performed in technical triplicate).



**Fig. S4.** Monitoring cell invasion in vivo a-b) Quantification of the post-extravasation invasion potential at 72 hours post injection. a) Representative example of the segmented image. Vasculature (blue) and WM983B melanoma cells (red) were segmented using ImageJ. Segmented images were further analyzed using CellProfiler: Image was divided into five regions (bins) with increasing distance from the vasculature region and the total cell area in each region was quantified. b) Percentage of cells (measured from their area) present in each bin was calculated and plotted for both conditions. Magenta = cells with spread lysosomes, blue = cells with clustered lysosomes. Each line represents mean value from three independent experiments ± SEM. Percentage (scattered) = 23.5 ± 2.0, 6.4 ± 0.9, 3.5 ± 0.5, 0.9 ± 0.2, 0.2 ± 0.1, respectively, n = 29 embryos. Percentage (clustered) = 20.9 ± 2.9, 3.4 ± 0.7, 1.6 ± 0.5, 0.74 ± 0.2, 0.2 ± 0.1, respectively, n = 12 embryos.