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Autophagy plays a critical role in the degradation of active RHOA, the control of cell cytokinesis and genomic stability

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Abstract

Degradation of signaling proteins is one of the most powerful tumor suppressive mechanisms by which a cell can control its own growth. Here, we identify RHOA as the molecular target by which autophagy maintains genomic stability. Specifically, inhibition of autophagosome degradation by the loss of the v-ATPase *a3* (*TCIRG1*) subunit is sufficient to induce aneuploidy. Underlying this phenotype, active RHOA is sequestered *via* p62 (SQSTM1) within autolysosomes, and fails to localize to the plasma membrane or to the spindle midbody. Conversely, inhibition of autophagosome formation by *ATG5* shRNA dramatically increases

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Development of methodology: AB, MC, AC, FP, SG, CB and BM conducted experiments; IR, MT, SB, and GFC established the PCT cell-lines from WT and v-ATPase $a3^{-/-}$ mice. **NSCLC diagnosis and collection:** MI and PH.
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localization of active RHOA at the midbody, followed by diffusion to the flanking zones. As a result, all of the approaches we examined that compromise autophagy (irrespective of the defect: autophagosome formation, sequestration or degradation) drive cytokinesis failure, multinucleation, and aneuploidy, processes that directly have an impact upon cancer progression. Consistently, we report a positive correlation between autophagy defects and the higher expression of RHOA in human lung carcinoma. We therefore propose that autophagy may act in part as a safeguard mechanism that degrades and thereby maintains the appropriate level of active RHOA at the midbody for faithful completion of cytokinesis and genome inheritance.

Keywords

Autophagy; RHOA; tumor suppression; cytokinesis; aneuploidy

Introduction

Cells are faced with the tasks of dividing, of keeping the genome intact, and even of dying when appropriate. At their heart, these cell fates are dictated by tumor-suppressor mechanisms. One such mechanism is autophagy, which is commonly mutated or downregulated in human cancers (1). Indeed, the essential autophagy gene *BECN1* is deleted or mutated in 40 to 75% of breast, ovarian, colon and prostate cancers. Consistently, the notion that autophagy suppresses tumor development came from the demonstration that allelic loss of *Becn1* predisposes mice to lymphomas, hepatocellular carcinomas, and lung carcinomas (2, 3). Likewise, defects in other autophagy genes (*Atg4c*, *Atg5, Uvrag*, *Ambra1,* and *Bif-1/Sh3glb1*) render cells or mice tumor prone (4-8).

Physiologically, autophagy ensures in all cell types the turnover of all organelles and most long-lived proteins by a pathway, which begins with the formation of a double-membrane compartment, termed a “phagophore” that sequesters them. The phagophore expands into a completed vesicle, an “autophagosome”, and subsequently, the autophagosome rapidly fuses with a lysosome to become an “autolysosome” where the content is finally degraded. Originally identified as a housekeeping process, emerging data suggest that constitutive autophagy (*i.e.*, under nutrient–rich conditions) might also fight cancer by limiting inflammation (9), facilitating senescence (10) or clearing signaling proteins (11). Likewise, recent studies reveal that both BECN1 and ATG5 function as ‘guardians’ of cellular genome. Epithelial cells with loss of *Becn1* or *Atg5*, display gene amplification, and aneuploidy (7, 8). In support, activation of autophagy was demonstrated to reduce genomic instability within hepatocarcinoma cells(12). However, despite the importance of aneuploidy in cancer development (13, 14), the mechanisms underlying how autophagy deficiency compromises genomic stability are still unknown.

To address this issue, we explored the possibility that signaling proteins essential for cell growth might be degraded by autophagy. Whereas significant advances have been made in the discovery of autophagy machinery, less is known about the nature of the autophagy substrates. Therefore, a key feature of our strategy was to inhibit the autophagy pathway at the degradation step, in order to achieve sequestration and accumulation of substrates within autolysosomal structures (Fig. S1A).

Materials and Methods

Cell culture and treatments

To inhibit the maturation of autophagosomes into degradative autolysosomes, renal cells derived from proximal convoluted tubules of wild-type (WT, $a3^{-/-}$) or the lysosomal v-

ATPase *a3/TCIRG1*-nullmice ($a3^{-/-}$) were isolated and immortalized with the pSV3 neo vector. The renal epithelial cell lines that do or do not express *a3* were referred to as WT and $a3^{-/-}$ cells, respectively. As controls, cells were stimulated with an inhibitor of v-ATPase activity, bafilomycin A1 (100 nM, bafA1;Sigma); or a weak base that raises intralysosomal pH, chloroquine (100 μM, CQ; Sigma). Alternatively, the formation of autophagosomes was inhibited at the initiation step by *Atg5* or *Atg7* short hairpin RNA (shRNA). As a further control, we analyzed the phenotype of *Atg5* KO MEFs (provided by N. Mizushima) (15), and *ATG5*-depleted A549 lung epithelial cells. We also prevented the sequestration of autophagy substrates within the autophagic vesicles by *p62* shRNA. For details on cell culture and shRNA sequences see supplemental information.

Clinical samples

Primary NSCLC (pairs of pathological and control tissues from the same patient) were obtained from patients in Nice (France) and collected by the Tumor Biobank of Nice Hospital (Nice CHU, agreement 2010–06).

Analysis of autophagy

The activity of the autophagy pathway was monitored by four hallmarks: *i*) the formation of autophagic vesicles; and the degradation of three well-established autophagy substrates: *ii*) membrane-associated LC3-II; *iii*) p62/SQSTM1; and *iv*) long-lived proteins.

Ploidy determination and chromosomal abnormalities by metaphase spread

At 70% confluency, cells were arrested with colchicine (Invitrogen) in metaphase. Chromosomes were stained with giemsa and ’150 mitotic figures per cell line were photographed and the number of chromosomes was counted by Metafer M-Search Metaphase Finder and Ikaros softwares (Metasystems).

Time-lapse video microscopy

For monitoring cell progression through mitosis, exponentially growing cells cultured in complete growth medium were imaged every 5 min during 18 h on an inverted microscope (Carl Zeiss) equipped with a CO_2-equilibrated chamber.

Analysis of the RHOA pathway

The activity of RHOA pathway was monitored by *i*) the levels of active GTP-bound RHO(RHOTEKINRHO Binding Domain Pull-down and ELISA-based G-LISA assays, Cytoskeleton Inc.); *ii*) the recruitment of RHOA to membranes; and *iii*) the downstream phosphorylation of myosin regulatory light chain (P-MLC) and reticulation of ACTIN cytoskeleton.

RHOA immunoprecipitation—Cells were lysed in RIPA and RHOA was immunoprecipitated with anti-RHOA antibody followed by western blotting with anti-ubiquitin, anti-p62, anti-LC3 and anti-RHOA antibodies.

RHOA stability—HEK 293 cells and A549 cells (Control, *ATG5*, or *p62* shRNA transduced cells) were transfected with FuGene^{HD} (Promega) and plasmids encoding the active (RHOA Q63) or inactive (RHOA N19) RHOA mutants. 20 h after transfection, cells were treated with cycloheximide (CHX; Sigma; C-4859; 10-20μg/mL) to stop *de novo* protein synthesis for 7-57 h, alone or in combination with proteasomal (MG132, Sigma; 10 μM) and lysosomal (CQ; 100 μM) inhibitors and the drop in the levels of RHOA mutants was assayed by anti-myc western blotting (Millipore; P01106; 1:1000).

Complete and detailed description of all methods employed are available as Supplementary Data.

Statistical analysis

When adequate, results are presented as means ± SD from the indicated number n of separate experiments. Statistical comparisons were done using Khi2 or Student T tests as appropriate. A p value <0.05 was considered significant.

Results

The V-ATPase a3-dependent autophagy defect is characterized by the formation of giant multinucleate cells

To gain a deeper insight into the role of autophagy, we established cell-lines from v-ATPase *a3/TCIRG1*-null mice (16). PCT (proximal convoluted tubule) cells were chosen as they express the highest level of v-ATPase (17); the a3 subunit is localized in the lysosomal limiting membrane (18). In agreement with the other reported defect of v-ATPase (19, 20), we show that the *a3* loss increased autophagy sequestration and simultaneously impaired autophagic degradation, as evidenced by the accumulation of ATG12–ATG5 conjugate, of autolysosomes and of autophagic substrates (long-lived proteins, LC3-II, and p62) (Fig. 1A and S1B). In contrast, *a3*-null cells seemed to have functionally intact proteasomes (Fig. S1C). Remarkably, this a3-dependent autophagy defect was characterized by an increase in cell sizeand a flattened morphology with the accumulation of vesicles and nuclei (Fig.1B-C), supporting the importance of autophagy in maintaining cell size (21, 22). By using different approaches, we excluded a role for senescence as a cause of enlarged morphology (Fig. S2). Consistently, FACS analysis revealed that $a3^{-/-}$ cells were dividing and displayed increased DNA content (3–4 N, Fig.S2C). Of interest, no subdiploid or subtetraploid cells were detected, indicating that the *a3* loss did not induce cell death. Subsequent karyotypes of $a3^{-/-}$ cells confirmed a near-triploid karyotype (group average, 131; Fig. 1D). Therefore, despite aneuploidy, the $a3^{-/-}$ cells continued to proliferate, and escaped apoptosis or senescence. All these abnormalities, which are hallmarks of cancer cells, were reminiscent of that reported for $Atg5^{-/-}$ or $Becn1^{-/+}$ cells (8). Collectively, these data support the notion that defects of the entire autophagy pathway [*i.e.* either at the step of formation ($Atg5^{-/-}$ or $Becn1^{-/+}$) or degradation (herein, $V\text{-}ATPase a3^{-/-}$) of autophagosomes] could result in aneuploidy.

The multinucleate phenotype of $a3^{-/-}$ cellsarises from cytokinesis failure

We hypothesized that the aneuploidy of $a3^{-/-}$ cells might arise through cytokinesis failure. Multiple mitotic defects leading to aneuploidy were indeed identified in $a3^{-/-}$ cells, as these cells often showed multipolar spindles and several chromosome segregation defects: improper attachment of chromosomes to spindle microtubules, lagging chromosomes, chromosome bridges connecting daughter cells, and micronuclei. Strikingly, a large proportion of $a3^{-/-}$ cells were connected *via* an asymmetric bridge, in contrast to the short intracellular bridge observed in the middle of the two WT daughter cells (Fig.S3).

Using real-time imaging, we showed that the WT cells completed cytokinesis in only 15 min (Fig. 2A, Movie S1). By contrast, the cytokinesis was incomplete upon v-ATPase inhibition by bafilomycin A1 treatment (Fig.2B) or *a3* loss (Fig.2C-G, Movies S2-S5). 72% of $a3^{-/-}$ cells that entered mitosis normally failed abscission (Fig. 2C) and instead remained connected by an intracellular bridge for up to eight h before separating (Fig.2D, Movie S2), re-entering mitosis synchronously (still bound to the sister cell, Fig.2E Movie S3) or collapsing back, forming a single binucleate cell (Fig.2F-G, Movies S4-S5). Moreover, we did not observe cell-cell fusion, cell engulfment and endoreplication during live-cell

imaging (n=200). Thus, impairment of cytokinesis at the membrane abscission step was the key event responsible for the formation of multinucleate *a3*$^{-/-}$ cells.

The inhibition of autophagy degradation by v-ATPase *a3* loss stabilizes RHOA-GTP within autolysosomes

We then explored which signaling proteins might be degraded by autophagy and could underlie this phenotype. One candidate was the small GTPase RHOA, that dictates cell shape and completion of cytokinesis *via* F-ACTIN reticulation (23). In this regard, a striking hallmark of *a3*$^{-/-}$ cells was a dramatic remodeling of ACTIN cytoskeleton with the loss of stress fibers and the formation of ACTIN patches (Fig. 3A and S4A,B). The current consensus is that fine-tuning of RHO activity involves the guanine nucleotide exchange factors (GEFs) that activate them, the GTPase-activating proteins (GAPs) that inactivate them, and the guanine nucleotide-dissociation inhibitors (GDIs) that maintain RHO inactive within the cytoplasm (23). Exciting findings have revealed that RHO GTPases, their upstream regulators and downstream targets might also be subjected to irreversible proteasome-dependent degradation (24-32).

Instead of proteasome, however, we determined that active RHOA was constitutively maintained at low levels by autophagy. Indeed, the active RHOA was barely detected at the plasma membrane of *a3*$^{-/-}$ cells but instead accumulated intracellularly, within autolysosomes positive both for the autophagy marker LC3 and lysosomal marker LAMP1 (Fig. 3B, S4B *inset*). Notably, the level of RHOA-GTP was elevated in resting *a3*$^{-/-}$ cells, as evidenced by the RHOTEKIN-binding pull-down assay, the recruitment of RHOA to cellular membranes and the downstream phosphorylation of myosin regulatory light chain (P-MLC, Fig. 3C and S4B).

A role for autophagy in controling RHOA-GTP level was then suggested by the shRNA-mediated inhibition of autophagosome formation: expression of *Atg5* shRNA increased the localization of active RHOA at the plasma membrane of *a3*$^{-/-}$ cells, which allowed ACTIN polymerization into filaments (Fig. 3D). Consistently, the *Atg5* shRNA-transduced *a3*$^{-/-}$ cells displayed a smaller size and tight cohesion within the colony (Fig. 3D). Importantly, the control of RHOA by autophagy was remarkably specific, as the related GTPase RAC (Fig. S4E), as well as the RHOA regulator, RHOGDI, were not affected (Fig. 3C). Together, these data highly suggest a working model in which autophagy might sequester and degrade active RHOA. Inhibition of v-ATPase by *a3* loss would stabilize RHOA-GTP within autolysosomal structures, protecting it from autophagy degradation, and at the same time this would preclude reticulation of ACTIN cytoskeleton (Fig. S1A).

p62-dependent autophagy specifically degrades active RHOA

As proof-of-concept, pharmacological inhibition of autophagy degradation by bafA1 or chloroquine(CQ) treatment similarly increased the levels of membrane-bound RHOA together with the autophagy substrates LC3-II and p62 (Fig. S5). This occurred in multiple cell types, including fibroblast, kidney, and lung epithelial cells (Fig. S5A). In contrast, impairment of proteasome by MG132 failed to alter the levels of the membrane-bound RHOA in every cell line tested (Fig. S5A). As expected, all features of *a3*$^{-/-}$ cells (*i.e.*, RHOA autolysosomal recruitment, F-ACTIN depolymerization, and increased cell size) were mimicked by bafA1, but not MG132 treatment, further supporting the model that activity of the RHOA pathway can be controlled by the autophagy-lysosome pathway (Fig. S5B–C). Consistently, the active, but not inactive, RHOA mutants were long-lived proteins selectively degraded by autophagy (Fig. 4). Indeed, upon inhibition of protein synthesis with cycloheximide (CHX), the active RHOA (RHOA^{+}, Q63) was degraded within 28 h and stabilized by inhibiting lysosomal degradation (CQ, Fig. 4A), or autophagosome formation

(*ATG5* shRNA, Fig. 4B and S6A-B) but not proteasomal activity (MG132, Fig. 4A). Inversely, the inactive form of RHOA ($RHOA^{-}$, N19) was unstable and as expected stabilized by inhibition of proteasome, but not by inhibition of lysosomal activity or of autophagy (Fig. 4A-B), in agreement with previous reports (24-30).

At this stage, it was of interest to address how autophagy may specifically targets the active RHOA. One adaptor by which autophagy acquires specificity is p62 (SQSTM1) that targets ubiquitinated substrates to the autophagy machinery (33). Of interest, p62 appears as a prime candidate for regulating RHOA as it is the sole autophagy adaptor involved in cell mitosis (34), cell spreading (35) and tumor growth (36-38). We observed that p62 not only co-immunoprecipitated (Fig. 4C) and co-localized (Fig. 4D) with ubiquitinated RHOA and LC3-II in CQ-treated cells but also was essential in the selective clearance of active RHOA by autophagy (Fig. 4B and D). Upon p62 silencing and CQ treatment, autophagosomes formed normally (as evidenced by LC3-II conversion, Fig. S6A), but the autophagic sequestration (Fig. 4D) and degradation (Fig. 4B) of active RHOA were defective. Collectively, these results strongly suggest a new signaling role for p62-dependent autophagy in the control of RHOA pathway.

Regulation of the amount of active RHOA at the midbody during cytokinesis by autophagy

The multinucleate phenotype of $a3^{-/-}$ cells raised the question of whether autophagy may control cytokinesis through regulation of RHOA. Autophagy was suggested to clear the midbody ring after cytokinesis completion (39, 40). Likewise, the depletion of several autophagy genes (*Atg5, Becn1, Uvrag, Bif-1* And *Vps34*) was demonstrated to lead to polynucleation, but the role of autophagy in controlling RHOA activation during cytokinesis was not documented (8, 41).

Although several key RHOA activators have been identified (42), little is known about the mechanisms that confine active RHOA at the midbody. Using the $a3^{-/-}$ cells, we observe that little, if any, active RHOA was at the equatorial furrow and was instead sequestered within autolysosomes, close to the midbody (Fig. 5A), likely as a result of increased sequestration. Inversely, when we blocked autophagosome formation by *ATG5* shRNA (Fig. S6A), RHOA was highly enriched at the equatorial furrow of cytokinetic A549 lung cancer cells (Fig. 5B and S7A). Notably, the RHO activity zone in *ATG5*-depleted cells was three times as wide and bright as in controls (control shRNA = 3.7 ± 0.8 μm; *ATG5* shRNA = 11.4 ± 2.6 μm; mean ± SD, Fig. 5C–D). Outside the cell equator, RHOA activity was also abnormally high at the cell cortex (Fig. 5B and S7). Accordingly, *Atg5* knockdown in mouse embryonic fibroblasts (Fig. 5E), and *ATG7* or *p62* depletion in A549 cells (Fig. S6) faithfully recapitulated the same phenotype with regard to the RHOA pathway. Likewise, closer examination indicated that the upstream regulators required for narrowed activation of RHOA such as the kinesin MKLP1, the RHOA GEF ECT2 and spindle microtubules concentrated at the midbody of cytokinetic cells (Fig. S8A-B) (43). It is also highly likely that the loss of *ATG5* would creates defects in the generation and the delivery of new membranes to the cleavage furrow, a process that is central to cytokinesis. As shown in Figure S8C, the delivery of endosomes to the midbody appeared notto be affected by *ATG5* depletion: the endosomes were delivered to the cleavage furrow and clustered on either side of midbody of *ATG5*-depleted cells, as observed in control cells. By contrast, *ATG5*-depleted cells exhibited in addition to RHOA a broader distribution of downstream F-ACTIN that triggers the formation of actomyosin ring (Fig. S7B). Our data therefore suggest that the degradation of RHOA by autophagy may function as a key, but hitherto uncharacterized, mechanism concentrating active RHOA at midbody.

Autophagy defects fuel chromosomal instability in lung cancer cells

Considering the apparent connection between autophagy and RHOA, a key issue is how defects in autophagy might affect cell behavior in a way relevant for cancer progression. Maintaining the appropriate amount of active RHOA at midbody is critical for faithful cytokinesis as it dictates the position, the formation and the contraction of actomyosin ring (42, 44). If our model were correct, one would expect that the inhibition of autophagy sequestration in autophagy-competent A549 tumor cells would be sufficient to disturb cytokinesis and thereby drive the genomic instability required for tumor progression. As shown in Figure 6, in control A549 cells, the clustering of RHOA in a narrow zone resulted in the formation of a unique and compact ring whose position and size remained constant throughout furrow ingression (Fig. 6A-B, upper panels). As a result, control cells successfully completed cytokinesis (Movie S6). Without functional autophagy, the subsequent hyperactivation of RHOA zone would jeopardize the assembly of an efficient contractile ring, as expected from the cytokinesis defects observed for active RHOA mutants (Fig. S4C). Indeed, *ATG5*-depleted A549 cells progressed through mitosis until the furrow started to constrict, then 52% of the cells demonstrated unstable and loose furrowing that suddenly fell apart, reformed and constricted more slowly than control furrows, delaying cytokinesis completion (Fig. 6A-B, lower panels, Movie S6). As result of cytokinesis failure, these autophagy compromises (*ATG5* shRNA and *p62* shRNA) were sufficient to increase the percentage of cellswith multiple nuclei (Fig. 6C), and the frequency of chromosomal gains and losses in nearly all of the chromosomes(Fig. 6D), one hallmark of aggressive cancer. Together, these findings highly suggested that autophagy may act in part as a master safeguard mechanism of active RHOA localization during cytokinesis for faithful genome inheritance.

Overexpression of RHOA is correlated with autophagy defects in lung carcinomas

The biochemical, cellular, and genomic changes (increased RHOA activity, increased cellular sizes, and aneuploidy) we consistently observed herein following autophagy inhibition (irrespective of particular autophagy defects) are noteworthy because they accompany the onset and progression of cancers. RHOA overexpression has been observed in many aggressive cancers, such as breast, colon, prostate, and lung cancers, but has been attributed largely to increased *RHOA* transcription (45). Consistent with a role of autophagy in RHOA degradation, we found that RHOA, together with p62 and LC3-II were overexpressed in late stages of non-small cell lung cancers (NSCLC; T; pTNM stage IIIA), compared to normal epithelia (N) and stage I adenocarcinomas (Fig. 7A-B). An impaired autophagic degradation rather than an increased transcription was correlated with RHOA overexpression, as the levels of RHOA expression and activity increased progressively with the accumulation of the autophagy substrates p62 and LC3-II (Fig. 7A), and no significant changes in *RHOA* mRNA expression were measured (Fig. 7A, right panel). Of interest, the inactivation of autophagy and the subsequent RHOA upregulation were not early events, but instead were late events in the progression of lung cancer, when these cancers acquire a more genomic instability. All tumor cells positive for p62 were also RHOA-positive (Fig. 7B). p62 overexpression, as a readout of an autophagy defect, was associated with a reduced or absent RHOA membrane localization, which instead displayed intracellular localization (T, right panels). In sharp contrast, normal bronchial epithelial cells that barely expressed p62 showed strong membrane expression of RHOA primarily at their apical ciliated cell surface (N, left panels).

Discussion

So far, most studies suggest that signal termination and irreversible progression through cell-cycle depend largely on the proteasomal degradation of key regulatory proteins (46).

Recently, Gao et al. provide the first evidence that autophagy negatively regulates Wnt signaling by degrading Dishevelled; however, this occurs under nutrient starvation (11). Therefore, the ability of autophagy to degrade signaling proteins under basal conditions and thereby to ensure tumor suppressive functions remains to be established.

We show here that autophagy promotes the degradation of active RHOA. In support of this, (*i*) RHOA–GTP was elevated in cells deficient in autophagosome clearance; (*ii*) importantly, RHOA failed to localize to midbody and instead accumulated within autolysosomes; (*iii*) a role for autophagy in regulating RHOA-GTP was then suggested by the shRNA-mediated inhibition of autophagosome formation: *ATG5* shRNA dramatically increased the localization of RHOA-GTP at the equatorial furrow of cytokinetic cells.One related RHO GTPase, RAC, was not targeted by autophagy. As a result, defects of the entire autophagy pathway [*i.e.* either at the formation (*ATG5* or *ATG7 shRNA*), sequestration (*p62 shRNA*) or degradation of autophagosomes (v-ATPase $a3^{-/-}$ cells)] similarly drive the formation of unstable and loose furrowing, disturbing cytokinesis completion and genome inheritance; processes that directly contribute to cancer development.

The regulation of RHOA is unique in that it involves GEF, GAP, and GDI proteins, along with the proteasome (47) and autophagy. Depending on its activation state, we provide the first lines of evidence that RHOA used distinct routes for degradation: while the proteasome degraded the cytosolic and inactive forms, the autophagy pathway specifically degraded the membrane-associated and active pool of RHOA. This is consistent with the recently reported degradation of two constitutively active RHO, RHOH and RHOB, within lysosomes (48, 49) and the redistribution of active RHOA to undefined perinuclear localization after treatment of enterocytes with an autophagy inducer, LPS (50). We therefore propose that autophagy may act as a tumor suppressor pathway in part by turningoff RHOA activation (Fig. 7C). In this model, the remarkable dynamics of autophagy together with its integration of extracellularcues might dictate the time and place where a RHOA is active, and able to interact with its downstream substrates. Accordingly, we reported the targeting of autolysosomes at midbody during cytokinesis, the same subcellular and temporal localizations where RHOA should be controlled. Therefore, autophagy might be critical for localized degradation of active RHOA at midbodyand thereby proper ACTIN dynamics during accurate completion of cytokinesis and faithful genome inheritance.

The development of a cancer depends on the ability of tumor cells to acquire growth advantages. Whatever the mutations, deletions, and epigenetic silencing of autophagy genes, our findings provide new information on how autophagy defects can drive tumor progression through deregulation of RHOA pathway. As autophagy is commonly downregulated in cancers (1) where RHOA is overexpressed (47), this new paradigm may be a general mechanism for the acquisition of aneuploidy and progression of human cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nat Rev Cancer. 2007; 7:961–7. [PubMed: 17972889]
2. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest. 2003; 112:1809–20. [PubMed: 14638851]
3. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci USA. 2003; 100:15077–82. [PubMed: 14657337]
4. Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, et al. Ambra1 regulates autophagy and development of the nervous system. Nature. 2007; 447:1121–5. [PubMed: 17589504]
5. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat Cell Biol. 2007; 9:1142–51. [PubMed: 17891140]
6. Mariño G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, Lopez-Otin C. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J Biol Chem. 2007; 282:18573–83. [PubMed: 17442669]
7. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev. 2007; 21:1621–35. [PubMed: 17606641]
8. Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, et al. Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev. 2007; 21:1367–81. [PubMed: 17510285]
9. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell. 2006; 10:51–64. [PubMed: 16843265]
10. Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, et al. Autophagy mediates the mitotic senescence transition. Genes Dev. 2009; 23:798–803. [PubMed: 19279323]
11. Gao C, Cao W, Bao L, Zuo W, Xie G, Cai T, et al. Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation. Nat Cell Biol. 2010; 12:781–90. [PubMed: 20639871]
12. Xie R, Wang F, McKeehan WL, Liu L. Autophagy enhanced by microtubule- and mitochondrion-associated MAP1S suppresses genome instability and hepatocarcinogenesis. Cancer Res. 2011; 71:7537–46. [PubMed: 22037873]
13. Geigl JB, Obenauf AC, Schwarzbraun T, Speicher MR. Defining ‘chromosomal instability’. Trends Genet. 2008; 24:64–9. [PubMed: 18192061]
14. Fujiwara T, Bandi M, Nitta M, Ivanova EV, Bronson RT, Pellman D. Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. Nature. 2005; 437:1043–7. [PubMed: 16222300]
15. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, et al. The role of autophagy during the early neonatal starvation period. Nature. 2004; 432:1032–6. [PubMed: 15525940]
16. Scimeca JC, Franchi A, Trojani C, Parrinello H, Grosgeorge J, Robert C, et al. The gene encoding the mouse homologue of the human osteoclast-specific 116-kDa V-ATPase subunit bears a deletion in osteosclerotic (oc/oc) mutants. Bone. 2000; 26:207–13. [PubMed: 10709991]

17. Hurtado-Lorenzo A, Skinner M, El Annan J, Futai M, Sun-Wada GH, Bourgoin S, et al. V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. Nat Cell Biol. 2006; 8:124–36. [PubMed: 16415858]
18. Toyomura T, Murata Y, Yamamoto A, Oka T, Sun-Wada GH, Wada Y, et al. From lysosomes to plasma membrane: localization of vacuolar type H+-ATPase with the a3 isoform during osteoclast differentiation. J Biol Chem. 2003; 278:22023–30. [PubMed: 12672822]
19. Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, Peterhoff CM, et al. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell. 2010; 141:1146–58. [PubMed: 20541250]
20. Juhasz G. Interpretation of bafilomycin, pH neutralizing or protease inhibitor treatments in autophagic flux experiments: Novel considerations. Autophagy. 2012; 8:1875–6. [PubMed: 22874642]
21. Hosokawa N, Harab Y, Mizushima N. Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size. FEBS Lett. 2006; 580:2623–9. [PubMed: 16647067]
22. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, et al. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell. 2005; 120:237–48. [PubMed: 15680329]
23. Jaffe AB, Hall A. RHO GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 2005; 21:247–69. [PubMed: 16212495]
24. Doye A, Mettouchi A, Bossis G, Clément R, Buisson-Touati C, Flatau G, et al. CNF1 exploits the ubiquitin-proteasome machinery to restrict RHO GTPase activation for bacterial host cell invasion. Cell. 2002; 111:553–64. [PubMed: 12437928]
25. Wang HR, Zhang Y, Ozdamar B, Ogunjimi AA, Alexandrova E, Thomsen GH, et al. Regulation of cell polarity and protrusion formation by targeting RHOA for degradation. Science. 2003; 302:1775–9. [PubMed: 14657501]
26. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. Regulation of the polarity protein Par6 by TGFβ receptors controls epithelial cell plasticity. Science. 2005; 307:1603–9. [PubMed: 15761148]
27. Chen Y, Yang Z, Meng M, Zhao Y, Dong N, Yan H, et al. Cullin mediates degradation of RHOA through evolutionarily conserved BTB adaptors to control actin cytoskeleton structure and cell movement. Mol Cell. 2009; 35:841–55. [PubMed: 19782033]
28. Bryan B, Cai Y, Wrighton K, Wu G, Feng XH, Liu M. Ubiquitination of RHOA by Smurf1 promotes neurite outgrowth. FEBS Lett. 2005; 579:1015–9. [PubMed: 15710384]
29. Asanuma K, Yanagida-Asanuma E, Faul C, Tomino Y, Kim K, Mundel P. Synaptopodin orchestrates actin organization and cell motility via regulation of RHOA signalling. Nat Cell Biol. 2006; 8:485–91. [PubMed: 16622418]
30. Boulter E, Garcia-Mata R, Guilluy C, Dubash A, Rossi G, Brennwald PJ, et al. Regulation of RHO GTPase crosstalk, degradation and activity by RHOGDI1. Nat Cell Biol. 2010; 12:477–83. [PubMed: 20400958]
31. Su L, Agati JM, Parsons SJ. p190RHOGAP is cell cycle regulated and affects cytokinesis. J Cell Biol. 2003; 163:571–82. [PubMed: 14610059]
32. Lynch EA, Stall J, Schmidt G, Chavrier P, D'Souza-Schorey C. Proteasome-mediated degradation of Rac1-GTP during epithelial cell scattering. Mol Biol Cell. 2006; 17:2236–42. [PubMed: 16481404]
33. Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. Autophagy. 2011; 7:279–96. [PubMed: 21189453]
34. Linares JF, Amanchy R, Greis K, Diaz-Meco MT, Moscat J. Phosphorylation of p62 by cdk1 controls the timely transit of cells through mitosis and tumor cell proliferation. Mol Cell Biol. 2011; 31:105–17. [PubMed: 20974803]
35. Kadandale P, Stender JD, Glass CK, Kiger AA. Conserved role for autophagy in RHO1-mediated cortical remodeling and blood cell recruitment. Proc Natl Acad Sci USA. 2010; 107:10502–7. [PubMed: 20498061]

36. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, et al. Autophagy suppresses tumorigenesis through elimination of p62. Cell. 2009; 137:1062–75. [PubMed: 19524509]
37. Duran A, Linares JF, Galvez AS, Wikenheiser K, Flores JM, Diaz-Meco MT, et al. The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis. Cancer Cell. 2008; 13:343–54. [PubMed: 18394557]
38. Inami Y, Waguri S, Sakamoto A, Kouno T, Nakada K, Hino O, et al. Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. J Cell Biol. 2011; 193:275–84. [PubMed: 21482715]
39. Pohl C, Jentsch S. Midbody ring disposal by autophagy is a post-abscission event of cytokinesis. Nat Cell Biol. 2009; 11:65–70. [PubMed: 19079246]
40. Kuo TC, Chen CT, Baron D, Onder TT, Loewer S, Almeida S, et al. Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity. Nat Cell Biol. 2011; 13:1214–23. [PubMed: 21909099]
41. Sagona AP, Nezis IP, Pedersen NM, Liestøl K, Poulton J, Rusten TE, et al. PtdIns(3)P controls cytokinesis through KIF13A-mediated recruitment of FYVE-CENT to the midbody. Nat Cell Biol. 2010; 12:362–71. [PubMed: 20208530]
42. Piekny A, Werner M, Glotzer M. Cytokinesis: welcome to the RHO zone. Trends Cell Biol. 2005; 15:651–8. [PubMed: 16243528]
43. Yüce O, Piekny A, Glotzer M. An ECT2-centralspindlin complex regulates the localization and function of RHOA. J Cell Biol. 2005; 170:571–82. [PubMed: 16103226]
44. Miller AL, Bement WM. Regulation of cytokinesis by RHO GTPase flux. Nat Cell Biol. 2009; 11:71–7. [PubMed: 19060892]
45. Chan CH, Lee SW, Li CF, Wang J, Yang WL, Wu CY, et al. Deciphering the transcriptional complex critical for RHOA gene expression and cancer metastasis. Nat Cell Biol. 2010; 12:457–67. [PubMed: 20383141]
46. Kerscher O, Felberbaum R, Hochstrasser M. Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu Rev Cell Dev Biol. 2006; 22:159–80. [PubMed: 16753028]
47. Sahai E, Marshall CJ. RHO-GTPases and cancer. Nat Rev Cancer. 2002; 2:133–42. [PubMed: 12635176]
48. Perez-Sala D, Boya P, Ramos I, Herrera M, Stamatakis K. The C-terminal sequence of RHOB directs protein degradation through an endo-lysosomal pathway. PLoS ONE. 2009; 4:e8117. [PubMed: 19956591]
49. Schmidt-Mende J, Geering B, Yousefi S, Simon HU. Lysosomal degradation of RHOH protein upon antigen receptor activation in T but not B cells. Eur J Immunol. 2009; 40:525–9. [PubMed: 19950172]
50. Cetin S, Ford HR, Sysko LR, Agarwal C, Wang J, Neal MD, et al. Endotoxin inhibits intestinal epithelial restitution through activation of RHO-GTPase and increased focal adhesions. J Biol Chem. 2004; 279:24592–600. [PubMed: 15169791]

Abbreviations

Atg	autophagy-related
bafA1	bafilomycin A1
CQ	chloroquine
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
LAMP	lysosomal-associated membrane protein
LC3	microtubule-associated protein 1 light chain 3
PCT	proximal convoluted tubule

Ⓟ-MLC	phospho-myosin light chain
RHOGDI	RHO guanine nucleotide-dissociation inhibitor-1
shRNA	short hairpin RNA
v-ATPase	vacuolar-ATPase

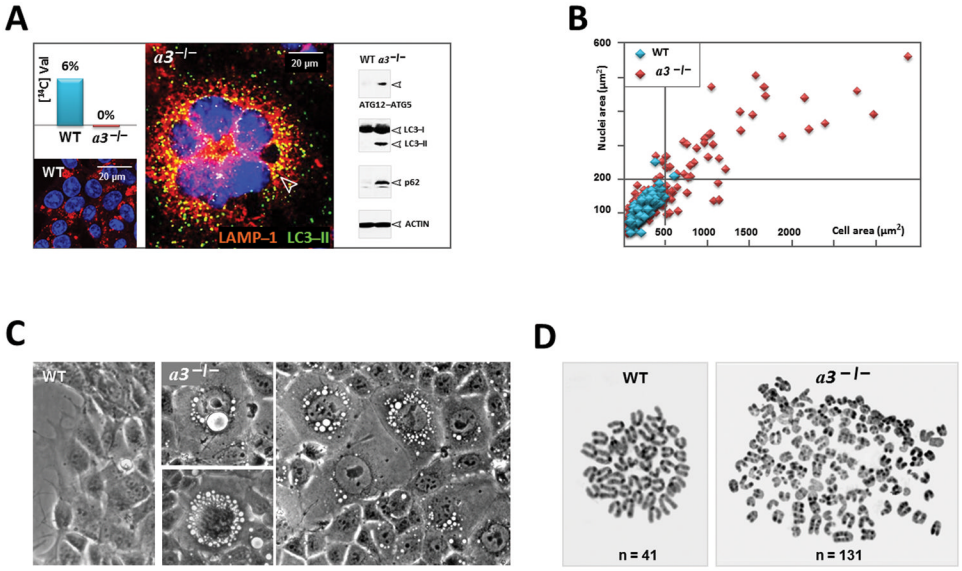

Figure 1. The a3-dependent autophagy defect is characterized by the formation of giant multinucleate cells

A, Accumulation of autolysosomes (LC3-II and LAMP1 positive; fluorescence images) that were defective in the degradation of long-lived proteins (upper left inset), LC3-II and p62 (right insets). Right: note the accumulation of the ATG12–ATG5 conjugate (a marker of autophagosome formation) in response to the *a3* loss.(See Fig. S1).

B, Cell and nuclei area of WT and $a3^{-/-}$ cells (n = 400).

C, Representative photomicrographs showing that the WT cells were all small (200 ± 47 μm^2) with one nucleus, while the *a3*-null cells were heterogeneous in size. 30-40% of $a3^{-/-}$ cells showed a gigantic size (200-7,300 μm^2), and a flattened shape with the accumulation of vesicles and nuclei.

D, Representative karyotype figures showing aneuploidy and structural abnormalities in $a3^{-/-}$ cells.

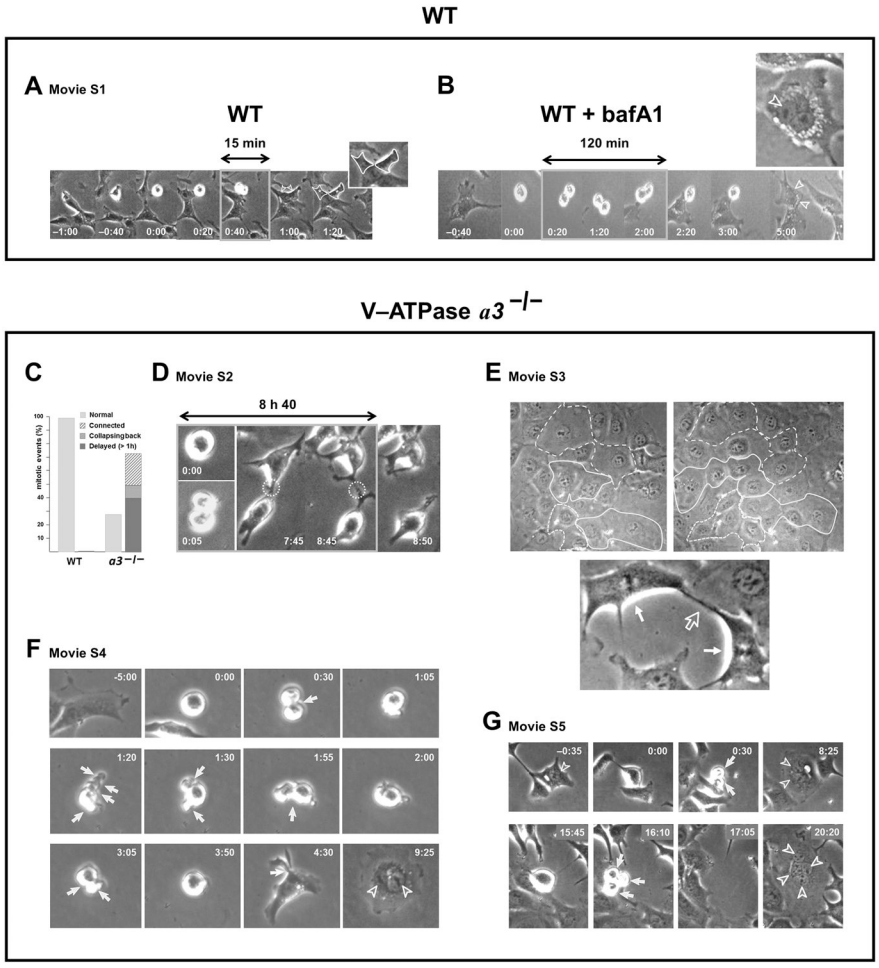

Figure 2. Cytokinesis is specifically impeded by v-ATPase inactivation

Control (A, treated with DMSO, Movie S1; *inset*: high magnification of the last frame where the daughter WT cells are outlined in white to help visualize their successful division), bafA1-treated WT cells (B, 100 nM, 8 h after the addition of bafA1) or $a3^{-/-}$ cells (C-G, Movies S2–S5), all under complete medium were followed for 18 h using phase-contrast time-lapse microscopy.

C, Frequency of cytokinesis failure in $a3^{-/-}$ cells(n = 125).

D, Still images of a mitotic $a3^{-/-}$ cell that remained connected by an intracellular bridge for up to eight h before abscission (Movie S2).

E, Pairs of $a3^{-/-}$ cells that entered mitosis synchronously are outlined in white (Movie S3). Lower panel: two mitotic cells (white arrows) remained connected by an intracellular bridge (empty arrow).

F, Still-images of an $a3^{-/-}$ cell that exited mitosis as a binucleated cell after 18 attempts of cleavage furrow formation. Note that this cell developed ectopic furrows, which led to the formation of a nuclear fragments (white filled arrows) that fused back to the cell (Movie S4).

G, Still-images of a mononucleate $a3^{-/-}$ cell that formed a tetranucleate cell after two rounds of abortive mitoses (Movie S5). Both giant and small $a3^{-/-}$ cells failed cytokinesis, resulting in multinucleation and differentially sized daughter cells. All daughter cells were viable throughout imaging (up to 18 h). White empty arrows indicate cleavage furrow formation, arrowheads indicate nuclei, and time points are in h:min from the initiation of metaphase (zero time point).

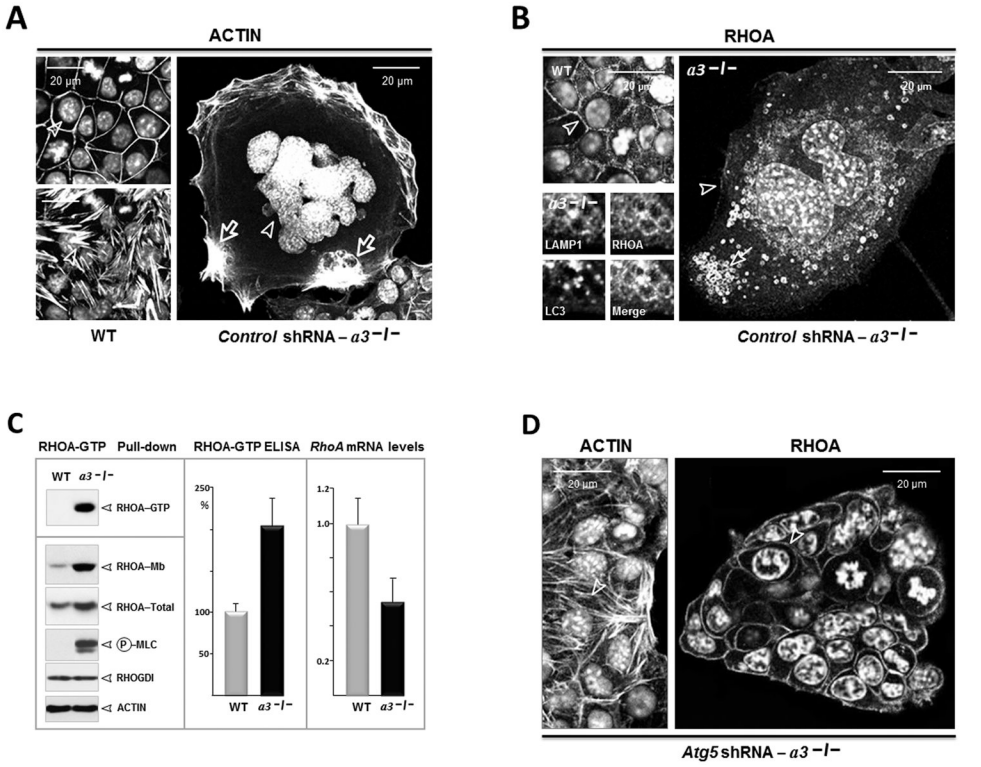

Figure 3.

The inhibition of autophagy degradation by v-ATPase *a3* loss stabilizes RHOA-GTP within autolysosomes.

A, Phalloidin labeling showed the loss of stress fibers in $a3^{-/-}$ cells that instead developed ACTIN patches (empty arrows).

B, Intracellular ring of RHOA (arrows) that colocalized with LC3-II and LAMP1 (*insets*, see also the color images in Fig. S4B left) in $a3^{-/-}$ cells in contrast to the WT cells that displayed RHOA at their plasma membrane (arrowhead).

C, Accumulation of RHOA-GTP in $a3^{-/-}$ cells was evidenced by RHOTEKIN binding (pull-down assay and G-ELISA kit; left and middle panels, respectively); the recruitment of active RHOA proteins to Triton X-100 insoluble cell membranes; and the downstream phosphorylation of myosin regulatory light chain (Ⓟ-MLC; lower panels). Bound proteins (upper panel) and total cell lysates (lower panels) were analyzed by western blotting. Note that *a3* loss increased the ratio of activated (membrane-associated):total RHOA from 2% (WT) to 4% ($a3^{-/-}$ cells). Western blots are representative of three independent experiments. RT-PCR analysis showing that RHOA-GTP accumulation was not due to an increased *RHOA* transcription (right panel).

D, *Atg5* shRNA rescues RHOA localization at the plasma membrane (arrowhead) of $a3^{-/\#x02212;}$ cells. Cells were infected with *Atg5* shRNA lentivirus and selected with media containing puromycin for 72 h. Shown are representative images where DAPI marks nucleus and F-ACTIN denotes the filamentous ACTIN stained by phalloidin.

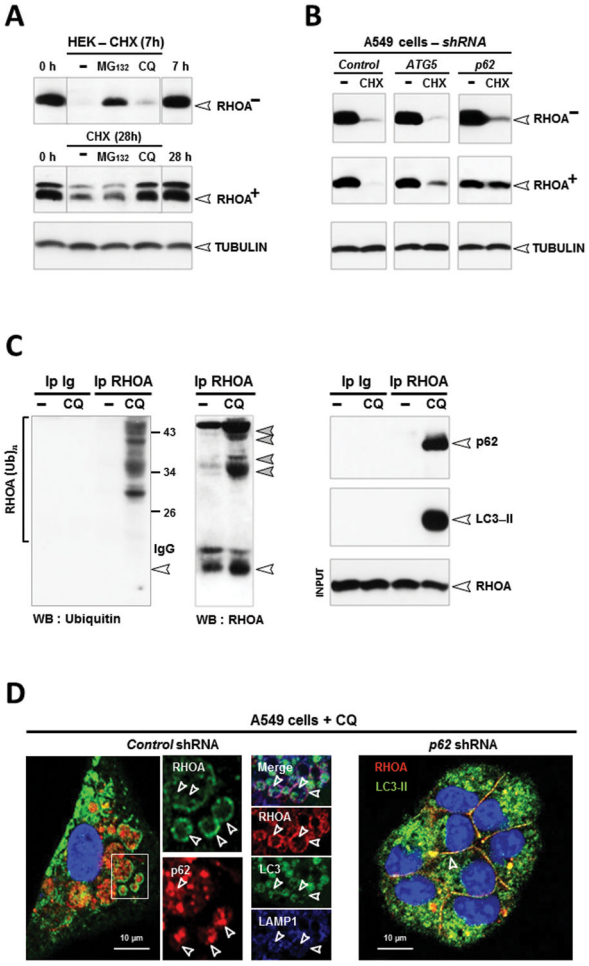

Figure 4. p62-dependent autophagy controls the levels of active RHOA

A, The active RHOA is specifically degraded by a lysosomal pathway. HEK cells were transfected with plasmids encoding the active (Q63, $RHOA^{+}$) or inactive RHOA (N19, $RHOA^{-}$) mutants, and incubated with cycloheximide (CHX; 10 μg/mL) for the indicated time in the presence or absence of CQ (100 μM) or MG132 (10 μM) to inhibit the lysosomal or proteasomal functions, respectively. Expression of RHOA mutants was analyzed by anti-myc western blotting. (See Fig. S5).

B, Inhibiting autophagy sequestration in A549 cells by *ATG5* or *p62* depletion stabilized the active but not the inactive RHOA mutant. Control, *ATG5*, or *p62* shRNA-transduced-A549 cells were transfected with the RHOA mutants, and treated with CHX (20 μg/mL) for 57 h. Equal protein loading was verified by anti-TUBULIN immunoblotting. The data are representative of at least 3 independent experiments. (See Fig. S6A–B).

C, The ubiquitin-binding protein p62/SQSTM1 acts as a receptor that targets the active RHOA to autophagy. Ubiquitinated RHOA species (gray arrowheads) formed a complex with p62 and LC3-II in CQ-treated A549 cells.

D, p62 affects RHOA localization. Left: RHOA colocalized with p62, LC3-II, and LAMP1 upon autophagy inhibition by CQ. Right: decreasing p62 levels by shRNA reduced colocalization of RHOA with LC3-II and rescued RHOA localization at the plasma membrane of CQ-treated cells.

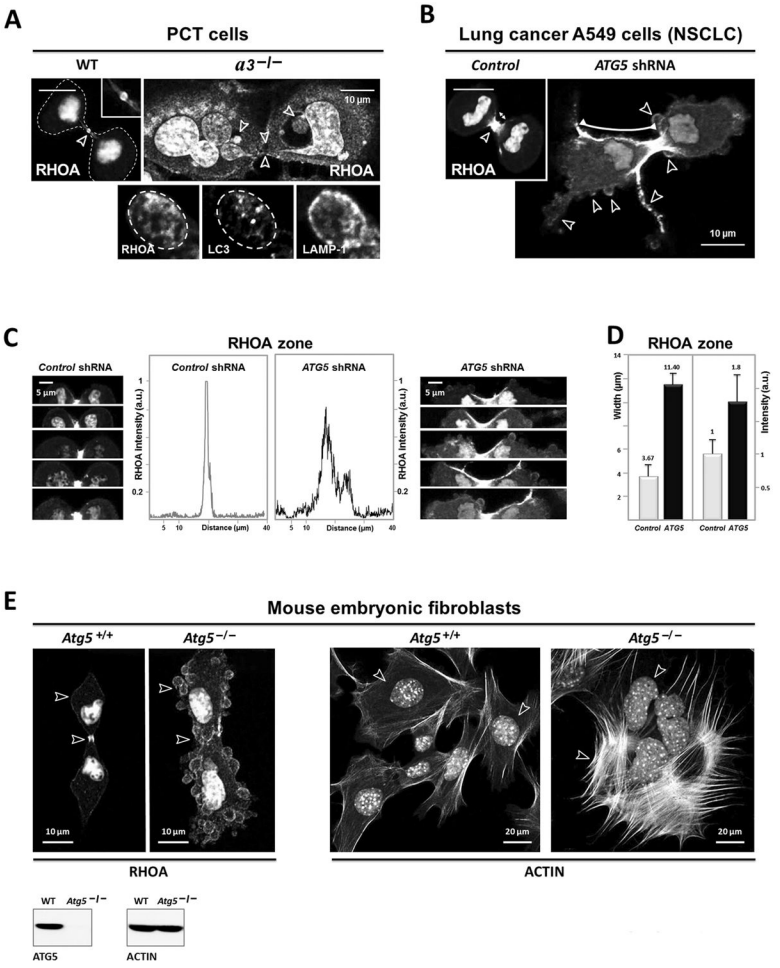

Figure 5. Autophagy defects disturb the clustering of active RHOA at the midbody during cytokinesis

A, Confocal images of cytokinetic *a3*$^{-/-}$ PCT cells showing that RHOA failed to localize to midbody (arrowhead) and instead accumulated within autolysosomes (LC3 and LAMP1 positive). *Inset*: RHOA-positive vesicles were not degraded and accumulated within the lumenal space of autolysosomes.

B, *ATG5* depletion in A549 tumor cells caused aberrant RHOA diffusion along the cell cortex during cytokinesis. RHOA, normally detected in a narrow zone at midbody of control cells, accumulated within a looser equatorial zone (arrows) and outside (arrowheads) of cleavage furrow of *ATG5*-depleted cells. (See Fig. S6A and S7).

C, RHOA staining at similar stages of furrowing in *control* (left) and *ATG5*-depleted (right) A549 cells. Representative intensity profile that measures RHOA zone width along the cell edge (middle).

D, The increases in the RHOA zone width ($P=5.34\ 10^{-8}$) and RHOA intensity (area under curve, $P=0.000266995$) reflect a net increase in RHOA activation at equatorial region of *ATG5*-depleted cells. Results are mean ± SD; n=15.

E, Compared to WT cells, *Atg5*$^{-/-}$ MEF cells showed severe RHOA diffusion over the entire cell surface, ectopic furrowing during cytokinesis, as well as enlarged nuclei and cell morphology, and enhanced F-ACTIN polymerization.

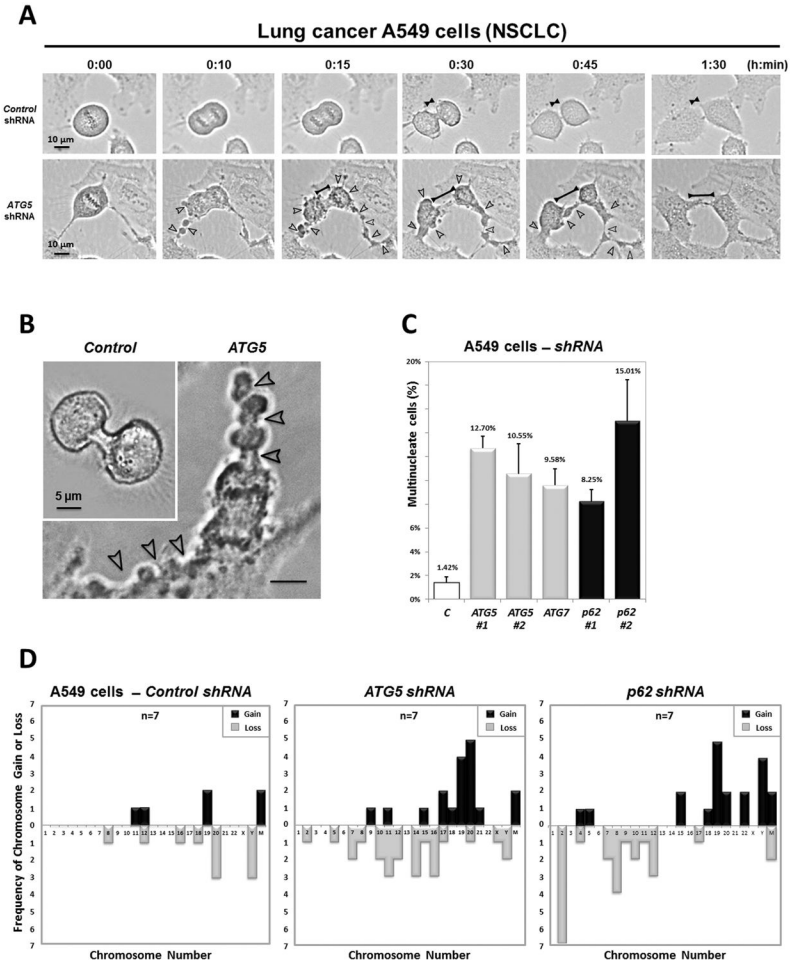

Figure 6. Autophagy defects cause genomic instability in lung cancer cells

A, Persistent ectopic furrowing as *ATG5*-depleted A549 cells entered cytokinesis. Phase-contrast images of control and *ATG5*-depleted A549 cells were acquired at the indicated times (h:min; Movie S6). Virtually all control cells showed normal compact furrowing during cytokinesis (85%; n=100, upper inset), whereas the majority (52%; $P=2.8\ 10^{-19}$; n=50) of telophase *ATG5*-depleted cells exhibited unstable and loose furrowing. The black arrowheads indicate ectopic furrows and the bar marks the width of the equatorial furrow.

B, Representative picture of a telophase *ATG5*-depleted cell that exhibited unstable furrowing, with numerous cortical constrictions and blebs (arrowheads), in contrast to the unique and compact furrow of a control cell.(See Fig. S7).

C, Defects at the stage of autophagosome formation (*ATG5* or *ATG7* shRNA) or the recruitment of autophagy substrates (*p62 shRNA*) resulted in a seven- to ten-fold increase of multinucleate cells compared with *control* shRNA-transduced cells. The different autophagy defects are indicated on the *x* axis (n =900). The y axis shows the percentage of multinucleate cells. (See Fig. S6C).

D, Frequency of whole chromosome gains (black) and losses (grey) in *control*, *ATG5*-depleted, and *p62*-depleted A549 cells (n = 7). Chromosome numbers are indicated on the *x* axis. Note that the chromosomal gains or losses occurred with low frequency in the autophagy-competent A549 tumor cells and were limited to fewer chromosomes in comparison with *ATG5*- depleted cells and *p62*-depleted cells, which showed widespread chromosomal gains and losses in nearly all of the chromosomes. Chromosome 2 loss (n=7) and chromosome 19gain (n=5) were recurrent in *p62*-depleted A549 cells.

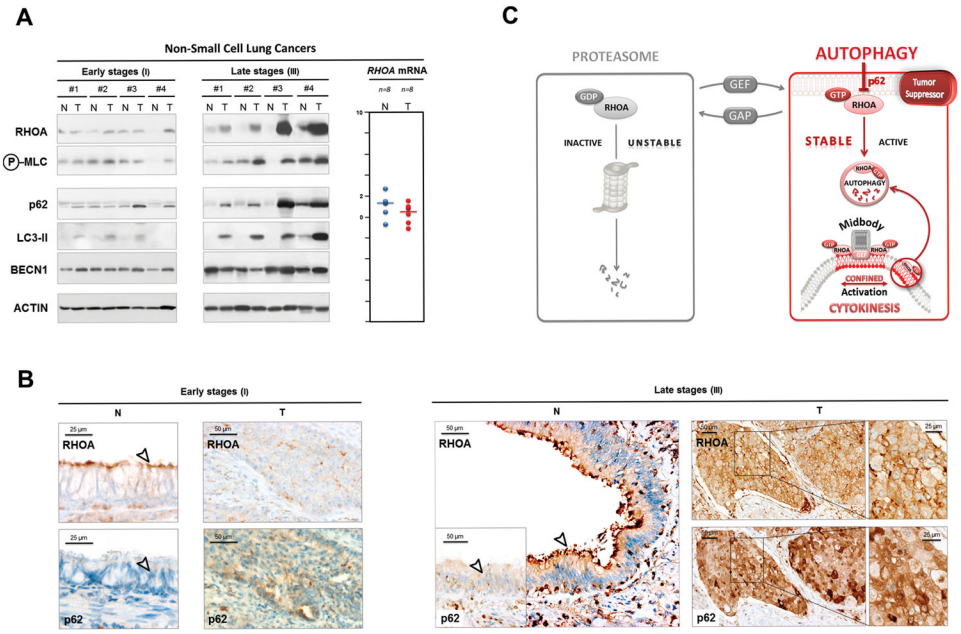

Figure 7. Correlation between RHOA protein levels and autophagy in human lung cancers

A, Immunoblotting of NSCLC samples (T, tumor: early stage n=4; late stage n=4; see Table S1) *versus* normal peritumoral tissues (N, normal) using the indicated antibodies. p62 serves as a positive control for autophagy impairment (36), and ACTIN as a loading control. Similar *RHOA* mRNA levels in tumor and normal tissues (right panel, not statistically significant).

B, Immunohistochemical staining revealed a high overexpression of RHOA and p62 in late stages of NSCLC. In line with $a3^{-/-}$ cell results (Fig. 3), the defect in lung cancer samples was a defect in autophagosome degradation with the concomitant accumulation of the three autophagy substrates LC3, p62, and active RHOA (RHOA recruitment to intracellular cell membranes; and downstream Ⓟ-MLC).

C, Proposed model by which autophagy sequesters and degrades the active RHOA that would otherwise diffuse from midbody to flanking zones. Together with RHO GEF, autophagy seems essential to confine RHOA activation at midbody, allowing the assembly of a unique and compact contractile ring, for successful cytokinesis and faithful genome inheritance.