Tissue-specific Autophagy Alterations and Increased Tumorigenesis in Mice Deficient in Atg4C/Autophagin-3*

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Atg4C/autophagin-3 is a member of a family of cysteine proteinases proposed to be involved in the processing and delipidation of the mammalian orthologues of yeast Atg8, an essential component of an ubiquitin-like modification system required for execution of autophagy. To date, the in vivo role of the different members of this family of proteinases proposed to be involved in the processing and delipidation of the mammalian orthologues of yeast Atg8, an essential component of an ubiquitin-like modification system required for execution of autophagy. To date, the in vivo role of the different members of this family of proteinases remains unclear.

This article has been withdrawn by the authors upon request from the Journal. The Journal raised questions regarding Fig.1C. In Fig. 1C, actin lanes appeared to be duplicated, and the original data for this panel were not available. The authors state that a new experiment was performed using RNA from mouse tissues in WT and Atg4C-deficient animals. The authors assert that all of the results reported in this article are valid, some of which are supported by a subsequent report by another group (Li et al. (2011) J. Biol. Chem. 286, 7327).

The mechanisms responsible for delivering cytoplasmic cargo to the lysosome are known collectively as autophagy and play an important role in the maintenance of cell homeostasis (1–4). This process has been observed in all eukaryotic cells, indicating the widespread occurrence of this evolutionarily conserved pathway. Autophagy can be classified into at least three different pathways: that is, macroautophagy, microautophagy, and chaperone-mediated autophagy (4, 5). Macroautophagy is the major lysosomal route for the turnover of cytoplasmic components and will hereafter be referred as autophagy. This process begins with a sequestration event consisting of an engulfment of cytoplasmic constituents by a membrane sac, called the isolation membrane. This structure results in a double-membrane vesicle called the autophagosome, containing bulk portions of cytoplasm, which eventually fuses with the lysosome. Finally, the inner membrane of the autophagosome is degraded by lysosomal hydrolases and recycled.

The knowledge of the molecular mechanisms underlying autophagy is crucial for the understanding and treatment of many human diseases such as tumors. Several studies have shown that these ubiquitin-like conjugation systems associated with autophagy are conserved in higher eukaryotes (10, 15–18). Thus, proteins structurally and functionally related to the diverse yeast Atg proteins have been described in mammalian cells, and their roles in the process of autophagy have been elucidated in some cases (15, 16, 19). Recently we have described and cloned the four human orthologues of the yeast proteinase Atg4 (20). These proteins are members of the C-54 family of cysteine proteinases and maintain a significant sequence similarity with yeast Atg4. Human Atg4 orthologues also exhibit the structural features characteristic of the yeast proteinase including the catalytic Cys residue and its surrounding amino acid sequences. All of these structural features are also absolutely conserved in the mammalian orthologues of yeast Atg8, an essential component of an ubiquitin-like modification system required for execution of autophagy. To date, the in vivo role of the different members of this family of proteinases remains unclear.

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The finding that the above-mentioned ubiquitin-like system is composed of four proteinases that may target at least six distinct substrates in mammals (LC3A, LC3B, LC3C, GATE-16, GABARAP, and ATG8L) (16, 21, 22) contrasts with the simplified yeast system involving a single proteinase with a specific substrate and indicates that this conjugation system has evolved to acquire a larger complexity during eukaryote evolution. Several works have shed some light on the involvement of Atg4 orthologues in the C-terminal processing and phosphatidylethanolamine conjugation of some of the Atg8 mammalian orthologues (22–24). However, to date the in vivo role of the different members of this family of cysteine proteinases remains to be characterized. Similarly, it is unclear if the complexity of mammalian Atg4 orthologues and their putative substrates simply derives from functional redundancy in this system, as described for other protease families (25, 26), or by contrast it corresponds to a different scenario in which some components of this proteolytic system have evolved to accomplish other functions distinct from autophagy.

Among the four mammalian Atg4 orthologues, Atg4C is the most widely distributed in human tissues (20). On this basis together with the fact that human Atg4C is able to complement the deficiency of Atg4 in yeast, studies were undertaken to generate a murine model defective for the Atg4C gene. In this work, we report the generation and phenotype analysis of mice deficient in this member of the Atg4 family of cysteine proteinases. We show that these mice exhibit a decrease in autophagy in response to starvation, the main autophagy proteinases. We show that these mice exhibit a tissue-specific decrease in autophagy in response to starvation in vivo. In addition, they also show a decreased locomotion that could be correlated with a decrease in autophagic flux. The Atg4C deficiency leads to a high incidence of fibrosarcomas induced by chemical carcinogens. Based on these results, we conclude that the Atg4C enzyme is not essential for a proper autophagic flux under normal circumstances but is required for an appropriate response to prolonged starvation in vivo. Finally, we propose that Atg4C could be involved in events associated with tumor progression.

**EXPERIMENTAL PROCEDURES**

**Targeting Vector Construction**—A genomic DNA clone was isolated from a mouse 129-SV/J genomic DNA library (Stratagene, La Jolla, CA), using a murine Atg4C cDNA fragment as probe. The genomic organization was determined by restriction analysis and subsequent subcloning of these fragments into pBluescript or pUC18. Plasmid pPN2T-Hgterm (kindly provided by Dr. C. Paszty, Lawrence Berkeley National Laboratory, Berkeley, CA) containing the pgk-neo and two pgk-tk (thymidine kinase) selection markers, was used to construct the Atg4C targeting vector. A 3.4-kb HindIII-BamHI fragment from the 5′-flanking region was used as the 5′-homologous region, whereas a 4.7-kb EcoRI-HincII fragment containing exons 5 and 6 was used as the 3′-region of homology. The 2.4-kb neo cassette was used as a positive marker and replaced a 10-kb fragment containing exons 1–4 of the Atg4C gene (Fig. 1A).

**Generation of Atg4C-deficient Mice**—The targeting vector was linearized by digestion with NotI, electroporated into HM1 embryonic stem cells, and selected for homologous recombination with G418 and ganciclovir. Positive clones were screened by Southern blot after PstI digestion of genomic DNA and probed with a radiolabeled 5′-external probe (Fig. 1B). A 12-kb fragment was detected from the wild-type allele, and an 8-kb fragment was detected from the corresponding mutant allele. The targeted embryonic stem cell clones were expanded and subsequently injected into blastocysts to generate chimeras. Chimeric males were mated with C57BL/6 female mice, and the offspring heterozygous for Atg4C were used to generate homozygous null mice. In all experiments homozygous Atg4C−/− mice and their corresponding wild-type mice were littermates derived from interbreeding of heterozygotes with a mixed background of C57Bl6/129Sv. In all cases mice genotypes were determined by Southern blot analysis of tail DNA.

**RT-PCR**—Total RNA was isolated from mouse tissues according to the method of Chomczynski and Sacchi (27). About half of the obtained product was transcribed using the RNA-PCR Core kit from SuperScript II. A PCR reaction was then performed with specific primers for 25 cycles of denaturation (62 °C, 20 s), annealing (62 °C, 20 s), and extension (72 °C, 20 s). As a control, 18S ribosomal RNA served as the internal standard for each amplification. 

**Western Blot Analysis**—Mice tissues were immediately frozen in liquid nitrogen after extraction and were homogenized in a 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and Complete protease inhibitor mixture (Roche Applied Science). Once homogenized, tissue samples were centrifuged at 12,000 rpm at 4 °C, and supernatants were collected. The protein concentration of the supernatant was evaluated by bicinchoninic acid technique (BCA protein assay kit, Pierce). 25 μg of protein sample was loaded on 13% SDS-polyacrylamide gels. After electrophoresis, gels were electrotransferred onto nitrocellulose filters, and then the filters were blocked with 5% nonfat dried milk in PBT (phosphate-buffered saline with 0.05% Tween 20) and incubated with primary antibodies in 5% bovine serum albumin in PBT. After three washes with PBT, filters were incubated for horseradish peroxidase-conjugated goat anti-rabbit IgG at a 1:10,000 dilution in 1.5% milk in PBT and developed with a West Pico enhanced chemiluminescence kit (Pierce). The antibodies against Atg4C, GATE-16, GABARAP, and LC3 were kindly provided by Dr. T. Ueno (Juntendo University, Tokyo). The antibodies against Atg4A, Atg4B, and Atg4D were from Abgene (Manchester, UK).

**Northern Blot Analysis**—Nylon filters containing 2 μg of poly(A) RNA of a wide variety of murine tissues were prehybridized at 42 °C for 3 h in 50% formamide, 5× saline/sodium phosphate/EDTA (1× saline/sodium phosphate/EDTA, 150 mM NaCl, 10 mM Na2HPO4, 1 mM EDTA, pH 7.4), 10× Denhardt’s solution, 2% SDS, and 100 μg/ml denatured herring 2

2 The abbreviations used are: RT, reverse transcription; GFP, green fluorescent protein; MCA, methylcholanthrene; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; GABARAP, γ-aminobutyric acid receptor-associated protein.
sperm DNA. Filters were then hybridized with radiolabeled probes for each full-length clone cDNA. Hybridization was performed for 20 h under the same conditions. Filters were washed with 0.1× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS for 2 h at 50 °C, and exposed to autoradiography. RNA integrity and equal loading were assessed by hybridization with an actin probe.

**Quantitative Real-time PCR**—Total RNA was extracted from mouse tissues using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized using 1–5 μg of total RNA, 0.14 mM oligo(dT) (22-mer) primer, 0.2 mM concentrations of each deoxynucleoside triphosphate, and Superscript II reverse transcriptase (Invitrogen). Quantitative reverse transcription-PCR was carried out in triplicate for each sample using 20 ng of cDNA, TaqMan® Universal PCR master mix (Applied Biosystems, San Francisco, CA), and 1 μl specific TaqMan® custom gene expression assay for Atg4A, Atg4C, and Atg4D (Applied Biosystems). To quantitate gene expression, PCR was performed at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s using an ABI Prism 7700 sequence detector system. As an internal control for the amount of template cDNA used,
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A.

B.

C.

[Graphs and images showing autophagy alterations and tumorogenesis in Atg4C\textsuperscript{+/−} mice with comparisons under different conditions.]
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gene expression was normalized to the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using the Mouse GAPD (GAPDH) Endogenous Control (VIC®/MGB Probe, Primer Ltd) TaqMan® Gene expression assay (Applied Biosystems). Relative expression of the distinct Atg4 genes was calculated according to manufacturer’s instructions. Briefly, Atg4A, Atg4B, Atg4C, and Atg4D expression was normalized to glyceraldehyde-3-phosphate dehydrogenase in wild-type or Atg4C−/− derived samples using the following formula: the mean values of 2−ΔΔCt gene (Atg4A, Atg4B, Atg4C, or Atg4D)−ΔCt gene (GAPDH) for three different wild-type animals were considered 100% for each Atg4 gene, and the same values for Atg4C−/− mice tissues were referred to those values according to Livak and Schmittgen (28).

Quantitative Analysis of GFP-LC3 Dots—Mutant mice deficient in Atg4C were crossed with transgenic mice overexpressing GFP-LC3 that provide an efficient in vivo marker for autophagy (29). To avoid autophagy induction, mice were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4. Tissues were harvested and further fixed with the same fixative solution for at least 4 h followed by treatment with 15% sucrose in PBS for 4 h, and then with 30% sucrose solution overnight. Tissue samples were embedded in Tissue-Tek OCT compound (Sakura Finetech Co. Ltd., Tokyo, Japan) and stored at −70 °C. Samples were then sectioned at 5-µm thickness with cryostat (CM3050 S, Leica, Deerfield, IL), air-dried for 1 h, washed in PBS for 5 min, dried at a ture for 30 min, and mounted with coverslips. The number of GFP-LC3 dots in 10 independent visual fields from at least five independent mice in each organ using a Leica TCS SP2 AOBS confocal fluorescence microscope.

Measurement of Locomotor Activity of Mice—To monitor the locomotor activity of mice, a sex-matched group of mice was housed in plastic cages inside the actimeter (Activity Monitor, Letica, Spain) and placed alone in the actimeter. Mice were washed once with ethanol, washed with PBS, and triturated with razor blades. Samples were then incubated with 600 ml of 4 mg/ml collagenase D (Roche Applied Science) and 4 mg/ml dispase II (Roche Applied Science) in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) for 45 min at 37 °C and 5% CO2. After filtering and washing, 6 ml of DMEM with 10% fetal bovine serum (Invitrogen), and 1% antimycotic-antibiotic (Invitrogen) were added, and the mixture was incubated at 37 °C and 5% CO2. Once extracted, cells were cultured in DMEM containing 10% fetal bovine serum at 37 °C and 5% CO2. Protein Degradation Assays—To label proteins, adult murine fibroblasts were incubated at 37 °C for 48 h in complete fresh DMEM (Sigma) containing 0.1% (v/v) fetal bovine serum (Invitrogen) and with 5 µCi/ml [3H]-lysine (Perkin Elmer, Woburn, MA). Before the proteolysis experiments were started, cells were washed once with DMEM lacking fetal bovine serum, 1% antimycotic-antibiotic (Invitrogen) were added, and the mixture was incubated at 37 °C for 24 h in DMEM containing 1% fetal bovine serum, 1% antimycotic-antibiotic (Invitrogen) and 50 µM 3-methyladenine to specifically inhibit autophagic degradation. Protein degradation was analyzed 1 h after the addition of the 3-methyladenine and for an additional period of only 3 h to ensure optimal inhibition and to avoid possible secondary effects of the inhibitor. All experiments were performed at least four times with duplicate samples and using independent cell lines.

Statistical Analysis—All experimental data are reported as the mean, and the error bars represent the experimental S.E. Statistical analysis were performed by the non-parametric Student’s t test excepting for MCA-tumor induction statistical analysis, which was evaluated using the Kaplan-Meier method (31) and compared with the log-rank test. Statistical analyses were made using the Prism program Version 4.0 (GraphPad Software, Inc).

RESULTS

Generation of Atg4C-deficient Mice—To examine the in vivo function of Atg4C, we generated mice with a targeted mutation in the Atg4C gene. A genomic clone encoding Atg4C was obtained from a mouse 129-SV/J genomic DNA library and

FIGURE 3. Analysis of LC3, GATE-16, and GABARAP status in Atg4C−/− mice. A, wild-type and Atg4C−/− mice of 12 weeks of age were fed ad libitum or starved for 24 h and then sacrificed. A variety of tissues from these mice were extracted and homogenized as described under “Experimental Procedures.” Panels show representative immunoblots against LC3, GATE-16, and GABARAP in multiple tissues from wild-type and Atg4C−/− mice. LC3-I (19 kDa), GATE16-I (17 kDa), and GABARAP-I (16 kDa) stand for the cytosolic forms of these proteins, whereas LC3-II (16 kDa) and GATE-16-II (15 kDa) stand for the lipidated forms of these proteins. The asterisk stands for a nonspecific band. B, densitometry analysis of the immunoblots against LC3 and GATE-16 in tissues from Atg4C−/− and Atg4C−/− mice. Bars represent the lipidation status of GATE-16 and LC3. At least four Atg4C−/− and 4 Atg4C−/− mice were used for this experiment. In the case of heart and diaphragm tissues, 12 Atg4C−/− and 12 Atg4C−/− mice were used. The asterisk indicates differences found to be statistically significant at p < 0.05. C, expression analysis of Atg4A, Atg4B, Atg4C, and Atg4D in diaphragms from wild-type and Atg4C−/− mice. The transcript expression levels are relative to Atg4C expression level, which was set at 100%.

WITHDRAWN
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A. no starvation 24 h starvation

Atg4C\(^{+/+}\)

Atg4C\(^{-/-}\)

B. no starvation

Atg4C\(^{+/+}\)

Atg4C\(^{-/-}\)

C. no starvation 24 h starvation

Atg4C\(^{+/+}\)

Atg4C\(^{-/-}\)

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WITHDRAWN

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used for construction of the targeting vector (Fig. 1A). This vector was designed to allow the replacement of exons 1, 2, 3, and 4 of the endogenous Atg4C gene with a neo cassette (Fig. 1A). The linearized targeting vector was electroporated into HM-1 embryonic stem cells, and 4 clones that were positive for homologous recombination were used to generate chimeric founder mice. Heterozygous mice from the F1 generation were used to identify Southern blot analysis and then crossed to obtain Atg4C−/− mice (Fig. 1B). RT-PCR analysis of total RNA from diverse tissues of wild-type and knock-out animals revealed that the Atg4C transcript was absent in Atg4C−/− mice (Fig. 1C). The same negative results were obtained by immunoblotting of these tissues (Fig. 1D), confirming the generation of an Atg4C-null allele.

Normal Development and Growth of Atg4C−/− Mice—Despite the Atg4C deficiency, mutant mice developed normally with males and females being fertile. Likewise, there were no gross detectable differences between the growth curves of wild-type and knock-out mice. In addition, the mutant mice showed no overt phenotype, and their long-term survival rates were indistinguishable from those of their wild-type littermates. Histological analysis of diverse tissues from adult Atg4C−/− animals revealed no observable differences with wild-type tissues (data not shown). Similarly, plasma levels of amino acids and major biochemical parameters (including glucose, cholesterol, triglycerides, uric acid, creatine kinase, aldosterone, and hepatic aminotransferases) were similar to those of wild-type littermates. Taken together, these findings suggest that Atg4C is dispensable for embryonic and adult mouse development as well as for normal physiological functions.

The above findings showing that the Atg4C-deficient mutation is not necessary for the induction of autophagy, which has been proved to be a requirement in the liver (32, 33). A functional redundancy in the Atg4 family of cysteine proteinases, which is thought to be shared by other protease families (25, 26, 34–36), could contribute to explain this observation. To test this possibility, we examined by Northern blot analysis the expression profile of these genes in a variety of mouse tissues including testis, spleen, kidney, skeletal muscle, liver, lung, brain, and heart (Fig. 2A). We observed that the expression pattern of the four Atg4 genes is very similar in murine tissues. This could explain at least in part the fact that the activity of Atg4C is not essential to generate the sufficient basal autophagy requested under normal circumstances in any of the tissues analyzed. Given this overlapping expression pattern in the Atg4 gene family, we examined whether the loss of Atg4C activity in vivo could modulate the levels of other Atg4 cysteine proteinases. To test this hypothesis, we first analyzed by quantitative RT-PCR the Atg4A, Atg4B, and Atg4D transcript levels in the livers of 48-h-starved Atg4C−/− and Atg4C+/+ mice. As shown in Fig. 2B, a consistent change in the relative expression levels of Atg4 genes was not detected. We next analyzed the protein levels of Atg4A, Atg4B, and Atg4D in Atg4C−/− and Atg4C+/+ tissues. As shown in Fig. 2C and in agreement with our quantitative PCR results, we did not observe any significant change in the protein levels of the different Atg4 enzymes in Atg4C−/− tissues. Finally, to evaluate a possible change in the subcellular distribution of these proteins in the absence of Atg4C, we analyzed the localization of the other Atg4 family members in Atg4C−/− cells, but we did not find any evidence supporting putative changes in the distribution of these proteins (data not shown). Taken together, these results do not provide evidence for a compensatory mechanism in Atg4C-deficient mice.

Atg4C Gene Disruption Leads to a Tissue-specific Decrease of Autophagy in Vivo—The fact that Atg4C−/− mice develop normally suggests that autophagy is not impaired in the absence of Atg4C. However, it should be emphasized that previous studies with other mutant mice exhibiting reduced autophagic activity have demonstrated that a decrease in this process is not necessarily accompanied by perinatal lethality (37, 38).

To test the possible role of Atg4C disruption in the regulation of autophagy in vivo, we first analyzed by quantitative RT-PCR the transcript levels in the livers of 48-h-starved Atg4C−/− and Atg4C+/+ mice (Fig. 2C). As expected, we observed a decrease in the transcript levels in the livers of 48-h-starved Atg4C−/− mice, whereas no significant change was detected in autophagosome membranes of wild-type animals, whereas we could not detect the lipidated form of GABARAP either in wild-type nor in Atg4C−/− mice, demonstrating that Atg4C activity is not essential for the processing of these proteins in vivo. However, we observed that the ratio of LC3-II/LC3-I, which has been widely used as an indicator of autophagic activity, was significantly decreased in the diaphragm muscle of starved Atg4C−/− mice (Fig. 3B). We also analyzed by quantitative RT-PCR the relative expression level of the distinct Atg4 cysteine proteinases in the diaphragm with the finding of a high relative expression of Atg4C in this tissue (Fig. 3C).

To confirm and extend these observations pointing to a tissue-specific reduction of autophagic responses in Atg4C−/− mice, we crossed these mutant mice with those expressing the transgene GFP-LC3 that provides an efficient marker for autophagy (29, 37, 40, 41). Fluorescence microscopic analysis of diverse tissues revealed that the number of punctate GFP-LC3 structures is equivalent in tissues from non-starved wild-type or Atg4C−/− mice (Fig. 4). Similarly, we were not able to detect any significant change in the number of GFP-LC3 punctate structures in the majority of tissues analyzed after 24 or 48 h of starvation.
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starvation (Fig. 4 and data not shown). However, in agreement with our immunoblotting results, we could detect a clear decrease in the number of GFP-LC3 punctate structures per cell area unit in the diaphragms from 24-h-starved Atg4C−/− mice as compared with their wild-type littermates. Although not statistically significant, the LC3 distribution in the diaphragm of starved Atg4C−/− mice showed a trend toward a reduction of autophagic activity as compared with their wild-type littermates, thus confirming our immunoblotting results.

Atg4C Gene Disruption Decreases Autophagy in Response to Starvation—To further investigate the effects of autophagy treatments performed on Atg4C−/− mice under confluent growing in normal conditions (Fig. 7A), we analyzed the degradation of long-lived proteins in fibroblasts derived from Atg4C−/− mice and their wild-type littermates fed ad libitum or subjected to 48 h starvation using a photocell actimeter, an apparatus that allows the recording of the movements and crossings of mice (42, 43). As shown in Fig. 5, the locomotor activity of non-starved Atg4C−/− mice was similar to control mice. However, after prolonged starvation, the locomotor activity of null mice was significantly reduced as compared with wild-type animals. Thus, it is tempting to speculate that the diminished capacity of inducing autophagy in diaphragm from Atg4C−/− mice leads to a reduced resistance to starvation that results in a reduced mobility in these mutant animals. Nevertheless, we cannot rule out the possibility that additional factors could contribute to explain the observed reduced resistance to starvation of Atg4C−/− mice.

Increased Susceptibility of Atg4C-deficient Mice to MCA-induced Fibrosarcomas—Many works have analyzed the putative correlations between the ability of cancer cells for developing autophagy and their neoplastic potential (44–46). The fact that beclin-1 haplo-deficient mice, which show a decrease in autophagy, also exhibit a high incidence of spontaneous tumors supports the idea that an autophagy decrease can confer an increased susceptibility to cancer (37, 38). On this basis and considering that Atg4C−/− disruption leads to a decrease in autophagy under demanding conditions, we were prompted to test the hypothesis that the absence of this proteinase could be associated with an increase in cancer susceptibility in these mutant mice. Because our first studies to examine this possibility indicated that Atg4C−/− mice do not develop more spontaneous tumors than their wild-type littermates (data not shown), we next focused on the comparative analysis of the susceptibility of these mice to tumors induced by chemical carcinogens. To this purpose, Atg4C mutant mice were induced to develop fibrosarcomas using a carcinogenesis protocol based on the intradermal injection of a single dose of the chemical carcinogen MCA, as previously described (47, 48). This chemical carcinogen promotes the transformation of mesenchymal fibroblasts into fibrosarcomas. As shown in Fig. 6A, tumors arose more rapidly and with a significantly higher incidence in Atg4C−/− mice than in wild-type animals. Differences in number of fibrosarcomas between mutant mice and wild-type controls were statistically significant. Histological analysis of fibrosarcomas generated in both wild-type and Atg4C−/− male mice revealed that most of them (80%) were partially infiltrative grade I fibrosarcomas (with absence of necrosis, undifferentiated, and with 2 or 3 mitosis by each high magnification field), and only exceptionally gave rise to more aggressive grade II tumors (Fig. 6B). These results suggest that the absence of Atg4C does not influence the late stages of tumor development in male mice.

To test the possibility that a decrease in autophagy due to Atg4C disruption leads to the higher incidence of fibrosarcomas observed in mutant mice, we studied the autophagic activity of adult murine fibroblasts, cells in which Atg4C is highly expressed (Fig. 7A). For that purpose, we analyzed the in vitro degradation of long-lived proteins in fibroblasts derived from Atg4C−/− mice and their wild-type littermates. As previously reported (30), we employed the autophagy inhibitor 3-methyladenine to measure the autophagy-mediated proteolysis in cultured cells. As expected, Atg4C−/− fibroblasts did not show changes in protein degradation under confluent growing in normal conditions (Fig. 7B). However, under serum and amino acids deprivation, Atg4C−/− cells showed a lower rate of 3-methyladenine sensitive protein degradation, which represented autophagic degradation, than the corresponding controls (Fig. 7C). To test if the observed reduction of autophagic degradation was due to the lack of Atg4C or to other causes, we transfected Atg4C−/− cells with an eukaryotic expression vector con-
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To date, the precise \textit{in vivo} role of the four members of the mammalian \textit{Atg4} family of cysteine proteinases remains to be characterized. So far, it is not known whether the existence of four mammalian orthologues of the yeast protease \textit{Atg4} corresponds to a functional redundancy or, by contrast, the components of this proteolytic system have acquired other functions distinct from autophagy during eukaryote evolution. As a first step to address this issue, we describe in this work the generation and phenotypic analysis of mutant mice deficient in \textit{Atg4C}, a member of the \textit{Atg4} family of cysteine proteinases that exhibits a wide tissue distribution and complements the deficiency of yeast \textit{Atg4} in autophagic processes. However, despite the wide expression pattern of \textit{Atg4C} under normal conditions, targeted disruption of this gene in mice does not cause any major abnormalities. Thus, \textit{Atg4C}^{-/-} mice develop normally, are fertile, and have long-term survival rates indistinguishable from those of wild-type mice. These observations suggest that \textit{Atg4C} gene disruption does not impair the ability of cells to undergo a proper autophagic response after birth, which has been proven to be essential for neonate viability.

To evaluate the involvement of \textit{Atg4C} in the processing and delipidation of \textit{Atg8} orthologues \textit{in vivo}, we first performed immunoblot analysis of their putative major substrates LC3, GATE-16, and GABARAP. These studies revealed that the lipidated forms of LC3 and GATE-16 were present in the majority of the analyzed tissues from both mutant and wild-type animals at a similar extent, whereas we could not detect the lipidated forms of GABARAP in any of the tissues analyzed. The absence of \textit{GABARAP} lipidation even under starvation conditions provides support to the idea that the putative physiological function of this protein is not related to autophagic processes. All these observations suggest that \textit{Atg4C} activity is not essential for the processing or delipidation of \textit{Atg8} orthologues. However, we found that under starvation conditions, the lipilation status of LC3 in the diaphragm, a continuous energy-consuming muscle, was diminished in mutant mice. This observation was confirmed by crossing \textit{Atg4C}^{-/-} mice with transgenic mice overexpressing GFP-LC3. \textit{Atg4C}^{-/-} mice showed a slight but significant decrease in the number of GFP-LC3 punctate structures, which was associated with a decrease in autophagy in diaphragm under starvation conditions.

The fact that \textit{Atg4C} activity is not essential for the development of autophagy under normal conditions might be explained by a functional redundancy in the \textit{Atg4} family of cysteine proteinases, as previously reported for other protease families from different catalytic classes (25, 35, 36, 51). Our finding of a considerable overlapping in the tissue distribution of the different mouse \textit{Atg4} orthologues should be consistent with the proposal of a functional redundancy in the \textit{Atg4} family of proteinases. However, despite this apparent redundancy observed in the \textit{Atg4} family, the finding that autophagy is reduced after 24 h of starvation in diaphragm from \textit{Atg4C}-deficient mice muscle suggests that only under very demanding conditions, \textit{Atg4C} activity is necessary for a proper autophagic response. Likewise, the reduced locomotor activity observed in \textit{Atg4C}^{-/-} mice after prolonged starvation could be a consequence of the decrease of autophagic activity in the diaphragm. Further works aimed to study metabolic and respiratory functions of...
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FIGURE 7. Analysis of the autophagic activity of Atg4C−/− and wild-type fibroblasts were metabolically labeled and chased as described under "Experimental Procedures." Confluent cells were then switched to complete (CF) or serum- and amino acid-deficient (KH) media with or without 3-methyladenine (3-methyladenine sensitive degradation) under serum and amino acid deprivation. To allow optimal inhibition and to avoid secondary effects, protein degradation was measured at four separate experiments under the control of independent cell lines for autophagic potential (44, 55–60). In this regard the activation of several tumor suppressor genes as Beclin-1, p53, PTEN, or p19ARF are associated with an up-regulation of autophagy, whereas their loss is often accompanied by a loss of autophagic potential (44, 55–60). On the other hand, several genes which act as oncogenes, including AKT, mTOR, or Ras, are reported inhibitors of autophagy, and their overexpression is associated with a reduced ability of tumor cells to undergo autophagy (52, 61–63). Furthermore, several recent studies have described a protective role for autophagy as an alternative cell death pathway that is activated in cancer cells that have lost their potential to undergo apoptosis (64–66). All these findings suggest that malignant transformation exposes cells to a stressing situation in which all their autophagic potential would be required. Accordingly, a reduction in the ability of inducing autophagy could eventually lead to a higher rate of tumorigenesis under compromising conditions. To test this hypothesis, we examined the susceptibility of Atg4C−/− mice to cancer development with the finding that these mice show a higher incidence of MCA-induced fibrosarcomas, which is consistent with the proposed tumor suppressor role for autophagy processes. Moreover, the fact that Atg4C loss leads to a reduced autophagic response in fibroblasts under stressing conditions such as starvation could contribute to explain the higher incidence of fibrosarcomas, which are fibroblast-derived tumors, observed in Atg4C mutant mice.

In summary, in this work we show that Atg4C activity is not essential for autophagy under normal conditions in vivo. However, under stressing conditions such as starvation, Atg4C disruption leads to a specific decrease of autophagy in fibroblasts, a tissue that is subjected to a continuous and high energy consumption, leading to a reduced resistance to fasting. In addition, the Atg4C−/− mice also show a higher incidence of MCA-induced fibrosarcomas as compared with their wild-type littermates, which could be correlated to the decrease in autophagy observed in Atg4C-deficient fibroblasts under nutritional stressful conditions. Further studies involving mice deficient in other components of the Atg4 protease family will help to define the precise in vivo role of each family member in both normal and pathological conditions including cancer.

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WITHDRAWN
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