

1 **Title: mTORC1 deregulation and increased invasiveness cohere with**
2 **dispersed endolysosomes in high-grade bladder cancer.**

3 **Running title: Endolysosome dysfunctions in the bladder cancer model**

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20

21 **Abstract**

22 Late endosomes/lysosomes (endolysosomes) emerge as a potential regulatory hub
23 during cancer. Here, we investigate the intracellular landscape of this organelle in a
24 collection of bladder cancer cell lines and normal human urothelium cells under
25 standardized culture conditions. We find that high-grade bladder cancer cells are
26 characterized by scattered endolysosomes that are accompanied by an altered cellular
27 pH homeostasis and major changes of mTORC1 regulation. Mechanistically, we reveal
28 that mTORC1 substrate specificity is altered, and mTORC1 responsiveness to
29 endolysosome positioning is lost in high-grade cancer cells compared to low-grade
30 cells, highlighting unexpected mechanisms of mTORC1 deregulation in the bladder
31 cancer model. Because endolysosome positioning was critical for invasion from 3D
32 spheroids, our results indicate that changes in their cellular positioning and ability to
33 support signaling, strongly impact cancer cell behavior. Thus, monitoring detailed
34 changes of endolysosomes at different steps of cancer disease reveals intricate spatial
35 and temporal dimensions of tumorigenesis.

36

1 **Statement of significance**

2 Our study reveals significant changes of endolysosomes in bladder cancer
3 progression, highlighting endolysosome dysfunction as a fundamental driving
4 progress in malignancies. The identified alterations in endolysosome positioning and
5 associated mTORC1 signaling regulation could help to stratify emerging therapeutic
6 strategies targeting the endolysosomal compartment.

1 Introduction

2 Malignant transformation is characterized by major alterations in the
3 intracellular landscape. However, with the exception of the nucleus, it is generally not
4 well understood to which extend intracellular organelles are altered during
5 carcinogenesis. In recent years, late endosomes/lysosomes (endolysosomes) have
6 emerged as a potential regulatory hub during cancer development (Perera et al., 2019;
7 Thelen and Zoncu, 2017; Hämälistö and Jäättelä, 2016). Endolysosomes are
8 heterogeneous acidic organelles that are functionally similar to yeast and plant
9 vacuoles. They are specialized in the degradation of extracellular molecules or
10 pathogens internalized by endocytosis or phagocytosis, and the intracellular recycling
11 of macromolecules and organelles sequestered by autophagy (Ballabio and
12 Bonifacino, 2020; Lawrence and Zoncu, 2019; Perera and Zoncu, 2016; Thelen and
13 Zoncu, 2017). In addition to this classic role in cellular clearance, several core
14 functions of endolysosomes are often deregulated in cancer. Endolysosomes
15 attenuate growth factor signaling (Pu et al., 2016), whose increase is a common
16 feature of many cancers. They are also the storage compartments for secretory
17 proteases that degrade extracellular matrix during invasion and regulate the trafficking
18 of adhesion molecules for cell migration (Castro-Castro et al., 2016; Steffan et al.,
19 2014). Significantly, the surface of endolysosomes is the cellular platform where the
20 mammalian Target of Rapamycin Complex 1 (mTORC1), a conserved
21 serine/threonine kinase complex, integrates chemically diverse nutrients and growth
22 factor signaling to adjust cellular metabolisms through either promotion of biosynthesis
23 or catabolism (Ballabio and Bonifacino, 2020; Thelen and Zoncu, 2017). One important
24 target of mTORC1 is the MiT/TFE family of transcription factors, which promote the
25 development of several cancers, including renal cell carcinoma, melanoma, and
26 sarcoma (Perera et al., 2019). The MiT/TFE family of transcription factors, including
27 transcription factor EB (TFEB) and MITF, are master regulators of lysosome
28 biogenesis and autophagy. It has been shown that a positive feedback mechanism
29 between mTORC1 and TFEB was sufficient to promote cancer growth in mouse
30 models (Calcagni et al., 2016; Di Malta et al., 2017), and it is well established that
31 MITF is an oncogene in melanoma (Perera et al., 2019). Moreover, endolysosomes
32 are related to multivesicular bodies (MVB) that secrete one class of extracellular
33 vesicles called exosomes that impact on tumor progression through exosome-
34 dependent altering of stromal cell fate (Hyenne et al., 2017). Thus, endolysosomes
35 seem to support several cellular pathways that are characteristic of tumors. Yet, it is
36 not known whether and how endolysosomes change during cancer.

1 Here, we investigate the intracellular landscape of the endolysosomal compartment in
2 a collection of bladder cancer cell lines as compared to normal human urothelium
3 (NHU) cells. Bladder cancer represents the ninth most frequently-diagnosed cancer
4 worldwide and fourth most common cancer in men in North America and Europe, thus
5 is an important health burden (Antoni et al., 2017). Employing cell culture on adhesive
6 micropatterns of defined geometry that allow normalization of cell shape, we find that
7 high-grade bladder cancer cells are characterized by scattered endolysosomes that
8 are accompanied by an altered cellular pH homeostasis, major changes of mTORC1
9 signaling regulation and increased invasiveness.

10 **Results**

11 ***High-grade cancer cell lines are specifically characterized by scattered, 12 peripheral positioning of endolysosomes***

13 Because of the importance of endolysosomes in cellular homeostasis and its
14 possible role in promoting cancer progression, we aimed at a systematic analysis of
15 endolysosome morphology in a collection of bladder cancer cell lines and their
16 comparison to primary normal human urothelium (NHU) cells. Bladder carcinomas are
17 highly diverse, from low-grade luminal-like subtypes that are not very aggressive, to
18 muscle-invasive bladder subtypes that are characterized by an aggressive behavior
19 (Biton et al., 2014). We have analyzed seven broadly studied bladder cancer cell lines,
20 namely MGHU3, RT4, RT112, KU19-19, T24, TCCSup and JMSU1. These cell lines
21 show the diverse characteristics of bladder carcinomas. Therefore, to access the
22 relation between the different tumor cell lines and compare them with NHU cells, we
23 performed a principal component analysis employing transcriptome data of these cells
24 (Figure 1A). As expected, the replicates of the NHU transcriptomes clustered together
25 and separately from the tumor cells. The low-grade bladder cancer cell lines MGHU3
26 and RT4 clustered in the upper left corner, positioning them away from high-grade
27 bladder cancer cells JMSU1, TCCSup and T24 that were found in the lower right
28 corner. The cell lines RT112 and KU19-19 were found between these two groups.
29 Next, to compare these different cells at the morphological level, we cultured them on
30 identical crossbow-shaped patterns. All tested cells were fully spread after 3h
31 incubation, visualized by the average projection of the actin cytoskeleton
32 (Supplementary Figure 1A), indicating that all cells adapted well to the micropatterns.
33 We visualized the endolysosomal compartment by immunofluorescence staining of the
34 lysosomal-associated membrane protein 1 (LAMP-1, CD107a, Figure 1B), acquired
35 images in 3D and segmented them to obtain quantitative information. No clear trend
36 in the average volume or the number of endolysosomes per cell was found among the

1 tested cell lines (Supplementary Figure 1B,C). Interestingly, the endolysosomal
2 volume was negatively correlated with their total number (Supplementary Figure
3 1B,C,D), implying that the balance between few large endolysosomes and many small
4 ones is differently regulated in individual bladder cancer cells. Because the total cell
5 spreading area is controlled by the micropattern and is similar between all cells, we
6 additionally calculated the nearest neighbor distance (NND) of all endolysosomes in
7 each cell. Interestingly, the average NND of NHU cells and low-grade bladder cancer
8 cell lines was smaller than the average NND high-grade bladder cancer cell lines
9 (Figure 1C), indicating that endolysosomes are more scattered throughout the cell in
10 high-grade bladder cancer cell lines. To better characterize the observed changes in
11 endolysosomes, we chose four cell lines for detailed analysis, namely MGHU3 and
12 RT112, KU19-19, and JMSU1. Invasion assays from spheroids into collagen matrix
13 confirmed that MGHU3 was the less invasive cell line (invasion at 5d), followed by
14 RT112 (invasion at 3d), KU19-19 and JMSU1 that both invaded at 1d, however
15 whereas KU19-19 invaded via collective migration of few leader cells from few sites,
16 JMSU1 escaped spheroids via single cells throughout the entire spheroid surface
17 (Supplementary Figure 1E). Similarly, in agreement to the notion that nucleus size
18 increases with transformation, the nuclei in micropatterned cells showed an increase
19 in size from NHU cells to JMSU1 cells (Supplementary Figure 1F). To further analyze
20 the average positioning of compartments we employed our original method based on
21 probabilistic density maps to visualize the smallest cellular volume containing 50% of
22 endolysosomes (Schauer et al., 2010). The corresponding density maps revealed
23 striking differences between NHU and cancer cell lines (Figure 1D): while in NHU and
24 MGHU3 cells endolysosomes were positioned centrally, they were found to be spread
25 out to the periphery in RT112, KU19-19 and JMSU1 cells with strongest phenotype in
26 high-grade cancer cell lines. To verify that positioning changes were not induced by
27 micropatterning, we analyzed endolysosomes in classical cell culture conditions and
28 classified them as peripheral, intermediate and perinuclear depending on their relative
29 positioning between the nucleus and plasma membrane (Figure 1E and
30 Supplementary Figure 1G). In agreement with our density map analysis, we found that
31 the percentage of peripheral endolysosomes significantly increased from MGHU3 to
32 JMSU1, with JMSU1 showing as much as 30% of peripheral endolysosomes.
33 Endolysosomes are acidic compartments that acquire their characteristic pH through
34 the transport of protons from the cytosol through V-type ATPases (Lawrence and
35 Zoncu, 2019). Endolysosomes change their positioning as a response to changes in
36 the cytosolic pH and acidic conditions disperse the endolysosomes to cell periphery in
37 a rapid and reversible manner (Korolchuk et al., 2011), although the underlying

1 biological significance is not well understood. Thus, we asked whether peripheral
2 positioning in bladder cancer cell lines are accompanied by acidification of the
3 cytoplasm. To test this, we incubated cells with the commercially available dye pHrodo-
4 green, whose fluorescence intensity increases with decreasing pH. We found that
5 cytoplasmic pH was indeed decreased in high-grade cancer cells as compared to low-
6 grade cell line MGHU3, the JMSU1 cells showing the lowest pH (Figure 1F,G). Our
7 analyses collectively indicate that the endolysosomal compartment shows differences
8 between high-grade and low-grade bladder cancer cell lines as well as NHU. The most
9 prominent alteration was a scattered, peripheral positioning of the endolysosomal
10 compartment, accompanied by intracellular acidification, that we found as a specific
11 feature of high-grade bladder cancer cell lines.

12

13 ***Altered endolysosomes reveal changes in mTORC1 substrates in cancer*** 14 ***progression***

15 Endolysosomes are the cellular signaling platform of the mammalian target of
16 rapamycin (mTORC1), the main regulator of cell proliferation and survival which plays
17 a key role in carcinogenesis (Calcagni et al., 2016; Di Malta et al., 2017). Because
18 mTORC1 signaling has been shown to be regulated by endolysosomes positioning
19 (Korolchuk et al., 2011; Perera and Zoncu, 2016), we wondered whether altered
20 endolysosome positioning across different bladder cancer cell lines affected mTORC1
21 signaling. We tested mTORC1 activity in bladder cancer cell lines monitoring the
22 phosphorylation of several direct downstream substrates. First, we analyzed eIF4E
23 Binding Protein (4EBP1) and p70-S6 Kinase 1 (S6K1) that are phosphorylated during
24 activation of protein synthesis under control of mTORC1. Interestingly, whereas
25 phosphorylation of 4EBP1 was high in high-grade cells KU19-9 and JMSU1 as
26 compared to MGHU3 and RT112 cells (Figure 2A), phosphorylation of S6K1 was
27 opposite: we detected low phosphorylation of S6K1 in high-grade bladder cancer cell
28 lines compared to MGHU3 and RT112 cells (Figure 2B). Note that the total protein
29 level of 4EBP1 was also upregulated in high-grade cell lines (Figure 2A) whereas the
30 total protein level of S6K remained the same in all cell lines. Employing rapamycin and
31 torin, which directly inhibit mTORC1 (Dumont and Su, 1995; Liu et al., 2010),
32 wortmannin that inhibits upstream signaling as well as starvation that switches off
33 mTORC1, we verified that both 4EBP1 and S6K1 phosphorylation was dependent on
34 mTORC1 activity (Supplementary Figure 2A-D). Moreover, we monitored cellular
35 localization of the transcription factors TFEB employing transfection of plasmid EGFP-
36 N1-TFEB and monitoring cells 48h after transfection. We found that TFEB was
37 retained in the cytosol in the majority of MGHU3 and RT112 cells whereas the

1 majority of TFEB was found translocated into the nucleus in high-grade bladder cancer
2 cell lines, KU1919 and JMSU1 (Figure 2C). This indicated that TFEB was stronger
3 phosphorylated by mTORC1 in low-grade bladder cancer cell lines in which P-TFEB
4 was retained in the cytoplasm. Again, as expected, translocation into the nucleus was
5 induced upon rapamycin treatment in all cell lines indicating mTORC1 specificity
6 (Supplementary Figure 2E). Overall, our results convey that mTORC1 activity is
7 maintained across all grades in bladder cancer cell lines, but that substrate specificity
8 of mTORC1 changes in different bladder cancer cell lines: MGHU3 and RT112 show
9 a high mTORC1 phosphorylation of S6K1 and TFEB that is retained in the cytosol.
10 High-grade bladder cancer cell lines, KU19-19 and JMSU1, show a high mTORC1
11 phosphorylation of 4EBP1 that is upregulated in these cell lines. Consistent with these
12 results, we found that mTORC1 localizes on endolysosomes in all analyzed bladder
13 cancer cell lines, which supports an active state of mTORC1 (Figure 2D).

14

15 ***mTORC1 signaling does not respond to endolysosomes positioning changes in***
16 ***high-grade bladder cancer cell line***

17 Next, we investigated how mTORC1 signaling responds to changes in
18 endolysosome positioning within different cell lines. Nutrient status, pH and growth
19 factors affect endolysosome positioning and impact on mTORC1 signaling (Ballabio
20 and Bonifacino, 2020; Thelen and Zoncu, 2017), indicating that endolysosomes
21 integrate different upstream signals via positioning for mTORC1 signaling (Korolchuk
22 et al., 2011). Thus, in order to directly test mTORC1 activity due to endolysosome
23 positioning, independent of the complex regulation by nutrients, we performed all
24 assays in full media and specifically altered endolysosome positioning via recruitment
25 or targeting of motor proteins. Dynein is the retrograde motor required for
26 endolysosomal transport towards the cell center (Pu et al., 2016). To change
27 positioning of endolysosomes, we induced recruitment of dynein on them employing
28 FKBP/FRB heterodimerization by the A/C heterodimerizer, a strategy that has been
29 previously validated (van Bergeijk et al., 2015). We engineered RT112 and JMSU1
30 cells, representing non-aggressive and aggressive cell lines, respectively, to stably
31 express FKBP-fused to Lamp1-mCherry and FRB-fused to the dynein adaptor BicD2
32 (Supplementary Figure 3A). In these cell lines, addition of A/C heterodimerizer to the
33 culture medium redistribute endolysosomes toward the cell center where they strongly
34 cluster (Supplementary Figure 3B). We monitored mTORC1 activity by visualizing
35 phosphorylation of 4EBP1 and nuclear translocation of TFEB in RT112 and JMSU1
36 cells. We found that whereas A/C heterodimerizer-induced clustering of
37 endolysosomes in RT112 cells decreased 4EBP1 phosphorylation, clustering of

1 endolysosomes in JMSU1 cells did not change 4EBP1 phosphorylation levels (Figure
2 3A, B). Similarly, we found monitoring TFEB that its nuclear translocation was
3 increased in RT112 cells but not in JMSU1 cells after A/C heterodimerizer-induced
4 endolysosome clustering (Figure 3C, D). To further confirm these results, we altered
5 endolysosome positioning by targeting the small GTPases Arl8b or Rab7, which
6 regulate the recruitment of molecular motors on endolysosomes (Supplementary
7 Figure 3C). Arl8b recruits kinesins for anterograde transport (Pu et al., 2016), and thus
8 gene silencing of Arl8b leads to retrograde movement of endolysosomes to the cell
9 center (Supplementary Figure 3D). Similar to the A/C heterodimerizer-induced
10 clustering of endolysosomes, siArl8b decreased phosphorylation of 4EBP1 in RT112
11 but not in JMSU1 (Supplementary Figure 3E, F). However, preventing recruitment of
12 dynein and thus anterograde movement of endolysosomes to the cell periphery by
13 silencing Rab7 (Pu et al., 2016) did not change the levels of phosphorylated 4EBP1 in
14 either cell line (Figure 3D-F). Finally, we investigated mTORC1's localization on
15 endolysosomes after changing their positioning. Consistent with our previous results,
16 we found that upon A/C heterodimerizer-induced clustering mTORC1 was lost from
17 endolysosomes in RT112 cells (Figure 3E). Contrary, in JMSU1 cells, mTORC1
18 remained on clustered endolysosomes (Figure 3F). Altogether, our results show that
19 mTORC1 recruitment, and thus mTORC1 signaling respond to endolysosomes
20 positioning in non-aggressive RT112 cells but not in high-grade JMSU1 cells.

21

22 ***Endolysosome positioning regulates invasion of bladder cancer cell lines***

23 Finally, we investigated whether endolysosomes positioning impacts the
24 invasive capacity of cells. We employed our FKBP/FRB engineered cell lines RT112
25 and JMSU1 to directly control endolysosome positioning and performed 3D invasion
26 assay based on collagen I matrix invasion from cell spheroids. Invasion from RT112
27 spheroids occurred via collective migration of few leader cells that invaded the collagen
28 matrix on average on day 3 (Supplementary Figure 1E), thus spheroids from RT112
29 cells were observed for 5 consecutive days (Figure 4A). A/C heterodimerizer treatment
30 in control did not affect endolysosome positioning, and FKBP/FRB engineered cells
31 showed comparable invasion behavior as control cells treated or not with A/C
32 heterodimerizer (Supplementary Figure 4A, B). Strikingly, we found that A/C
33 heterodimerizer-induced endolysosome clustering significantly reduced invasion from
34 spheroids in RT112 cells, increasing the time of invasion to five days and the fraction
35 of non-invasive spheroids from 20% to almost 90% (Figure 4B, C). We did not observe
36 alteration in spheroid growth, indicating that under this condition cell proliferation was
37 not affected. To confirm that endolysosome positioning can impact on invasion we

1 additionally targeted several cellular regulators that regulate endolysosome
2 positioning. When targeting *Arl8b* to displace endolysosomes towards the cell center
3 in RT112 cells, we again observed a significant decrease in spheroid invasion (Figure
4 4D and Supplementary Figure 4C). Contrary, when we displaced endolysosomes
5 towards the cell periphery by silencing *Rab7*, invasion was significantly earlier than in
6 control cells (Figure 4E and Supplementary Figure 4C).

7 In striking difference, invasion from JMSU1 spheroids occurred via escape of single
8 cells that invaded the collagen matrix after 3h (Supplementary Figure 1E, 4D).
9 Contrary to RT112 cells, we did not observe any delay in invasion in JMSU1 cells upon
10 A/C heterodimerizer-induced clustering of endolysosomes (Supplementary Figure 1E),
11 indicating that invasion of JMSU1 was independent of endolysosome positioning.
12 Taken together these results indicate that endolysosomes are critical players in cell
13 invasion and that their changes during cancer progression, characterized by changes
14 in their cellular positioning and ability to support signaling, strongly impact cancer cell
15 behavior.

16

17 **Discussion**

18 Endolysosome dysfunction as a driving progress of cancer has been previously
19 proposed (Perera et al., 2019; Zoncu et al., 2011; Perera et al 2016) and our results
20 confirm that the endolysosomal compartment shows consistent alterations in a
21 collection of bladder cancer cell lines. Surprisingly, we did not find an increase in the
22 average number or volume of endolysosome in the bladder cancer model but rather
23 changes of endolysosomal positioning that were more scattered and peripheral than
24 in NHU cells. A general enlargement of the endolysosomal compartment could have
25 been expected, because excessive endolysosomal activity has been proposed as a
26 recurrent feature in cancer (Perera et al., 2019). Indeed, increased lysosome
27 biogenesis and abundant lysosomes were observed in several cancer models, alveolar
28 soft part sarcoma, pancreatic ductal adenocarcinoma and melanoma that all show
29 hyperactivation of *MiT/TFE* transcription factors and upregulation of the *mTORC1*
30 regulatory small GTPases *RagD* that expands the endolysosomal compartment (Di
31 Malta et al., 2017; Perera et al., 2019). In agreement with our results, we also did not
32 find up-regulation of *Rag* genes in the transcriptome data of the bladder cancer cell
33 lines (data not shown). However, changes in endolysosome positioning in bladder
34 cancer cell lines were associated with alterations of the fundamental *mTORC1*
35 signaling pathways, shown to be involved in cancer progression (Bar-Peled et al.,
36 2013; Di Malta et al., 2017). First, we found that *mTORC1* was active in all cell lines
37 despite of different endolysosome positioning. Yet, whereas low-grade cell lines

1 MGHU3 or non-aggressive RT112 show high mTORC1 phosphorylation activation
2 towards S6K1 and TFEB, high-grade bladder cancer cell lines, KU19-19 and JMSU1,
3 show high mTORC1 activity towards 4EBP1. It should be noted, that the total levels of
4 4EBP1 were additionally increased in high-grade cells, indicating dysregulation of
5 mTORC1 signaling by substrate competition. Although 4EBP1 and S6K1 both
6 contribute to the regulation of translation, 4EBP1 has the higher affinity towards
7 mTORC1 (Choo and Blenis, 2009). Thus, the preferential phosphorylation of 4EBP1
8 in high-grade cancer cell lines could allow to simultaneously keep maintenance of the
9 cellular translation machinery and the nuclear translocation of TFEB that both seem to
10 be regulated mutually exclusive by mTORC1 in non-transformed cells (Perera et al
11 2016). Given the fact that 4EBP1 has been suggested to be a tumor suppressor and
12 its overexpression was shown to be associated with an unfavorable prognosis in a
13 recent meta-analysis (Zhang et al., 2017), including bladder cancer, our results
14 suggest that shifting substrates could be a strategy to fuel the overgrowth of bladder
15 cancers. In the future, it will be important to further investigate whether this mechanism
16 could be a driving progress in bladder cancer and potentially other cancer models.

17 Second, we found that mTORC1 signaling is specifically decoupled from
18 endolysosome positioning in high-grade bladder cancer cell lines. Through a
19 sophisticated machinery that dynamically assembles mTORC1 on the surface of
20 endolysosomes, signals from nutrients in the cytoplasm, inside endolysosomes as well
21 as outputs downstream of growth factor signaling are integrated (Thelen and Zoncu,
22 2017). Notably, this machinery is coordinated with endolysosome positioning, as
23 nutrient status or growth factors simultaneously change endolysosome positioning
24 (Ballabio and Bonifacino, 2020; Thelen and Zoncu, 2017). An emerging picture is that
25 dynamic endolysosome positioning helps to integrate the many upstream signals for
26 mTORC1 via spatial compartmentalization, although the exact relation between
27 endolysosome positioning and mTORC1 signaling remains controversial (Walton et
28 al., 2019): whereas mTORC1 was shown to signal from peripheral endolysosomes in
29 some studies (Korolchuk et al., 2011), others suggest the presence of active mTORC1
30 on central endolysosomes (Walton et al., 2019). Although mTORC1 signaling was
31 present in low-grade cell lines with moderate central endolysosome positioning, we
32 found that central clustering of endolysosomes via enforced recruitment of minus-end
33 motor proteins leads to loss of mTORC1 and attenuation of downstream signaling in
34 non-aggressive bladder cancer cell lines. Indeed, mTORC1 dissociation from
35 endolysosomes in nutrient deficient or starvation conditions often correlates with
36 displacement of endolysosomes to the cell center (Korolchuk et al., 2011; Perera and

1 Zoncu, 2016). Interestingly, we did not observe a loss of mTORC1 from clustered
2 endolysosomes in high-grade cancer cell lines, indicating that mTORC1 responses are
3 uncoupled from endolysosome positioning. As nutrient status and endolysosome
4 positioning are tightly linked, the loss of the spatial compartmentalization of mTORC1
5 signaling may help cancer cells to evade metabolic checks on anabolism and
6 proliferation. Future studies will address by which mechanisms mTORC1 is retained
7 on central endolysosomes in high-grade bladder cancer cells.

8 Finally, we found that endolysosomes are important for invasion of bladder cancer cells
9 in the 3D spheroid model. Interestingly, invasion of non-aggressive cell line RT112,
10 which shows intermediate upregulation of 4EBP1 (compared to low-grade MGHU3
11 cells) and responsiveness of mTORC1 signaling to endolysosome positioning, was
12 controlled by placement of endolysosomes. Whereas central positioning led to
13 decreased invasiveness, peripheral positioning had the opposite effect. Invasion was
14 observed from isolated loci after several days and showed phenotypes typical for
15 collective cell migration. Contrary, high-grade cancer cell line JMSU1, which shows a
16 high expression of 4EBP1 and no responsiveness of mTORC1 signaling to
17 endolysosome positioning, invaded rapidly (after 3h) by single cell escape and
18 invasiveness was not regulated by endolysosome positioning. Our study proposes that
19 monitoring detailed changes of the endolysosome compartment could reveal intricate
20 spatial and temporal dimensions of tumorigenesis (Figure 4F). Besides the alterations
21 in mTORC1 signaling that we observed in bladder cancer cells, important for cellular
22 homeostasis and nutrient balance, endolysosome positioning regulates protease
23 secretion/proteolysis (Castro-Castro et al., 2016; Steffan et al., 2014), migration (Pu et
24 al., 2016) and remodeling of tumor environment through the release of exosomes
25 (Hyenne et al., 2017). Indeed, proteins and mechanisms implicated in endolysosome
26 positioning are often found to be deregulated in the progression of different kind of
27 cancers (Dykes et al., 2016; Steffan et al., 2014). Thus, it is tempting to speculate that
28 altered endolysosomes could link dysregulation of metabolism, signaling or/and
29 trafficking to invasiveness and migration that characterize cancer cell behavior.

30 One important question to address in the future is why endolysosomes change
31 positioning in high-grade bladder cancer cell lines. It was previously reported that
32 endolysosomes are more peripheral due to acidification of the extracellular tumor
33 microenvironment (Steffan et al., 2014; Walton et al., 2019) that is a common feature
34 of cancer. Interestingly, we found that endolysosome positioning changes are
35 accompanied by a decrease in intracellular pH. As both extracellular and intracellular
36 pH regulate endolysosome displacement (Walton et al., 2019) and are closely coupled

1 to cellular metabolism and its regulation via mTORC1, it will be critical to investigate in
2 detail how metabolic rewiring, common to all cancer cells, is interconnected with pH
3 homeostasis and endolysosome positioning.

4 In conclusion our study revealed characteristic changes in endolysosome
5 positioning that were associated with unexpected outcomes of mTORC1 signaling in
6 a collection of bladder cancer cell lines. Particularly, we revealed that mTORC1
7 substrate specificity is altered, and responsiveness to endolysosome positioning is lost
8 in high-grade bladder cancer cell lines as compared to low-grade cancerous cells.
9 Importantly, our results suggest that endolysosome positioning is critical for cell
10 invasion from 3D spheroids. Our study proposes that monitoring detailed changes of
11 the endolysosome compartment at different steps of cancer disease could reveal
12 intricate spatial and temporal dimensions of tumorigenesis. Targeting lysosomal
13 function is emerging as a promising avenue in several malignancies (Hämälistö and
14 Jäätelä, 2016) and a full understanding of the basic biological processes underlying
15 transitions during cancer progression will be critical to improve cancer detection,
16 prevention, and the rational design of more effective and less toxic therapeutic
17 strategies.

18

19 **Methods**

20 *Cell culture*

21 Bladder cancer cells lines MGHU3, RT112, KU19-19, JMSU1, T24 and TCCSup were
22 grown in RPMI medium (Life Technologies, Carlsbad, CA, USA), supplemented with
23 10% Fetal Bovine Serum (FBS; Eurobio, Courtaboeuf, France). RT112 and JMSU1
24 cells stably expressing Lamp1-mCherry-FKBP and BicD2-HA-FRB were obtained via
25 viral transduction. For A/C heterodimerizer induced endolysosome clustering in cells
26 stably expressing the FKBP-FRB system, 0.5 μ M of A/C heterodimerizer (635056;
27 Takara) was added in complete media for 1 hour at 37 °C. Normal human urothelium
28 (NHU) cells were from Jennifer Southgate (University of York, UK). NHU were grown
29 in KSFMC medium according to (Southgate et al., 1994, 2002). For experiments with
30 inhibitors, the day after cell seeding, respective drugs were added for 2 hours at 37°C.
31 The concentration of inhibitors used were as follows: Rapamycin (20 μ M), Wortmannin
32 (1 μ M), Torin (1 μ M). For starvation experiment, the day after cell seeding, the media
33 was removed and cells were washed once with EBSS (Earle's Balanced Salt Solution)
34 and incubated in EBSS for 4hours before lysate preparation.

35

36 *Cell transfection*

37 For gene depletion studies, 200 000 cells were transfected in 6 well plate with 25

1 pmol.mL⁻¹ siRNA (siLuc: 5'-CGTACGCGGAATACTTCGA-3'; siRab7: 5'-
2 CACGTAGGCCTTCAACACAAT-3' and 5'-CTGCTGCGTTCTGGTATTTGA-3';
3 siArl8b: 5'- GAUAGAAGCUUCCCGAAAU-3'; Sigma-Aldrich) using Lipofectamine
4 RNAiMAX Transfection Reagent (5 µL.mL⁻¹; Life Technologies). Cells were incubated
5 72 h prior to further manipulations. Efficiency of siRNA gene silencing was verified by
6 Western Blot on cell lysate after three days of transfection. In invasion assays, the
7 siRNA was added into the collagen mix. For plasmid transfection, 100 000 cells were
8 transfected in a 12 well plate on sterilized coverslips (12mm) using Lipofectamine LTX
9 with Plus reagent (Invitrogen). pEGFP-N1-TFEB plasmid was a gift from Shawn
10 Ferguson (Addgene plasmid # 38119; <http://n2t.net/addgene:38119>;
11 RRID:Addgene_38119n (Roczniak-Ferguson et al., 2012)). Cells were transfected
12 using 1 µg of plasmid for 48 hours before PFA fixation and imaging.

13

14 *PCA analysis*

15 Micro array data were analyzed with R (3.5.2). The annotation was performed using
16 affy package (1.58.0) with a custom CDF from brain array (huex10st, genome version
17 23). Normalization was done with RMA algorithm and batch effect corrected with
18 ComBat. The PCA was computed from these normalized and corrected data.

19

20 *Micropatterned coverslips preparation and cell seeding*

21 Micropattern production was as previously described (Schauer et al., 2010) using
22 photo-lithography methods. Briefly, coverslips were coated with Poly-L-Lysine(20)-
23 grafted[3.5]-Polyethyleneglycol(2) (PLL-g-PEG) from SuSoS (Dübendorf, Switzerland)
24 at a final concentration of 0.1 mg.mL⁻¹ in 10 mM HEPES (pH 7,3) solution. Coverslips
25 were exposed to deep UV during 5 min using a photomask containing arrays of
26 crossbows (37 µm diameter, 7 µm thick). Prior to cell seeding, the patterned surface
27 was incubated for 1h with a mixture of 50 µg/ml fibronectin (Sigma-Aldrich, St. Louis,
28 MO, USA), 5 µg/ml concanavalin A (Sigma-Aldrich, St. Louis, MO, USA) and 1 µg/ml
29 fibrinogen–Cy5 (Invitrogen). Cells were seeded on micropatterns in RPMI medium
30 supplemented with 20 mM HEPES (Life Technologies) for 4 h prior the experiment.

31

32 *Immunofluorescence, image acquisition and processing*

33 Cells were fixed with 4 % formaldehyde for 15 min at room temperature, washed three
34 times with PBS and permeabilized in PBS/0.2% BSA/0.05% saponin. Cells were then
35 incubated with the primary antibodies (mouse monoclonal antibody against
36 Lamp1/CD107a (555798, BP Pharmingen™), rabbit mAb against mTOR (7C10,
37 #2983, Cell Signaling Technology)) and Alexa Fluor 488- or Cy3- coupled secondary

1 antibodies (Jackson ImmunoResearch) for 1 h. Actin was visualized by FluoProbes
2 547H (557/572nm) coupled Phalloïdin (Interchim) and nuclei with 0.2 $\mu\text{g}.\text{ml}^{-1}$ 4',6-
3 diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Slices were mounted in Mowiol
4 (Sigma-Aldrich). Images from immunolabelled cells were acquired with an inverted
5 wide field Deltavision Core Microscope (Applied Precision) equipped with highly
6 sensitive cooled interlined charge-coupled device (CCD) camera (CoolSnap Hq2,
7 Photometrics). Z-dimension series were acquired every 0.5 μm . For each experiment,
8 several tens of cells were imaged and aligned using the coordinates of the center and
9 the angle of rotation of the micropattern (determined on ImageJ (Bethesda, MD, USA)
10 as previously described (Schauer et al., 2010). To extract the 3D spatial coordinates
11 of intracellular structures, images were segmented with the multidimensional image
12 analysis (MIA) interface on MetaMorph (Molecular Devices, Sunnyvale, CA, USA)
13 based on wavelet decomposition.

14

15 *Kernel density estimation*

16 The coordinates of the segmented structures were processed for density estimation
17 programmed in the ks library in R according to (Schauer et al., 2010). For visualizing
18 kernel density estimates, we used probability contours and the extension libraries
19 mvtnorm, rgl, and miscd.

20

21 *Invasion assay*

22 Cells were trypsinized and 10^4 cells/ml were re-suspended in RPMI medium containing
23 10% FBS and 1% Penicillin-Streptomycin (Life Technologies). Then 100 μl of cell
24 suspension was plated in 48-well plates coated with 1% agarose (Life Technologies)
25 and incubated for 3 days. In each well, a spheroid was formed from 10^3 cells. Next,
26 the spheroids were plated on Lab-Tek chambers (Sigma), in a mixture of collagen I
27 from rat tail (Corning) at a final concentration of 2 $\text{mg}.\text{ml}^{-1}$, PBS, sodium hydroxide
28 (NaOH) and serum-free medium. For siRNA experiments, the medium was replaced
29 with the transfection mixture (Opti-MEM medium from Life Technologies, Lipofectamin
30 RNAiMAX and siRNA). For inducible cargo trafficking assays, 0.5 μM of A/C
31 heterodimerizer (635056, Takara) was added to the collagen mixture and the culture
32 medium. The spheroids were monitored for 5 consecutive days by using an inverted
33 Leica microscope (Wetzlar, Alemanha) equipped with camera device using 4x
34 objective.

35

36 *Cytoplasmic pH testing:*

37 Cells (200,000) were seeded in 35mm Fluorodishes and stained with pHRodo Green

1 (Invitrogen) according to the manufacturer's protocol along with 50nM LysoTracker
2 Deep Red (Invitrogen) and incubated at 37°C for 1 hour. Cells were washed with
3 complete RPMI media and imaged live using an inverted wide field Deltavision Core
4 Microscope. For calculating the RFU (Relative Fluorescence Unit) of cytoplasmic pH,
5 25,000 cells were seeded in black bottom 96 well cell culture plates and stained with
6 pHRodo Green as before for 1 hour at 37°C. Cells were then washed with complete
7 RPMI 1640 media and readings were taken using CLARIOstar plate reader (BMG
8 LABTECH) using excitation/emission of 509/533.

9

10 *Western Blot*

11 Cells were seeded in a 12 well plate (250,000) one day prior to the experiment. To
12 prepare lysates, cells were lysed in loading buffer (β -Mercaptoethanol (6%),
13 Bromophenol blue (0.02%) Glycerol (30%), SDS (Sodium dodecyl sulfate) (10%), Tris-
14 Cl (250 mM, pH 6.8), and 1X Protease Inhibitor Cocktail) on ice, boiled at 95°C for 5
15 min and stored at -20 °C before further use. On the day of western blot, lysates were
16 thawed and passed through a syringe to shred genomic DNA. Equal volume of lysates
17 from each cell line was loaded on a 10% or 12% polyacrylamide gel. Proteins were
18 then resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes
19 which was then blocked with 5% BSA in TBST (for phospho-antibodies) or 5% milk in
20 TBST (for all other antibodies). Membranes were incubated with respective primary
21 antibodies at 4°C overnight with constant shaking. Concentration of the primary
22 antibody used were as follows: Phospho P-70 (Thr389)-S6K (CST: 9205S, 1:1000 in
23 5% BSA in TBST), P-70 S6K (CST: 9202S, 1:1000 in 5% milk in TBST),
24 Phospho(Ser65)-4EBP1 (CST: 9451, 1:1000 in 5% BSA in TBST), 4EBP1(CST: 9452,
25 1:1000 in 5% milk in TBST), GAPDH (Sigma: G9545, 1:10,000 in 5% milk in TBST).
26 The next day, blots were washed with TBST (3X5 min) and incubated with respective
27 species specific HRP secondary antibodies (concentration of 1:10,000 was used for
28 all secondary antibodies) for 1hour at room temperature. Blots were washed again as
29 before with TBST and developed using ECL western blotting substrate.

30

31 *Statistical analysis*

32 The statistical analysis of endolysosome volume, number and normalized NND was
33 performed with R (3.6.0). For NND analysis, the centroids distance between structures
34 was calculated from a constant number of endolysosomes that was randomly sampled
35 from each cell. The statistical analysis was based on the Kruskal-Wallis test with Dunn
36 post-hoc test with Sidak correction for multiple comparisons correction.

37 For all experiment, a large number of cells were monitored from 3 to 6 independent

1 experiments. Two-sided Student t-tests were performed on averages to assess the
2 significance of difference. To compare the fraction of non-invasive spheroids a logrank
3 (Matel-Cox) test was performed in Prism software. Additionally, to compare the global
4 distribution of cell population, χ^2 tests were performed (R function “chi-square()²”). In
5 this case, results from independent experiments were individually compared and
6 combined for representation and statistical analysis.

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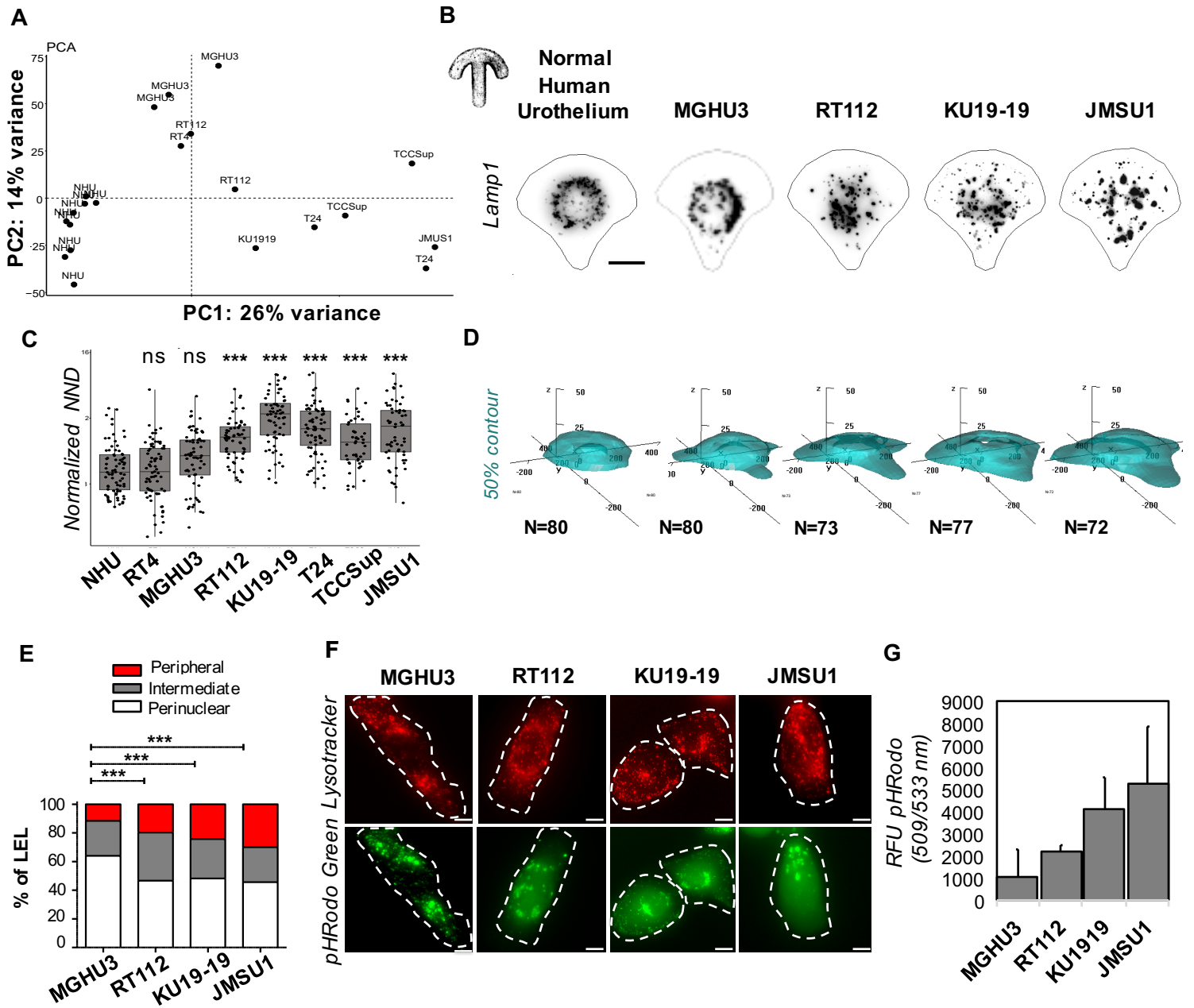


Figure 1

1 **Figure 1. High-grade cancer cell lines are specifically characterized by scattered,**
2 **peripheral positioning of endolysosomes**

3 A. Principal component analysis of transcriptome data of normal human urothelium
4 (NHU) cells and the bladder cancer cell lines RT4 (ATCC® HTB-2™), MGHU3 (Lin et
5 al., 1985), RT112 (Marshall et al., 1977), KU19-19 (Tachibana et al., 1997), T24
6 (n=72), TCCSup (Nayak et al., 1977), JMSU1 (Morita et al., 1995). B. Representative
7 images of endolysosomes visualized by immunofluorescence staining against the
8 lysosomal-associated membrane protein 1 (LAMP-1, CD107a) in NHU, MGHU3,
9 RT112, KU19-19 and JMSU1 single cells cultured on crossbow-shaped adhesive
10 micropatterns for better comparison. Scale bar is 10 μ m. C. Distribution of nearest
11 neighbor distance (NND) between endolysosomes in NHU (n=80), RT4 (n=73),
12 MGHU3 (n=80), RT112 (n=64), KU19-19 (n=77), T24 (n=72), TCCSup (n=48) and
13 JMSU1 (n=72). Adjusted p-values of testing against NHU condition are RT4: 0.9999,
14 MGHU3: 0.1501; RT112: <0.0001; KU19-19: <0.0001; T24: <0.0001; TCCsup:
15 <0.0001; JMSU1 : <0.0001 in a Kruskal-Wallis test with Dunn post-hoc test with Sidak
16 correction for multiple comparisons. ns p >0.01 and *** p < 0.0001. D. 3D probabilistic
17 density maps of endolysosomes in NHU (n=80), MGHU3 (n=80), RT112 (n=64), KU19-
18 19 (n=77) and JMSU1 (n=72). The 50% contour visualizes the smallest cellular volume
19 containing 50% of endolysosomes. E. Endolysosome distribution in classical cell
20 culture conditions, classified into peripheral (red), intermediate (grey) and perinuclear
21 (white) positioning based on their relative positioning between the nucleus and plasma
22 membrane for n>60 cells per cell line analyzed (see also Supplementary Figure 1G),
23 *** p < 0.001 in a χ^2 test. F. Representative images of endolysosomes visualized by
24 lysoTracker (red) and intracellular pH sensor pHrodo-green (green) in MGHU3,
25 RT112, KU19-19 and JMSU1. Scale bar is 5 μ m. G. Quantification of pH sensor
26 pHrodo-green (in relative fluorescence units emission at 509/533 nm) in MGHU3,
27 RT112, KU19-19 and JMSU1, error bars represent s.d. of three independent
28 experiments.

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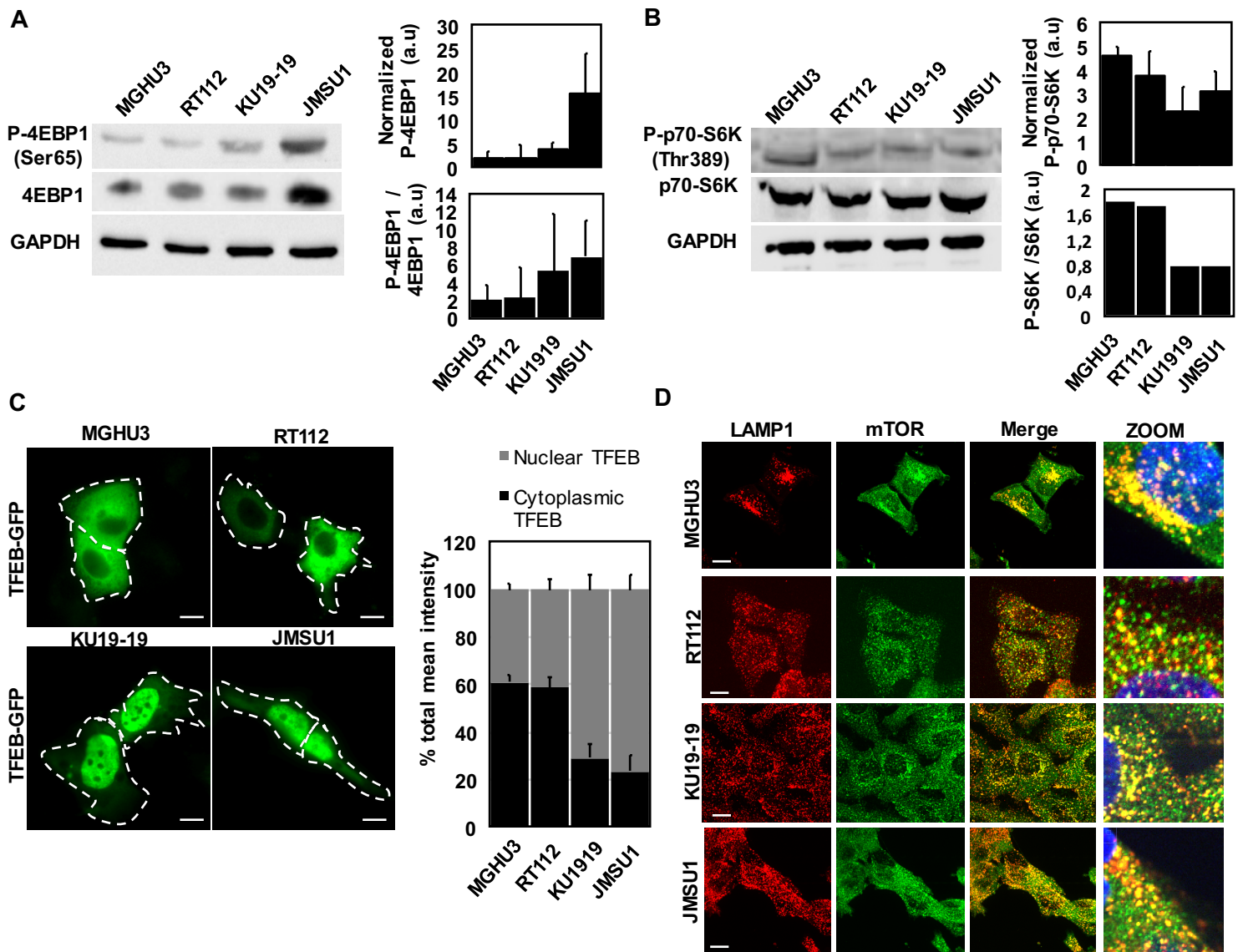


Figure 2

1 **Figure 2. Altered endolysosomes reveal changes in mTORC1 substrates in**
2 **cancer progression**

3 A. Western Blot analysis of eIF4E Binding Protein (4EBP1) phosphorylation (P-4EBP1
4 Ser65) in MGHU3, RT112, KU19-19 and JMSU1 and quantification of relative
5 phosphorylated P-4EBP1 to GAPDH and total 4EBP1 protein levels. Error bars show
6 s.d. of three independent experiments. B. Western Blot analysis of p70-S6 Kinase 1
7 (S6K1) phosphorylation (P-p70-S6K Thr389) in MGHU3, RT112, KU19-19 and JMSU1
8 and quantification of relative phosphorylated P-4EBP1 to GAPDH and total S6K1
9 protein levels. Error bars show s.d. of three independent experiments. C.
10 Representative images of MGHU3, RT112, KU19-19 and JMSU1 cells transfected with
11 TFEB-GFP (green) for 48h and quantification of relative fluorescent intensity between
12 nucleus (grey) and cytoplasm (black). Scale bars are 10 μ m. Error bars show s.d. of
13 three independent experiments. D. Representative images of endolysosomes
14 visualized by immunofluorescence staining against the lysosomal-associated
15 membrane protein 1 (LAMP-1, CD107a) and mTORC1 visualized by
16 immunofluorescence staining against mTOR in MGHU3, RT112, KU19-19 and
17 JMSU1. The zoom shows the merged image of both proteins. Scale bars are 10 μ m.
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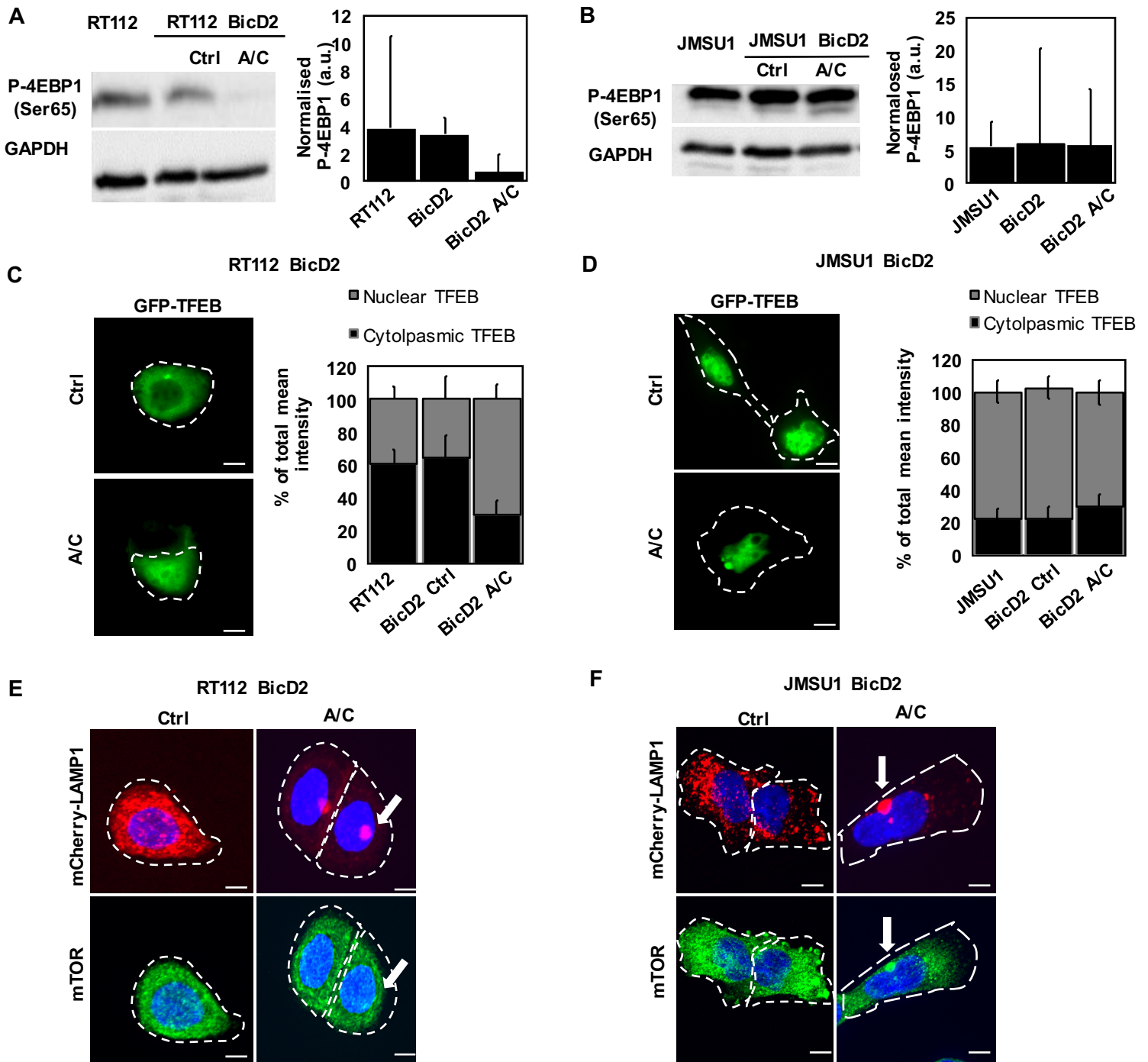


Figure 3

1 **Figure 3. mTORC1 signaling does not respond to endolysosomes positioning**
2 **changes in high-grade bladder cancer cell line**

3 A. Western Blot analysis of eIF4E Binding Protein (4EBP1) phosphorylation (P-4EBP1
4 Ser65) in RT112 cells stably expressing FKBP-fused to Lamp1-mCherry and FRB-
5 fused to the dynein adopter BicD2 (RT112 BicD2, see also Supplementary Figure 3A)
6 in control condition (DMSO, Ctrl) and after addition of A/C heterodimerizer (A/C) and
7 quantification of relative phosphorylated P-4EBP1 to GAPDH levels. Error bars show
8 s.d. of three independent experiments. B. Western Blot analysis of eIF4E Binding
9 Protein (4EBP1) phosphorylation (P-4EBP1 Ser65) in JMSU1 cells stably expressing
10 FKBP-fused to Lamp1-mCherry and FRB-fused to the dynein adopter BicD2 (JMSU1
11 BicD2) in control condition (DMSO, Ctrl) and after addition of A/C heterodimerizer
12 (A/C) and quantification of relative phosphorylated P-4EBP1 to GAPDH levels. Error
13 bars show s.d. of three independent experiments. C. Representative images of RT112
14 BicD2 cells transfected with TFEB-GFP (green) for 48h in control condition (DMSO,
15 Ctrl) and after addition of A/C heterodimerizer (A/C) and quantification of relative
16 fluorescent intensity between nucleus (grey) and cytoplasm (black). Scale bars are 10
17 μm . Error bars show s.d. of three independent experiments. D. Representative images
18 of JMSU1 BicD2 cells transfected with TFEB-GFP (green) for 48h in control condition
19 (DMSO, Ctrl) and after addition of A/C heterodimerizer (A/C) and quantification of
20 relative fluorescent intensity between nucleus (grey) and cytoplasm (black). Scale bars
21 are 10 μm . Error bars show s.d. of three independent experiments. E. Representative
22 images of mCherry-LAMP1 endolysosomes and mTORC1 visualized by
23 immunofluorescence staining in RT112 BicD2 cells in control condition (DMSO, Ctrl)
24 and after addition of A/C heterodimerizer (A/C). Scale bars are 5 μm . F. Representative
25 images of mCherry-LAMP1 endolysosomes and mTORC1 visualized by
26 immunofluorescence staining in JMSU1 BicD2 cells in control condition (DMSO, Ctrl)
27 and after addition of A/C heterodimerizer (A/C). Scale bars are 5 μm .

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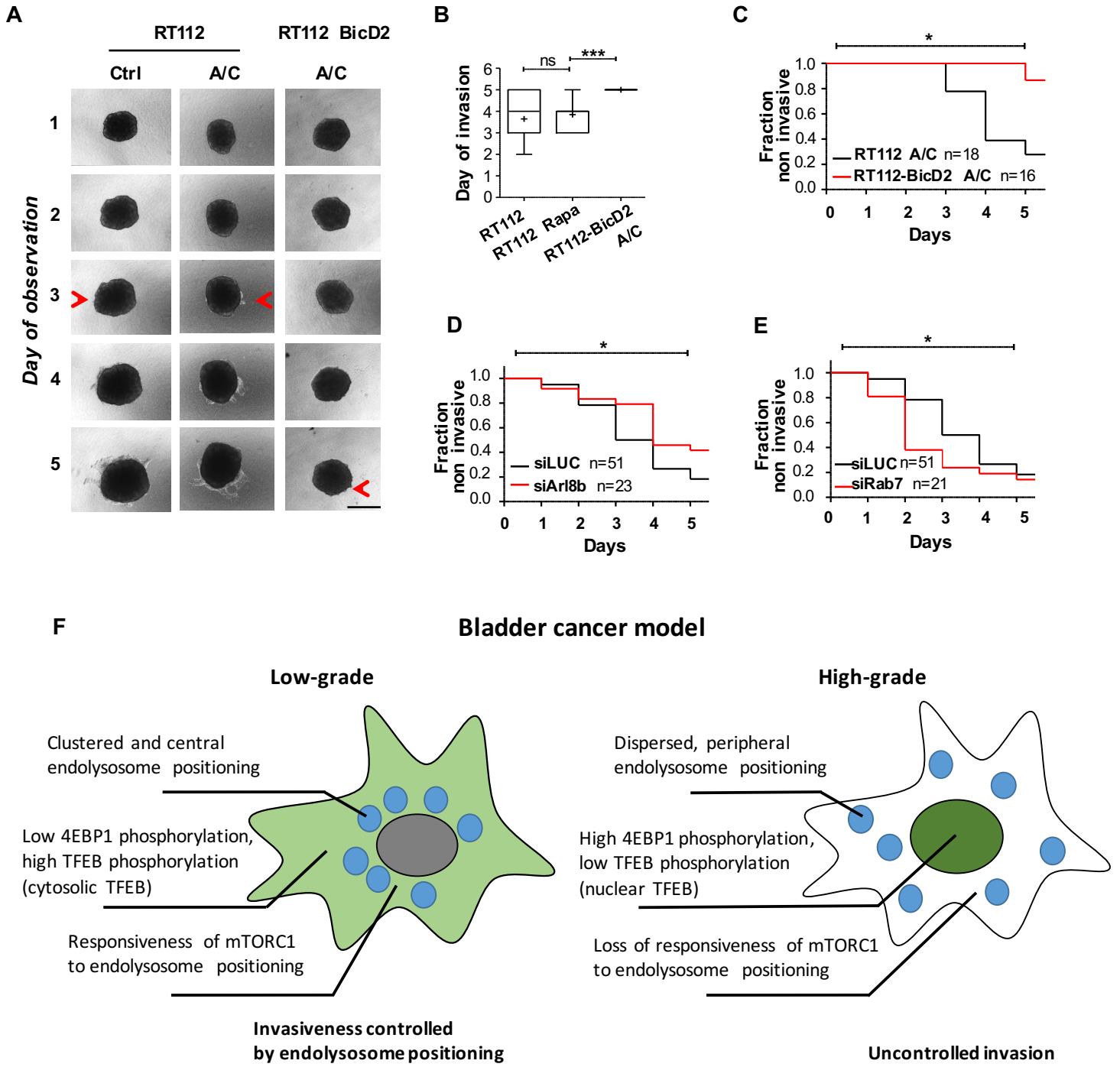


Figure 4

1 **Figure 4. Endolysosome positioning regulates invasion of bladder cancer cell**
2 **lines**

3 A. 3D invasion of RT112 and RT112 BicD2 spheroids into collagen I matrix in the
4 presence or absence of A/C heterodimerizer (A/C). Red arrows represent invading
5 cells. Scale bar is 0.5 mm. B. Average day of invasion of RT112 and RT112 BicD2
6 spheroids in the presence or absence of A/C heterodimerizer (A/C). *** $p < 0.001$ in a
7 student t-test. C. Invasion rate of RT112 (black, n=18) and RT112 BicD2 (red, n=16)
8 spheroids in the presence of A/C heterodimerizer as a function of the time (observed
9 at the interval of 1d). * $p < 0.05$ in a logrank test. D. Invasion rate of RT112 siLUC
10 (black, n=51) and RT112 siArf8b (red, n=23) spheroids as a function of the time
11 (observed at the interval of 1d). * $p < 0.05$ in a logrank test. E. Invasion rate of RT112
12 siLUC (black, n=51) and RT112 siRab7 (red, n=21) spheroids as a function of the time
13 (observed at the interval of 1d). * $p < 0.05$ in a logrank test. F. Schematic representation
14 of endolysosome dysfunction in the bladder cancer model: changes of the
15 endolysosome compartment at different steps of cancer disease reveal intricate spatial
16 and temporal dimensions of tumorigenesis. Endolysosomes are represented as blue
17 circles, TFEB localization is represented by green color either in the cytosol or nucleus.

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1 **Supplementary Figure 1:**

2 A. Average intensity projections of the actin cytoskeleton visualized by phalloidin of n
3 cells of normal human urothelium (NHU) and bladder cancer cell lines RT4 (ATCC®
4 HTB-2™), MGHU3 (Lin et al., 1985), RT112 (Marshall et al., 1977), KU19-19
5 (Tachibana et al., 1997), T24 (n=72), TCCSup (Nayak et al., 1977), JMSU1 (Morita et
6 al., 1995). B. Distribution of the average volume of endolysosomes in NHU (n=80),
7 RT4 (n=73), MGHU3 (n=80), RT112 (n=64), KU19-19 (n=77), T24 (n=72), TCCSup
8 (n=48) and JMSU1 (n=72). Adjusted p-values of testing against NHU condition are
9 RT4: 0.2475; MGHU3: <0.0001; RT112: 0.0095; KU19-19: 0.0220; T24: 0.9957;
10 TCCsup: 0.0006; JMSU1: <0.0001 in a Kruskal-Wallis test with Dunn post-hoc test
11 with Sidak correction for multiple comparisons. ns p > 0.01, * p < 0.01, ** p < 0.001
12 and *** p < 0.0001. C. Distribution of the average numbers of endolysosomes per cell
13 in NHU (n=80), RT4 (n=73), MGHU3 (n=80), RT112 (n=64), KU19-19 (n=77), T24
14 (n=72), TCCSup (n=48) and JMSU1 (n=72). Adjusted p-values of testing against NHU
15 condition are RT4: <0.0001; MGHU3: <0.0001; RT112: 0.9997; KU19-19: 0.8379; T24:
16 0.8755; TCCsup: 0.3407; JMSU1: <0.0001 in a Kruskal-Wallis test with Dunn post-hoc
17 test with Sidak correction for multiple comparisons correction. ns p > 0.01 and *** p <
18 0.0001. D. Correlation analysis between average endolysosomal volume and average
19 numbers per cell shows a weak ($R^2=0.19$) but significant association (p-value < 0.001
20 in a t-test for correlation). E. Average day of invasion of MGHU3, RT112, KU19-19,
21 and JMSU1 in invasion assays from 3D spheroids into collagen matrix, and
22 representative images of 3D spheroids from KU19-19 (upper panel) and JMSU1 (lower
23 panel) at 1 day after matrix embedding. White arrow indicates invasion of collagen
24 matrix by escaping cells. F. Average nucleus size of NHU (n=80), MGHU3 (n=80),
25 RT112 (n=64), KU19-19 (n=77) and JMSU1 (n=72) cells; *** p < 0.0001 in a χ^2 test. G.
26 Schematic representation of the classification analysis of endolysosome distribution in
27 classical cell culture conditions, based on their relative positioning between the
28 nucleus and plasma membrane as quantified in Figure 1E.

29

30 **Supplementary Figure 2:**

31 A. Western Blot analysis of eIF4E Binding Protein (4EBP1) phosphorylation (P-4EBP1
32 Ser65) and p70-S6 Kinase 1 (S6K1) phosphorylation (P-p70-S6K Thr389) in MGHU3,
33 RT112, KU19-19 and JMSU1 in control conditions (full media) and after treatment with
34 Wortmannin at 1 μ M for 2 h. B. Western Blot analysis of 4EBP1 and S6K
35 phosphorylation in MGHU3, RT112, KU19-19 and JMSU1 in control conditions (full
36 media) and after treatment with Rapamycin at 20 μ M for 2 h. C. Western Blot analysis
37 of 4EBP1 and S6K phosphorylation in MGHU3, RT112, KU19-19 and JMSU1 in

1 control conditions (full media) and after treatment with Torin at 1 μ M for 2 h. D. Western
2 Blot analysis of 4EBP1 and S6K phosphorylation in MGHU3, RT112, KU19-19 and
3 JMSU1 in control conditions (full media) and after starvation in EBSS for 4h. E.
4 Representative images of MGHU3, RT112, KU19-19 and JMSU1 cells transfected with
5 TFEB-GFP (green) for 48h and treated with Rapamycin at 20 μ M for 2 h. Scale bars
6 are 5 μ m.

7

8 **Supplementary Figure 3:**

9 A. Schematic representation of the FKBP/FRB heterodimerization system that allows
10 dynein recruitment on endolysosomes induced by A/C heterodimerizer addition
11 according to (van Bergeijk et al., 2015). RT112 and JMSU1 cells were engineered to
12 stably express FKBP-fused to Lamp1-mCherry and FRB-fused to the dynein adopter
13 BicD2 to move endolysosomes to the cell center upon A/C heterodimerizer addition.
14 B. Representative images of endolysosomes visualized by mCherry-LAMP1 in RT112
15 BicD2 single cells cultured on crossbow-shaped adhesive micropatterns in control
16 condition (DMSO, Ctrl) and after addition of A/C heterodimerizer (A/C). Scale bar is 10
17 μ m. Right panel shows corresponding 3D probabilistic density maps of endolysosomes
18 in control condition (grey) and after addition of A/C heterodimerizer (cyan) for N>60 for
19 each condition. The 50% contour visualizes the smallest cellular volume containing
20 50% of endolysosomes. C Western Blot analysis of Rab7 and Arl8b in RT112 and
21 JMSU1 cells in control condition (siLUC) and upon targeting Rab7 (siRab7) or Arl8b
22 (siArl8b) D. Representative images of endolysosomes visualized by
23 immunofluorescence staining against the lysosomal-associated membrane protein 1
24 (LAMP-1, CD107a) in RT112 single cells cultured on crossbow-shaped adhesive
25 micropatterns in control condition (siLUC) and upon targeting Rab7 (siRab7) or Arl8b
26 (siArl8b). Scale bar is 10 μ m E. Western Blot analysis of eIF4E Binding Protein
27 (4EBP1) phosphorylation (P-4EBP1 Ser65) in RT112 cells in control condition (siLUC)
28 and upon targeting of Rab7 (siRab7) or Arl8b (siArl8b) and quantification of relative
29 phosphorylated P-4EBP1 to GAPDH levels. F. Western Blot analysis of eIF4E Binding
30 Protein (4EBP1) phosphorylation (P-4EBP1 Ser65) in JMSU1 cells in control condition
31 (siLUC) and upon targeting of Rab7 (siRab7) or Arl8b (siArl8b) and quantification of
32 relative phosphorylated P-4EBP1 to GAPDH levels.

33

34 **Supplementary Figure 4:**

35 A. Representative images of endolysosomes visualized by immunofluorescence
36 staining against the lysosomal-associated membrane protein 1 (LAMP-1, CD107a) in

1 RT112 single cells cultured on crossbow-shaped adhesive micropatterns in control
2 condition (DMSO) and in the presence of A/C heterodimerizer (A/C). B. Invasion rate
3 of RT112 spheroids in the absence (black, n=18) and presence (red, n=16) of A/C
4 heterodimerizer as a function of the time (observed at the interval of 1d). n.s. in a
5 logrank test. C. 3D invasion of RT112 spheroids into collagen I matrix in control
6 conditions (siLUC) or after siRNA targeting of Arl8b (siArl8b) or Rab7 (siRab7). Red
7 arrows represent invading cells. Scale bar is 0.5 mm. D. 3D invasion of JMSU1 and
8 JMSU1 BicD2 spheroids into collagen I matrix in the presence or absence of A/C
9 heterodimerizer (A/C) observed at 3h interval. Scale bar is 0.5 mm.

10

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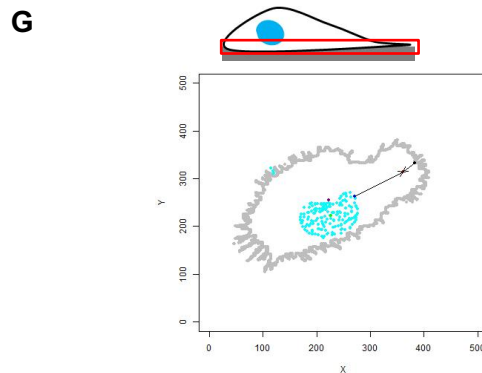
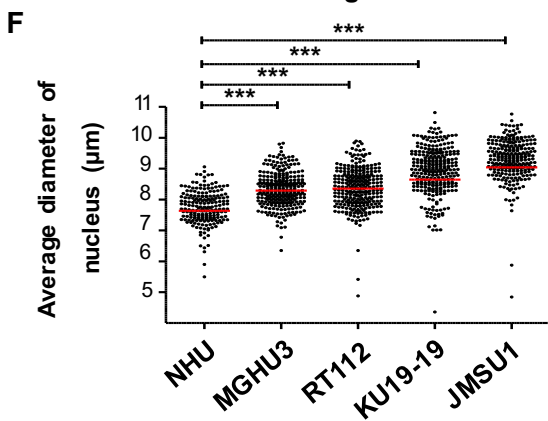
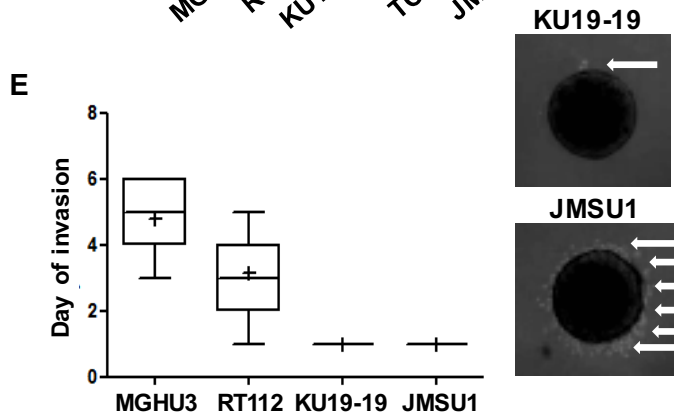
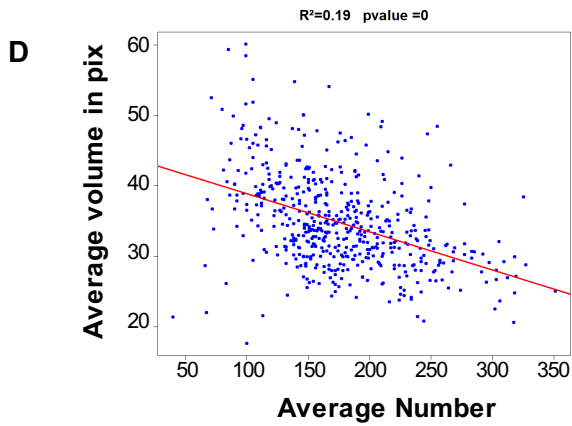
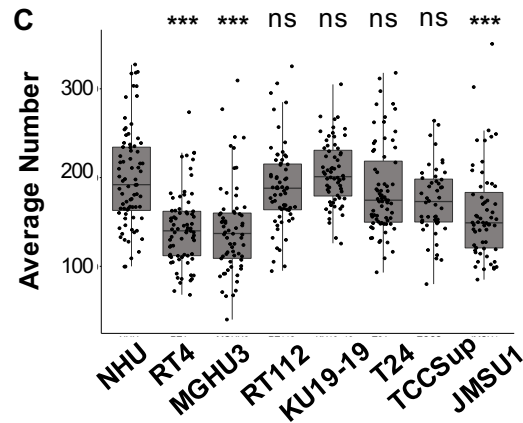
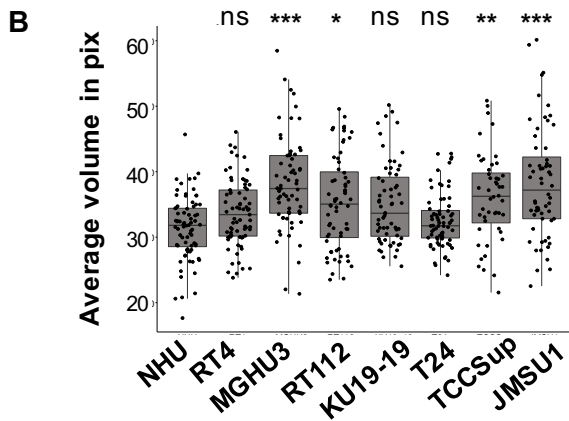
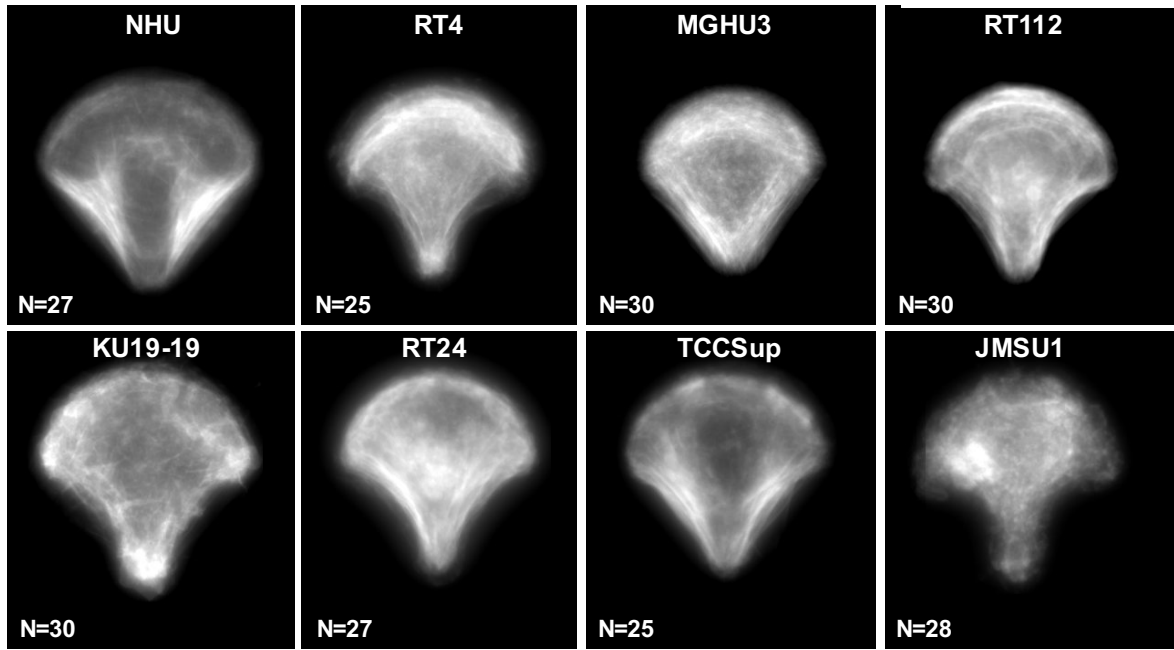
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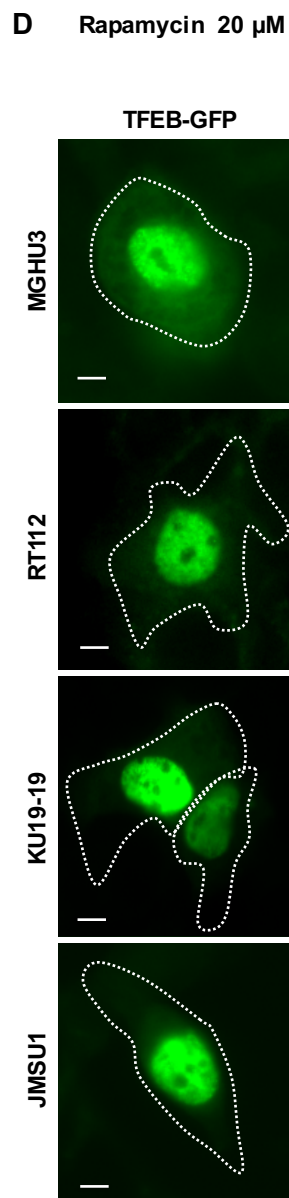
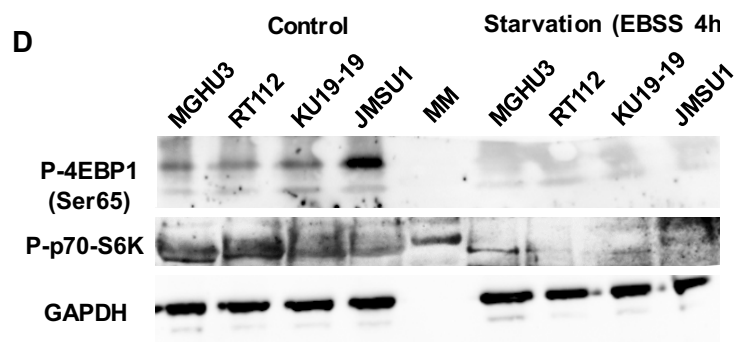
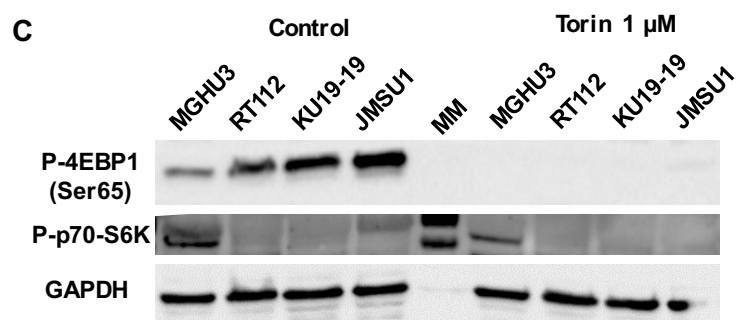
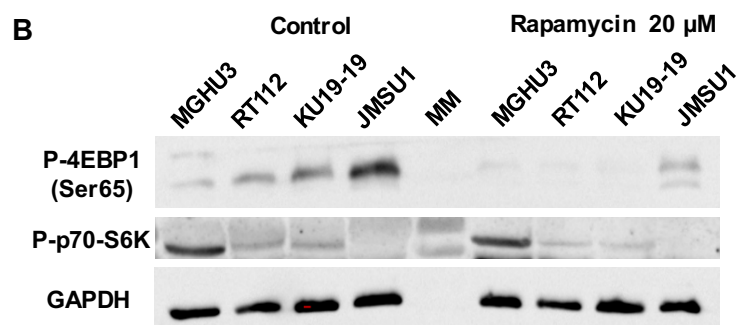
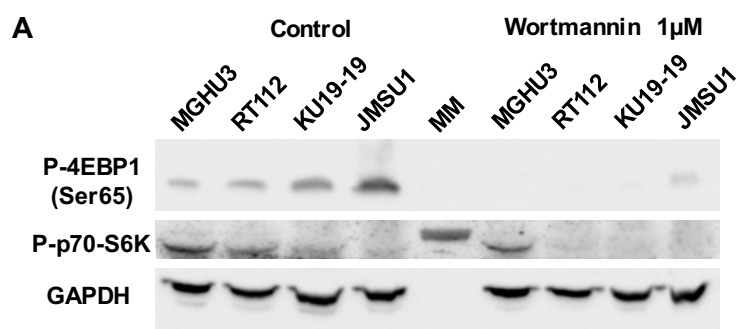
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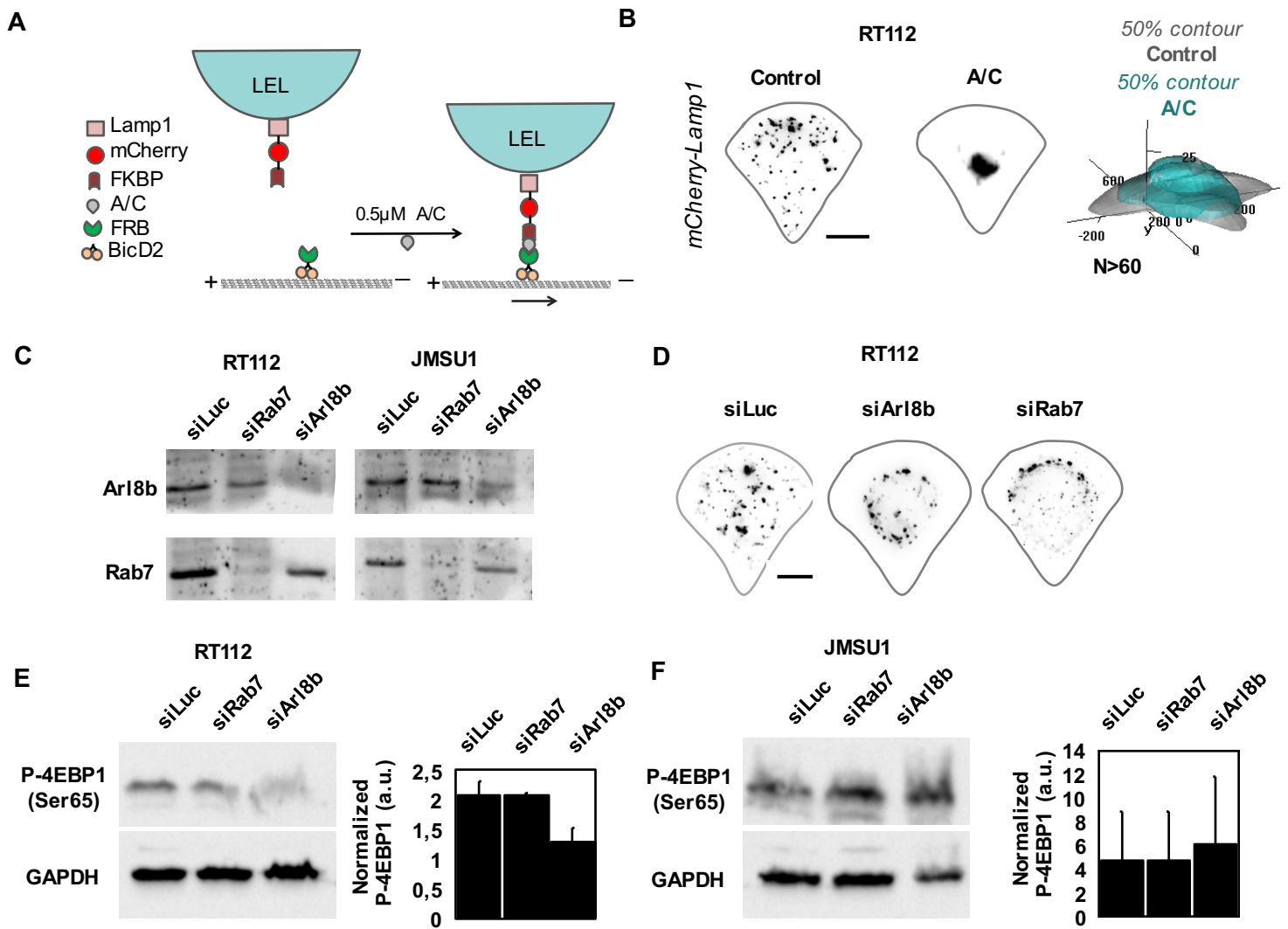
A
Average Intensity Projection of Phalloidin



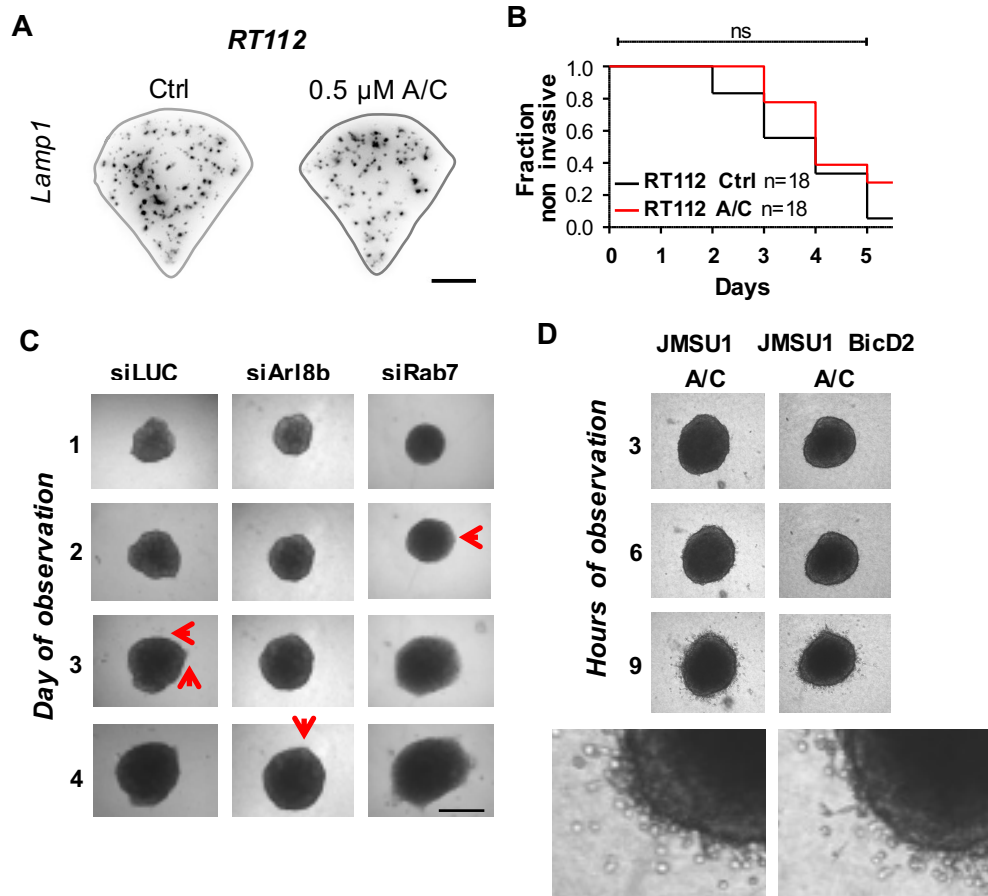
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4