Atg5-dependent autophagy contributes to the development of acute myeloid leukemia in an MLL-AF9-driven mouse model

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Acute myeloid leukemia (AML) is a hierarchical hematopoietic malignancy originating from leukemic stem cells (LSCs). Autophagy is a lysosomal degradation pathway that is hypothesized to be important for the maintenance of AML as well as contribute to chemotherapy response. Here we employ a mouse model of AML expressing the fusion oncogene MLL-AF9 and explore the effects of Atg5 deletion, a key autophagy protein, on the malignant transformation and progression of AML. Consistent with a transient decrease in colony-forming potential in vitro, the in vivo deletion of Atg5 in MLL-AF9-transduced bone marrow cells during primary transplantation prolonged the survival of recipient mice, suggesting that autophagy has a role in MLL-AF9-driven leukemia initiation. In contrast, deletion of Atg5 in malignant AML cells during secondary transplantation did not influence the survival or chemotherapeutic response of leukemic mice. Interestingly, autophagy was found to be involved in the survival of differentiated myeloid cells originating from MLL-AF9-driven LSCs. Taken together, our data suggest that Atg5-dependent autophagy may contribute to the development but not chemotherapy sensitivity of murine AML induced by MLL-AF9.

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Atg5 deletion during primary transplantation extends the survival of mice burdened with MLL-AF9-driven AML. In order to specifically assess the in vivo role of autophagy in the malignant transformation and progression of AML, we took advantage of transgenic mice expressing a tamoxifen-inducible Cre recombinase (CreERT2) in combination with floxed Atg5 alleles (Atg5FL/−). Therefore, the transplantation of cells from donor mice with the genotypes of Atg5WT/WT; CreERT2−/− (Atg5WT) and Atg5FL/−;CreERT2−/− (Atg5FL) to congenic C57BL/6J recipients followed by tamoxifen treatment induces the specific in vivo deletion of Atg5 in Atg5FL donor cells.

To determine whether Atg5 deletion affects MLL-AF9-induced transformation of hematopoietic progenitors, we transduced c-kit+ BM cells with dMLL-AF9 retrovirus and subjected them to a serial colony-forming unit assay in methylcellulose containing 4-hydroxytamoxifen (4OHT) to delete Atg5 in vitro. When compared with vehicle control, Atg5−/− cells treated with 4OHT demonstrated a transient but significant decrease in colony-formation potential during the second passage (Figure 1g), suggesting that autophagy may contribute to MLL-AF9-driven leukemogenesis. We confirmed that a 5-day continuous treatment with 4OHT is required to complete Atg5 deletion (Figure 1h).

Next we assessed the role of autophagy in MLL-AF9-driven AML development and progression in vivo. Atg5WT and Atg5FL c-kit+ BM cells were transduced with dMLL-AF9 and expanded for three rounds of methylcellulose culture (Figure 2a). Cells were then intrafemorally transplanted to sublethally irradiated C57BL/6J recipients and treated with tamoxifen after 10 days in order to assess the role of Atg5 during primary transplantation. Mice transplanted with Atg5WT or Atg5FL BM cells expressing MLL-AF9 became moribund with a median survival of 77.5 days and 104 days, respectively, demonstrating a prolonged survival for the Atg5FL group of mice (Figure 2b). PCR confirmed that Atg5 was efficiently deleted in the splenocytes of mice transplanted with Atg5FL cells (Figure 2c). We observed evidence of anemia and myeloid blasts in peripheral blood, as well as significant perivascular infiltration of blasts to the liver for both groups of mice (Figure 2d). Splenomegaly was consistently observed and no differences were observed between the two groups at morbidity (Figure 2e). Flow cytometric analysis of the peripheral blood, spleen, and BM revealed that all three hematopoietic tissues show high percentages of CD11b+ myeloid cells (Figure 2f). No differences were observed in blast morphology, liver infiltration, or myeloid cell proportions between mice transplanted with Atg5WT and Atg5FL cells at morbidity. This data suggest that autophagy facilitates the initiation of MLL-AF9-driven AML in our model.

In order to overcome experimental limitations regarding stem cell heterogeneity and oncogene dosage, as well as to control for toxicities associated with Cre recombinase and tamoxifen, we performed a series of experiments where mice were transplanted with Atg5WT or Atg5FL donor cells transduced with dMLL-AF9, each followed by vehicle or tamoxifen treatment. Mice transplanted with a single pool of Atg5WT cells and treated with vehicle or tamoxifen succumbed to AML without a significant difference in median survival, suggesting that active Cre recombinase or tamoxifen does not
affect the survival of leukemic mice in the absence of Atg5 deletion (Figure 3a). However, mice transplanted with a single pool of Atg5^{FL} cells expressing dMLL-AF9 and treated with either vehicle or tamoxifen demonstrated a modest but significant survival advantage for tamoxifen-treated mice (Figure 3b), consistent with our previous findings (Figure 2b). Weekly in vivo bioluminescent imaging of these Atg5^{FL} mice revealed that total leukemia burden was
Figure 1  Verification of the dMLL-AF9 vector expressing luciferase and GFP-LC3. (a) C-kit+ BM cells or malignant leukemia cells driven by exogenous pMIG-MLL-AF9 were cultured in cytokine-supplemented medium and treated with vehicle or 100 nM BafA1 for 6 h and subjected to immunoblotting. Quantified LC3-II levels were normalized against β-actin and its respective vehicle-treated control. Western blotting is representative of two independent experiments. (b) Schematic representing the retroviral construct containing MLL-AF9, luciferase (Luc), and GFP-LC3 with their respective promoters. (c) The luminescence of PHOENIX/Eco cells 24 h after calcium transfection of the pMSCV-empty (Empty), pMSCV-Luc-IRES-YFP (Luc), or dMLL-AF9. Error bars represent S.D. of three replicates. (d) Green fluorescence of PHOENIX/Eco cells 24 h after calcium transfection with the indicated vectors was detected with the Olympus CKX41 microscope using the Olympus DP20 camera and the Olympus CellSens software (original magnification ×40). (e) Luminescence of non-transduced or dMLL-AF9-transduced c-kit+ BM cells (c-kit+ BM+dMLL-AF9) compared with MOLM13 cells stably expressing pMSCV-Luc-IRES-YFP (MOLM13-Luc). Error bars represent S.D. of three replicates. (f) Non-transduced or dMLL-AF9 transduced c-kit+ BM cells were seeded to methylcellulose for three passages at 5 days each and counted for the number of colonies. Error bars represent S.D. of three replicates. (g) One day after c-kit+ BM were transduced with dMLL-AF9, cells were seeded to methylcellulose medium containing 100 nM 4OHT for three rounds of serial replating. Black bars represent the mean ± S.D. Statistics were calculated by ANOVA with multiple comparisons. **P<0.01. (h) Atg5FL c-kit+ BM were treated with 100 nM 4OHT for the indicated days, and control Atg5WT c-kit+ BM cells were treated with 100 nM 4OHT for 5 days. Genomic DNA was extracted and analyzed by PCR.

Figure 2  Development of AML in mice transplanted with Atg5WT and Atg5FL MLL-AF9-BM cells transduced with dMLL-AF9 and treated with tamoxifen. (a) A schematic representing the strategy by which the role of Atg5-dependent autophagy was assessed during primary transplantation in MLL-AF9-driven AML. (b) The Kaplan–Meier survival curve of mice transplanted with Atg5WT (n=8) and Atg5FL (n=7) MLL-AF9 cells. The P-value for the log-rank test between the two groups is shown. (c) Genomic DNA extracted from the splenocytes of a representative Atg5WT and six Atg5FL moribund mice from panel (b) were analyzed by PCR for the status of Atg5 alleles. (d) Representative figures of peripheral blood smears stained by May Grünwald–Giemsa (top) and liver section stained by hematoxylin and eosin (bottom) of moribund mice from panel (b). (e) The spleen weight of moribund mice from panel (b) for Atg5WT (n=8) and Atg5FL (n=6) mice. Error bars represent S.E.M. (f) The Annexin V−7-AAD−CD11b+ myeloid cells are shown as percentage of all Annexin V−7-AAD− cells according to flow cytometry in the indicated hematopoietic tissues of moribund mice from panel (b). Error bars represent S.E.M. of six mice.
decreased over time in Atg5 FL mice treated with tamoxifen relative to vehicle-treated mice (Figures 3c and d). Therefore, we concluded that the prolonged survival of mice burdened with AML following primary transplantation is an effect specifically associated with Atg5 deletion.

We examined various hematopoietic progenitor populations in moribund Atg5 FL mice to determine whether LSCs were affected by Atg5 deletion. LSCs were previously characterized to be exclusively within the c-kit+Sca-1- population in this model of MLL-AF9-driven AML, where CD16/32 is highly enriched but CD34 expression is dispensable. Data from our laboratory has shown that the c-kit+Sca-1- population of MLL-AF9-induced AML cells is almost exclusively CD16/32- and CD34- (unpublished observations) and we therefore refer
to these cells as phenotypic LSCs. We observed the number of LSCs to be higher in the BM of tamoxifen-treated animals but not in the spleen (Figure 3e). Other progenitor cell populations were not found to be different between the vehicle- and tamoxifen-treated mice. Atg5 deletion also had no effect on lymphocyte, F4/80+ monocytes, or c-kit+ myeloid cells in the peripheral blood, BM, or spleen at morbitity (Figure 3f). Thus, although in vivo Atg5 deletion during primary transplantation resulted in prolonged survival, leukemic mice lacking Atg5 are similar in disease presentation at morbitity compared with mice with intact Atg5.

**Atg5 ablation increases apoptosis of differentiated malignant myeloid cells.** We sought to confirm that tamoxifen treatment to Atg5FL cells resulted in functional ablation of autophagy in three ways, by PCR, immunoblotting, and immunofluorescence. The status of the Atg5 allele was examined by PCR in primary splenocytes from moribund Atg5FL mice treated with vehicle (Atg5FL+) or tamoxifen (Atg5KO) (Figure 3b) and confirmed that tamoxifen treatment efficiently deleted Atg5 (Figure 4a). In order to determine whether Atg5 deletion resulted in functional inhibition of LC3 lipidation in vitro, we treated both Atg5FL and Atg5KO cells with BafA1 to detect LC3-II by immunoblotting. Under BafA1 treatment, an accumulation of LC3-II was observed in Atg5FL cells but was absent in Atg5KO MLL-AF9 leukemic cells, suggesting that Atg5 is functionally ablated (Figure 4b). We then examined puncta formation by the GFP-LC3 reporter in Atg5FL and Atg5KO MLL-AF9 leukemia cells by fluorescence microscopy. Digtinin treatment prior to fixation facilitates the visualization of GFP-LC3 puncta by releasing cytoplasmic soluble GFP-LC3. Under BafA1 treatment, a significant accumulation of GFPp puncta was observed in Atg5FL cells with digitonin treatment (Figure 4c). In contrast, digitonin-treated Atg5KO cells demonstrated a lack of GFP signal. These three lines of evidence collectively indicate that LC3 lipidation was functionally ablated in Atg5KO cells.

As autophagy is important for the clearance of damaged mitochondria, we tested whether Atg5KO cells have altered mitochondrial respiration. Surprisingly, we did not detect a difference in basal respiration or spare respiratory capacity between Atg5FL and Atg5KO cells (Figure 4d). We detected no difference in the extracellular acidification rate (ECAR) during this assay, suggesting that lactate production might not be changed in Atg5-deficient cells (data not shown). The BM microenvironment is hypoxic and hypoxia is a known inducer of cytoprotective autophagy. Therefore, we therefore assayed whether autophagy contributes to the in vitro proliferation of Atg5FL and Atg5KO AML cells under both normoxic and hypoxic conditions. Interestingly, both Atg5FL and Atg5KO cells proliferated more rapidly under hypoxia through unknown mechanisms (Figure 4e). We observed a marginal but statistically significant delay in the proliferation of Atg5KO cells compared with Atg5FL cells under normoxia with a doubling time of 13.3 and 16.2 h, respectively. However, no significant difference in proliferation was observed under hypoxia with a doubling time of 10.2 and 11.6 h, respectively. Correspondingly, no differences were observed in the cell cycle of in vitro cultured Atg5KO and Atg5KO cells (Figure 4f). We instead noted an increase in the percentage of Sub-G0 cells in Atg5KO cells, indicating that the apoptosis program could be altered in leukemia cells lacking autophagy. Indeed, flow cytometric analysis of cells stained with Annexin V and 7-AAD confirmed that leukemic cells lacking autophagy demonstrated an increased proportion of cells with ongoing apoptosis (Figure 4g).

AML is a hierarchical malignancy originating from LSCs, and a compromise in the viability of LSCs could explain the mechanism by which Atg5 deletion prolongs the survival of mice burdened with MLL-AF9-driven AML during primary transplantation. Surprisingly, only a very marginal decrease in the viability of LSCs was detected for in vitro cultured Atg5KO cells compared with control (Figure 4h). Apoptosis in both c-kit+ and c-kit- myeloid populations was compared between Atg5FL and Atg5KO AML cells and was observed to be enhanced in c-kit+ myeloid cells, which are differentiated cells that lack leukemia-initiating potential (Figure 4h). This data collectively suggest that autophagy-deficient AML cells demonstrate enhanced apoptosis in differentiated malignant leukemia cells originating from LSCs in vitro.

**Atg5 deletion after secondary transplantation does not alter the chemotherapy response of MLL-AF9-driven AML.** The potential of functional autophagy inhibition as a therapeutic strategy has not yet been determined in MLL-AF9-driven AML. We chose to mimic autophagy inhibitor treatment by treating animals with tamoxifen in vivo after transplantation of malignant Atg5FL cells that were not previously exposed to tamoxifen. In this secondary transplant model, no significant difference in survival was observed between vehicle- and tamoxifen-treated mice, with median survivals of 34.5 and 36 days, respectively (Figure 5a). However, a significant decrease in the frequency of c-kit CD11b+ myeloid cells were observed in the peripheral blood of tamoxifen-treated mice during progression (Figure 5c), consistent with changes observed during in vitro culture (Figure 4g).

Autophagy has been postulated as a mechanism of chemotherapy resistance in AML. Therefore, autophagy’s role in chemotherapy resistance was also determined in vivo during secondary transplantation. Following vehicle or tamoxifen treatment, animals bearing malignant AML were administered with either PBS or a chemotherapy regimen with cytarabine and doxorubicin, mimicking the treatment of patients at diagnosis presenting malignant AML. We observed that MLL-AF9-driven AML responded to chemotherapy as both vehicle- and tamoxifen-treated mice receiving chemotherapy demonstrated prolonged survival compared with controls (Figure 5a). Additionally, chemotherapy treatment drastically reduced the WBC counts of animals 1 day following treatment termination (Figure 5b). However, no differences were observed between vehicle- and tamoxifen-treated animals in survival (41.5 and 42 days, respectively), WBCs, or myeloid markers during progression or at end point (Figures 5a–c, data not shown). We confirmed that Atg5FL and Atg5KO malignant AML cells responded similarly to chemotherapy in vitro (Figure 5d, Table 1). These data indicate that autophagy may not contribute to the chemotherapy response of MLL-AF9-driven murine AML.
Several targeted therapies are being investigated for the treatment of AML and we evaluated whether the potency of these agents could be enhanced by autophagy deletion. As autophagy is important for the clearance of mitochondria, we tested whether cells lacking autophagy were sensitized to agents which activate mitochondrial apoptosis. Both Atg5FL and Atg5KO MLL-AF9-driven AML cells cultured in vitro were completely resistant to Bcl-2 and Bcl-XL inhibitors ABT-199...
and ABT-737 (Table 1). On the other hand, Atg5KO leukemic cells were sensitized to maritoclax, a small molecular antagonist of Mcl-1. We also observed a significant sensitization of Atg5KO leukemic cells to vorinostat, a histone deacetylase inhibitor, similar to previous findings.

**Discussion**

Numerous studies have now described autophagy as essential for hematopoietic homeostasis, and several have suggested an important role for autophagy in myeloid differentiation or proliferation. Studies have revealed that both human and mouse HSCs demonstrate high levels of autophagic flux. Indeed, the deletion of key autophagy protein FIP200 in mouse hematopoietic cells abrogated the self-renewal of fetal HSCs, leading to severe anemia and perinatal lethality. Similarly, knocking down ATG5 and ATG7 function in human CD34+CD38− HSCs also drastically reduced their frequency, and deletion of Atg5 or Atg7 in...
Studies have shown that mice lacking Atg5 or Atg7 LC3 lipidation could have a compensatory role in this model of First, it is possible that autophagy pathways independent of but not to progression could be due to several possibilities. contribution of Atg5-dependent autophagy to AML initiation in the viability of LSCs or maintenance of AML during autophagy in leukemia initiation, autophagy was not involved with deleted Atg5 relative to controls during primary trans- survival advantage in vivo on oncogene (Figure 1g) and observed a statistically significant of BM cells immediately following introduction of the MLL-AF9 detected a transient decrease in the colony-forming potential capacity of HSCs immediately following introduction of the MLL-AF9 oncogene (Figure 1g) and observed a statistically significant survival advantage in vivo for mice harboring leukemia cells with deleted Atg5 relative to controls during primary trans-plantation (Figures 2 and 3).

Although we clearly demonstrate the contributions of autophagy in leukemia initiation, autophagy was not involved in the viability of LSCs or maintenance of AML during secondary transplantation (Figures 4h and 5a–c). The specific contribution of Atg5-dependent autophagy to AML initiation but not to progression could be due to several possibilities. First, it is possible that autophagy pathways independent of LC3 lipidation could have a compensatory role in this model of AML. Studies have shown that mice lacking Atg5 or Atg7 in HSCs demonstrate temporary myelomonocytic proliferation with aberrant maturation. In this model of MLL-AF9-driven AML, LSCs resemble immature myelomonocyes. A separate study has already suggested that myeloid cells might rely on Atg5-independent autophagy for survival and differentiation. Second, MLL-AF9 expression might attenuate autophagy’s role as a tumor suppressor by inactivating p53. MLL fusion oncogenes have previously been described to functionally suppress p53. In pancreatic tumors, autophagy’s role in suppressing malignant transformation has been linked to p53 function. Abrogating p53 resulted in a metabolic shift within tumors, reprogramming autophagy to a pro-tumorigenic role.

To our knowledge, we are the first to describe a role of Atg5-dependent autophagy in the initiation of MLL-AF9-driven AML. This is in some contrast to a recent study by Watson et al., who report an important role for Atg5 in murine MLL-ENL-driven AML. The in vitro homozgous deletion of Atg5 following introduction of MLL-ENL oncogene led to significant cell death, suggesting that functional autophagy is essential to maintain MLL-ENL-driven LSCs. In contrast, in our model using MLL-AF9-driven leukemia with homozygous Atg5 deletion, we detected a transient decrease in colony-formation potential during leukemia initiation and no significant LSC cell death hereafter (Figures 1g and 4h). Apoptosis was increased in differentiated myeloid cells originating from malignant LSCs lacking Atg5 (Figures 4h and 5c). Nonetheless, this difference in differentiated myeloid cells was not reflected at end point in any of our studies and did not correlate with survival. Thus the role of autophagy in the survival of differentiated myeloid cells originating from LSCs is unlikely related to its roles in the development of MLL-AF9-driven AML. Watson et al. further observed that heterozygous deletion of Atg5 facilitated AML initiation and development. In these cells, increased in vitro and in vivo proliferation of autophagy knockdown cells was accompanied by increased glycolysis that is likely due to reprogrammed mitochondrial function, evidenced by increased mitochondrial spare capacity. Although autophagy did have a role in the initiation of AML in our model, we did not observe a difference in the mitochondrial spare capacity or ECAR of malignant MLL-AF9 cells with homozygously deleted Atg5 (Figure 4d). Thus it is unlikely that Atg5 deletion reprogrammed mitochondrial respiration in this MLL-AF9-driven model of murine AML as it did in MLL-ENL-driven AML.

Autophagy is known to participate in treatment response of AML but its roles are controversial. Autophagy is traditionally implicated in therapy resistance in leukemia, as suppressing autophagy might lead to mitochondrial dysfunction and reactive oxygen species (ROS) production to enhance treatment response. On the other hand, several studies have shown that autophagy induction enhances treatment response. One proposed mechanism by which autophagy induction could enhance therapeutic response involves the apparent ability of autophagy to degrade key pro-tumorigenic proteins, including Flt3, PML-RARA, and Bcl-Abl. In our murine model of MLL-AF9-driven AML, Atg5-dependent autophagy was dispensable for in vitro and in vivo treatment response to the chemotherapeutics cytarabine and doxorubicin (Figure 5). However, AML cells lacking Atg5 were sensitized to maritoclax and vorinostat in vitro (Table 1). Autophagy deficiency has previously been described to enhance vorinostat sensitivity through ROS production. However, cytarabine and maritoclax have also been described to facilitate ROS production to induce cell death, making this an unlikely mechanism by which autophagy protects against maritoclax and vorinostat cytotoxicity. AML cells have previously shown to be dependent on Mcl-1 for survival, and it is possible that Atg5-deficient AML cells demonstrate increased Mcl-1 dependency, an idea which could be further explored.

Taken together, our study provides insight as to the role of Atg5-dependent autophagy in the development of MLL-AF9-driven murine AML. The in vitro deletion of Atg5 transiently decreased the colony-forming capacity of BM cells expressing MLL-AF9, consistent with in vivo data where Atg5 deletion significantly delayed MLL-AF9-induced AML initiation. Atg5 deficiency in malignant AML cells was also observed to promote apoptosis in differentiated malignant myeloid cells. Conversely, Atg5-mediated autophagy was not involved in the maintenance or chemotherapeutic sensitivity of malignant AML. The role of autophagy in the pathogenesis of AML remains for further investigation in order to determine whether and how autophagy should be modulated in AML for therapeutic benefit.
Materials and Methods

Animal studies. All animal studies were approved and followed the Penn State College of Medicine IACUC guidelines. C57BL/6J and B6.129-G/R(ROSA)26Sgc(foxP3ERT2(Ds)R)56 mice were purchased from Jackson Laboratories (Sacramento, CA, USA), and the B6.1295-Agt56 mice strain was obtained from RIKEN Bioresource Center, Ibaraki, Japan. All animals were bred at the Penn State College of Medicine and genotyped as previously described. 56-57 Age- and sex-matched animals were used for all animal studies, and all studies were carried out without blinding. Sample sizes were chosen by simulation to ensure adequate power to detect a median survival difference of 5 days.

Primary transplantation studies were largely performed as previously described. 5 Briefly, magnetically sorted (Miltenyi no. 130-091-224; Bergisch Gladbach, Germany) c-kit+ BM cells were spinoculated with concentrated Ecotropic retrovirus with 8 μg/ml polybrene at 1400 x g for 2 h at 32 °C once per day for 2 days. Cells were then seeded to methycellulose medium (Stemcell Technologies no. 03334; Vancouver, British Columbia, Canada) at 1 x 10^5 cells/ml for three passages of 5 days each. The cells were next transplanted intraperitoneally to 500 gY sublethally irradiated recipient mice under ketamine/xylazine anesthesia at 5 x 10^7 cells per animal. When applicable, animals were randomized to groups based on transplantation time. Animals suffering from labored breathing, lethargy, or any other signs of morbidity were defined as end point and killed by CO2 asphyxiation followed by cervical dislocation and necropsy.

For secondary transplantation, primary splenocytes from primary transplantation leukemia mice were incubated with red blood cell (RBC) lysis buffer (15.5 mM NH4Cl, 1.2 mM NaHCO3, 10 μM Na2EDTA, pH 7.2) for 15 min at 4 °C. Splenocytes were then washed twice and cryopreserved in 90% FBS and 10% DMSO in liquid nitrogen. Splenocytes from at least three different mice were thawed and pooled. Live cells were isolated by centrifugation with Histopaque-1119 (Sigma-Aldrich T5648; St. Louis, MO, USA) at 400 x g for 30 min at 4 °C. In all, 200 000 cells were then intrafetorally transplanted to non-irradiated recipient mice.

In vivo luminescence imaging was performed using the IVIS Lumina Series III (PerkinElmer; Waltham, MA, USA). Animals were intrafetorally injected with 150 mg/kg of D-luciferin in PBS and imaged under isoflurane anesthesia. Images were normalized with Living Image V4.1 (PerkinElmer), and the total flux (p/s) of the whole animal was used for quantification.

Drug treatment. Free-base tamoxifen (Sigma-Aldrich T5648) was prepared at 20 mg/ml and administered by oral gavage at 200 mg/kg as previously described. 58 Free-base tamoxifen (Sigma-Aldrich T5648) was prepared at 20 mg/ml and administered by oral gavage at 200 mg/kg as previously described. 58 Drug treatment.

Plasmids and cell culture. The pMSCV-luc-ires-YFP plasmid was received from Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). The construction of the pMSCV-MLL-AF9-αEF1-πFP-2GA-FPC-LC3 plasmid was carried out by replacing the luc-ires-YFP open reading frame in the pMSCV-luc-ires-YFP vector with the following components: ML-AF9 from pMIG-ML-AF9 (Dr. Robert Paulson, Penn State University Park, PA, USA); EF1α from pCDH- MCS-CES1-Puro (System Biosciences CD510B; Palo Alto, CA, USA); Luciferase from pMSCV-luc-ires-YFP; P2A from pULTRA (Dr. Malcolm Moore, Memorial Sloan Kettering, Addgene (Cambridge, MA, USA) no. 24129); GFP-LC3 from pMXs-VP-EGFP-LC3 (Dr. Noboru Mizushima, University of Tokyo, Tokyo, Japan).

The Atgs75–77 and Atgs1–23 mouse embryonic fibroblasts were kindly provided by Dr Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan) and cultured in DMEM medium with 20% FBS and 1% antibiotic-antimycotic solution (Mediatech, Manassas, VA, USA). Primary murine leukemia and hematopoietic cells were cultured in IMDM supplemented with 20% FBS, 1% antibiotic-antimycotic solution (Mediatech), 50 ng/ml SCF (PeproTech 250-03; Rocky Hill, NJ, USA), 20 ng/ml IL-3 (PeproTech 213-13), and 10 ng/ml IL-6 (PeproTech 216-16) and maintained between 0.2 and 1 x 10^6 cells/ml unless otherwise stated. For luminescence studies, luminescence was measured immediately after adding 75 μg/ml D-luciferin to cell culture on the BMG ClarioStar (BMG Labtech; Ortenberg, Germany).

Coloncy-formation assay. Cells were seeded at 500 viable cells/ml, according to the trypan blue exclusion assay, to methycellulose medium (Stemcell Technologies no. 03334) and cultured for 6 days before manual counting of colonies under light microscopy. For serial plating, cells were washed twice before re-seeding at 500 viable cells/ml to fresh methycellulose medium. For 4OH-T treatment, 100 μM 4OH-T or 0.1% ethanol (vehicle) was added to methycellulose medium before cells were seeded.

PCR. Genomic DNA was extracted from primary splenocytes using the DNeasy Blood and Tissue Kit (Qiagen no. 69504; Hilden, Germany) according to the manufacturer’s recommendations. PCR was then performed with 10 ng of genomic DNA, Perfect Taq Plus MasterMix (SPRI no. 2200095; Qiaogen, MD, USA) and previously published primers 57 to detect wild-type, floxed, and deleted Atg5 alleles.

Histology. Tissues were fixed in 10% neutral-buffered formalin for 24 h and then stored in 70% ethanol. Soft tissues were mounted in paraffin, sectioned, and stained by hematoxylin and eosin. Peripheral blood smears were fixed with methanol for 1 min. May Grünwald-Giemsa stain was performed using the May Grünwald solution (Sigma-Aldrich 32826) and the modified Giemsa stain (Sigma-Aldrich GS1L) according to the manufacturer’s recommendations.

Flow cytometry. Peripheral blood was collected by cardiac puncture from moribund mice during necropsy. BM was flushed from the femur and tibia with a 27-gauge needle, and the spleen was dissociated through 40 μm nylon mesh. For live animals, peripheral blood was collected by saphenous vein puncture into the Safe-T-Fill EDTA capillary blood collection system (RAM Scientific no. 077051; Yönkks, NY, USA). RBCs were lysed in RBC lysis buffer for 15 min on ice for spleen and BM or at room temperature for 20 min for peripheral blood. Cells were washed and blocked with either 2% unlabeled mouse CD16/32 (BioLegend no. 101326; San Diego, CA, USA) or 0.5% of BV711-CD16/32 (BioLegend no. 101337) in flow cytometric buffer (PBS, 2% FBS, 100 μM EDTA, 0.1% sodium azide) for 10 min on ice. Cells were then labeled with fluorochrome-conjugated antibodies listed below for 15 min on ice. Cells were washed twice with flow cytometric buffer and fixed with Fixation Buffer (BioLegend no. 420801) on ice for 20 min. Cells were washed twice and analyzed immediately on the BD Fortessa flow cytometer (BD, Franklin Lakes, NJ, USA) or stored in 90% FBS and 10% DMSO at –80 °C for later analysis.

The following antibodies were used at the indicated concentrations in this study: Annexin V/FTTC, 1:50 (BD no. 556423); 7-AAD, 1:20 (BD no. 559925); CD11b/BDV711, 1:200 (BioLegend no. 101241; San Diego, CA, USA); Gr-1/BDV785, 1:200 (BioLegend no. 101241); CD19/BDV650, 1:100 (BioLegend no. 115541); Gr-1/AF700, 1:200 (BioLegend no. 108422); TER-119/AC-Py-C7, 1:200 (BD no. 560509); CD3/BDV785, 1:200 (BioLegend no. 100231); c-kit/PE, 1:200 (BioLegend no. 105807); CD45/BV421, 1:50 (BioLegend 103133); Lineage Cocktail/V450, 1:200 (BD no. 561301); CD48/PE-Cy7, 1:200 (BD no. 560731); Sca-1/APC, 1:200 (BioLegend no. 100111); CD150/BV605, 1:200 (BioLegend); and CD34-AF700, 1:50 (BD no. 560518).

For cell cycle analysis, cells were fixed with 70% ethanol overnight at 4 °C. Cells were then washed twice in flow cytometric buffer and resuspended in flow cytometry buffer with 50 μg/ml propidium iodide (BioLegend no. 421301) and 20 μg/ml RNase A (Qiagen no. 19101) for flow cytometric analysis.

Immunofluorescence microscopy. Exponentially growing cells were prepared on microscope slides through the CytoSpin 4 Cyto centrifuge (Thermo Scientific, Leeesport, PA, USA) at 100 000 cells/well. Slides with digestion treatment were incubated with 250 μg/ml diglotion for 2 min and washed with PBS. Slides were fixed in 4% paraformaldehyde in PBS at room temperature for 20 min and washed three times with PBS. Slides were then mounted in Prolong Gold mounting medium with DAPI (Thermo Scientific no. P36841), and immunofluorescence microscopy was performed as previously described. 59

Mitochondrial stress test. A total of 100 000 cells were seeded in XF medium supplemented with 4.5 g/l glucose and 2 mM l-glutamate to the XF96 cell culture plate coated with Cell-Tak (Corning no. 354240, Manassas, VA, USA) according to the manufacturer’s recommendations. The assay was run on the XF96e Flux Analyzer (Seahorse, Santa Clara, CA, USA) with 1 μM of the indicated
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compounds added to the cells from the XF CellMito Stress Test Kit (Seahorse) according to the manufacturer's recommendations.

Cell proliferation assay. In all, 10,000 cells were seeded per well to 96-well plates in 100 μl of IMDM supplemented with 20% FBS, 1% antibiotic–antimycotic solution, 50 ng/ml SCF, 20 ng/ml IL-3, and 10 ng/ml IL-6. Cells under hypoxia treatment were incubated in the humidified Invitrogen 302 Hypoxia Workstation (Baker Ruskinn, Pencoed, UK) at 1.8% O₂, 5% CO₂, and 37 °C. At the indicated time points, 10 μl of PrestoBlue reagent (Thermo Scientific no. A13262) was added and incubated for 1 h at 37 °C. Fluorescence was measured according to the manufacturer's recommendations on the BMG ClarioStar.

Statistical analysis. Sample sizes were estimated with power calculations. All statistics were performed as indicated by GraphPad Prism (version 6.00 for Windows, GraphPad Software, La Jolla CA, USA).

Conflict of Interest

The authors declare no conflict of interest.

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1. Döhner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. N Engl J Med 2015; 373: 1136–1152.
2. Somerville TCP, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. Cancer Cell 2006; 10: 257–268.
3. Barabé F, Kennedy JA, Hope KJ, Dick JE. Modeling the initiation and progression of human acute myeloid leukemia. Nat Rev Cancer 2010; 10: 594–606.
4. Wang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Genes Dev 2013; 27: 1022–1046.
5. Mortensen M, Soilleux EJ, Djordjevic G, Tripp R, Lutteropp M, Sadighi-Akha E et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Cell 2016; 165: 227–247.
44. Rosenfeldt MT, O’Prey J, Morton JP, Nixon C, MacKay G, Mrowinska A et al. P53 status determines the role of autophagy in pancreatic tumour development. Nature 2013; 504: 296–300.

45. Evangelisti C, Evangelisti C, Chianni F, Lonetti A, Buontempo F, Neri LM et al. Autophagy in acute leukemias: a double-edged sword with important therapeutic implications. Biochim Biophys Acta 2015; 1853: 14–25.

46. Liu L, Yang M, Kang R, Wang Z, Zhao Y, Yu Y et al. HMGB1-induced autophagy promotes chemotherapy resistance in leukemia cells. Leukemia 2011; 25: 23–31.

47. Piya S, Kornblau SM, Ruvolo VR, Mu H, Ruvolo PP, McQueen T et al. Atg7 suppression enhances chemotherapeutic agent sensitivity and overcomes stroma-mediated chemoresistance in acute myeloid leukemia. Blood 2016 (e-pub ahead of print).

48. Nahimana A, Attinger A, Aubry D, Greaney P, Ireson C, Thougaard AV et al. The NAD biosynthesis inhibitor APO866 has potent antitumor activity against hematologic malignancies. Blood 2009; 113: 3276–3286.

49. Larrue C, Saland E, Boutzen H, Vergez F, David M, Joffre C et al. Proteasome inhibitors induce FLT3-ITD degradation through autophagy in AML cells. Blood 2016; 127: 882–892.

50. Elzinga BM, Nyhan MJ, Crowley LC, O’Donovan TR, Cahill MR, McKenna SL. Induction of autophagy by Imatinib sequesters Bcr-Abl in autophagosomes and down-regulates Bcr-Abl protein. Am J Hematol 2013; 88: 455–462.

51. Wang Z, Cao L, Kang R, Yang M, Liu L, Zhao Y et al. Autophagy regulates myeloid cell differentiation by p62/SQSTM1-mediated degradation of PML-RARα oncoprotein. Autophagy 2011; 7: 401–411.

52. Isakson P, Bjaras M, Boe SO, Simonsen A. Autophagy contributes to therapy-induced degradation of the PML/RARA oncoprotein. Blood 2010; 116: 2324–2331.

53. Mates JM, Segura JA, Alonso FJ, Márquez J. Oxidative stress in apoptosis and cancer: an update. Arch Toxicol 2012; 86: 1649–1665.

54. Bossis G, Sarry J-E, Kifagi C, Ristic M, Saland E, Vengez F et al. The ROS/SUMO axis contributes to the response of acute myeloid leukemia cells to chemotherapeutic drugs. Cell Rep 2014; 7: 1815–1823.

55. Glaser SP, Lee EF, Trounson A, Boultet P, Wei A, Fairlie WD et al. Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. Genes Dev 2012; 26: 120–125.

56. Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L et al. Restoration of p53 function leads to tumour regression in vivo. Nature 2007; 445: 661–665.

57. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Mgishima R et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 2006; 441: 885–889.

58. Feil S, Valtecheva N, Feil R. Inducible Cre mice. Methods Mol Biol 2009; 503: 343–363.

59. Young MM, Takahashi Y, Khan O, Park S, Hori T, Yun J et al. Autophagosomal membrane serves as platform for intracellular death-inducing signaling complex (DISC)-mediated caspase-8 activation and apoptosis. J Biol Chem 2012; 287: 12455–12468.

60. Lou E, Fujisawa S, Morozov A, Barlas A, Romin Y, Dogan Y et al. Tunneling nanotubes provide a unique conduit for intercellular transfer of cellular contents in human malignant pleural mesothelioma. PLoS One 2012; 7: e33093.

61. Hara T, Takamura A, Kishi C, Iemura S, Natsume T, Guan J-L et al. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. J Cell Biol 2008; 181: 497–510.