ATP-Dependent Lon Protease Controls Tumor Bioenergetics by Reprogramming Mitochondrial Activity

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SUMMARY

We generated mice deficient in Lon protease (LONP1), a major enzyme of the mitochondrial quality control machinery. Homozygous deletion of Lonp1 causes early embryonic lethality, whereas its haploinsufficiency protects against colorectal and skin tumors. Furthermore, LONP1 knockdown inhibits cellular proliferation and tumor and metastasis formation, whereas its overexpression increases tumorigenesis. Clinical studies indicate that high levels of LONP1 are a poor prognosis marker in human colorectal cancer and melanoma. Additionally, functional analyses show that LONP1 plays a key role in metabolic reprogramming by remodeling OXPHOS complexes and protecting against senescence. Our findings demonstrate the relevance of LONP1 for cellular and organismal viability and identify this protease as a central regulator of mitochondrial activity in oncogenesis.

INTRODUCTION

Mitochondria are subcellular organelles of eukaryotic cells responsible for generating the bulk of cellular energy in the form of ATP through oxidative phosphorylation (OXPHOS; Friedman and Nunnari, 2014). Mitochondria are also involved in other pathways of intermediate metabolism, generate and regulate reactive oxygen species (ROS), maintain homeostasis and calcium buffering, and participate in essential cellular processes such as apoptosis (Wallace et al., 2010). Consistent with the wide diversity of functional roles played by these organelles, mitochondrial dysfunctions are associated with aging and pathological processes such as cancer and neurological diseases (López-Otin et al., 2013; Wallace, 2005). To exert their functions properly, mitochondria have developed a quality control system composed of proteases and chaperones that regulate the assembly, folding, and turnover of proteins, as well as the removal of damaged proteins (Tatsuta, 2009). In mammals, there are four major ATP-dependent proteases that participate in mitochondrial quality control: Yme1 and m-AAA proteases localized in the inner membrane, and ClpP and Lon located in the matrix (Rugarli and Langer, 2012). Additionally, there are several ATP-independent proteases that collaborate in mitochondrial homeostasis (Cipolat et al., 2006; Quiros et al., 2012).

Lon protease (LONP1) is a conserved serine peptidase that contributes to protein quality control processes from bacteria to eukaryotic cells (Lu et al., 2003). LONP1 plays an important role in the degradation of misfolded and damaged proteins, and supports cell viability under oxidative, hypoxic, and endoplasmic reticulum-stress conditions (Venkatesh et al., 2012). There are several proposed substrates for this enzyme in mammals, such as aconitase, cytochrome c oxidase isoform COX4-1, steroidogenic acute regulatory protein, and 5-aminolevulinic acid synthase (Bota and Davies, 2002; Fukuda et al., 2007; Granot et al., 2007; Tian et al., 2011). LONP1 is also a DNA-binding protein that participates in mtDNA maintenance and gene expression regulation (Liu et al., 2004). Moreover, LONP1 degrades mitochondrial transcription factor A (TFAM), regulating mtDNA copy number and metabolism to maintain the TFAM/mtDNA ratio necessary to control replication and transcription (Lu et al., 2013; Matsushima et al., 2010).

Consistent with the crucial role of LONP1 in the control of mitochondrial function under stress conditions, changes in the expression levels of this protease gene are associated with several human diseases (Venkatesh et al., 2012). Moreover, LONP1 levels are increased in different tumors and tumor cell lines (Bernstein et al., 2012; Hu et al., 2005; Kita et al., 2012). Interestingly, downregulation of LONP1 in some tumor cells causes apoptosis and cell death (Bota et al., 2005), indicating...
a possible addiction of tumor cells to LONP1 function, as occurs with other intracellular proteases associated with cancer (Fraile et al., 2012; Freij et al., 2011).

To further analyze the in vivo roles of this mitochondrial protease in both normal and pathological conditions, including cancer, we have generated mutant mice deficient in Lonp1. Homozygous deletion of Lonp1 causes early embryonic lethality but Lonp1−/− heterozygous mice are fertile and viable, thereby facilitating cancer susceptibility studies that have shown that these haploinsufficient animals are protected against colon and skin carcinomas induced by chemical carcinogens. Moreover, knockdown of Lon protease in colorectal and melanoma cells inhibits in vivo tumor growth and metastasis, respectively, whereas LONP1 overexpression promotes tumorigenesis. Finally, we provide evidence that LONP1 regulates metabolic reprogramming and controls tumor bioenergetics by remodelingOXPHOS complexes.

RESULTS

Lonp1 Deletion Causes Embryonic Lethality in Mice

Mice heterozygous for the targeted allele of the mitochondrial Lon protease (Lonp1+/−) were viable and fertile with no obvious abnormalities. These mice were intercrossed to generate homozygous mice deficient in Lonp1 (Figure S1A). However, we did not detect homozygous pups at weaning (Figure S1B), suggesting that Lonp1 homozygous deletion causes embryonic lethality. To further assess this hypothesis, we analyzed and genotyped embryos at different stages of development, from 3.5 to 9.5 days postcoitum (dpc). We found that at blastocyst stage (3.5 dpc), the number of embryos of the different genotypes followed the expected Mendelian ratios. However, at 8.5 dpc, only one of the analyzed viable embryos carried the homozygous mutation, whereas at 9.5 dpc none of them carried the mutation in homozygosis (Figures 1A and S1B). Moreover, analysis of nonviable mutant embryos at 8.5 dpc showed a clear reduction in size when compared with their littermate controls, looking like 7.5 dpc embryos and suggesting that their development was arrested. These differences were maintained at 9.5 dpc (Figure 1A). To clarify the embryonic lethality of Lonp1−/−, we performed comparative histological analysis of embryos from both genotypes. Lonp1−/− embryos from 5.5 and 6.5 dpc showed normal size and development (Figure 1B). However, at 7.5 dpc, Lonp1 null embryos showed a marked growth decrease, being much smaller than their littermate controls and exhibiting incomplete formation of embryo cavities, thereby pointing to a developmental arrest at the egg-cylinder early gastrulation stage (Figure 1B).

To further characterize the cause of lethality observed in Lonp1 null embryos, we first tried to generate ES cells or murine embryonic fibroblasts with homozygous deletion of Lonp1. However, after repeated experiments, we were unable to obtain any Lonp1−/− deficient cell line. To evaluate the putative relationship between mtDNA deficiencies and embryonic lethality in Lonp1 null mice, we analyzed mtDNA copy number in Lonp1-null embryos during development. We observed that at 3.5 dpc, there were no differences between control, heterozygous, and Lonp1 null embryos (Figure 1C). However, analysis at 7.5 dpc showed a decrease in mtDNA copy number in Lonp1-deficient embryos compared with control and heterozygous embryos (Figure 1C). To further evaluate the influence of LONP1 deficiency in embryo development, we analyzed the in vitro growth of embryos. We isolated preimplantation embryos by uterine flushing at the blastocyst stage (3.5 dpc). As shown in Figures 1D and S1B, almost all blastocysts obtained from intercrossed heterozygous mice were normal and viable, and Lonp1-null blastocysts were indistinguishable from the corresponding controls. We then cultured blastocysts for 5 days in embryonic stem cell media, finding that virtually all analyzed embryos developed normally. However, Lonp1−/− deficient embryos showed a smaller size in the inner cell mass and trophectoderm cells compared with littermates (Figure 1E). Taken together, these results indicate that LONP1 is essential for in vivo and in vitro embryo development. Embryonic absence of this protease leads to marked growth retardation and arrest likely due to mitochondrial dysfunction and loss of mtDNA, with subsequent failure to accomplish the energy requirements necessary for embryonic development.

In marked contrast to embryonic lethality of Lonp1−/− mice, Lonp1+/− animals developed normally and did not display any obvious pathological alterations. Analysis of Lonp1 expression in heterozygous mice indicated a 50% reduction at both RNA and protein levels in these animals (Figures 1F and 1G). These findings demonstrate that LONP1 haploinsufficiency is compatible with embryonic and adult mouse development, as well as with normal growth and fertility, thus opening the possibility to perform long-term studies of cancer susceptibility in Lonp1+/− mice.

Lonp1-Haploinsufficient Mice Are Protected against Colorectal Cancer and Chemically Induced Skin Tumors

To analyze the role of LONP1 in tumor development, we induced colon carcinoma in Lonp1+/− and Lonp1+/+ mice, using azoxymethane (AOM) and dextran sulfate (Figure 2A). All Lonp1 wild-type mice, but only 72% Lonp1 heterozygous mice, developed colorectal tumors (Figure 2B). Moreover, Lonp1+/+ mice had significantly fewer tumors than control animals (Figures 2C and 2D). There was also a decrease in the colon length in Lonp1 control mice compared with heterozygous mice (Figure 2E). Quantitative analysis of the clinical score during the carcinogenesis protocol revealed that Lonp1+/− mice exhibited milder clinical features than wild-type animals (Figure 2F).

To characterize the putative role of LONP1 in tumor development, we used an additional carcinogenesis protocol to induce skin tumors. We subjected Lonp1−/− heterozygous mice and their wild-type littermate controls to a 7,12-dimethylbenzanthracene and tetradecanoylphorbol acetate (DMBA/TPA) protocol that induces skin papillomas. Lonp1−/− heterozygous mice developed significantly fewer and smaller papillomas during the treatment and displayed a significantly lower incidence of the appearance of papillomas (Figures 2G–2I). These results indicate that LONP1 has a key role in the development of colon and skin tumors, and a decrease in Lonp1 levels protects against colorectal carcinoma and papilloma formation in mice.

LONP1 is Upregulated in Colon Cancer and Promotes Tumor Growth

To clarify the role of this mitochondrial protease in colorectal cancer, we studied LONP1 expression in different human
Figure 1. Lonp1 Deletion Causes Embryonic Lethality
(A) Representative images of Lonp1+/+ and Lonp1−/− embryos at 8.5 and 9.5 dpc. Scale bar represents 1 mm. Right: PCR analysis of Lonp1 embryos’ genotypes. (B) Histological analysis of embryos from 5.5 to 7.5 dpc of control and Lonp1-deficient embryos. EX, extraembryonic part; EM, embryonic part; epc, ectoplacental cavity; exc, exocoelomic cavity; ac, amniotic cavity. Scale bar represents 100 μm. (C) Analysis of mtDNA quantity expressed as a percentage of levels in control embryos at 3.5 dpc and 7.5 dpc embryos. Data are presented as mean ± SEM (n = 10–20), **p < 0.01. (D) Representative images of blastocysts (3.5 dpc embryos) obtained after heterozygous intercrosses of Lonp1 mice. (E) Lonp1-null embryos showed reduced size and delayed development in vitro compared with control blastocysts. ICM, inner cell mass; TB, trophoblastic cells. Scale bar represents 50 μm. (F) qPCR and (G) western blot analysis of LONP1 expression in murine embryonic fibroblasts derived from Lonp1+/+ and Lonp1+/− mice. qPCR data are presented as RQ value ± SD, **p < 0.01.
colorectal cancer cell lines (HCT116, HCT15, HT29, SW480, SW620, DLD-1, and RKO), and in a colon epithelial cell line (FHC). Quantitative PCR and western blot analysis showed a high expression of LONP1 in all colon cancer cell lines compared to the control epithelial colon cells (Figures 3A and 3B). To analyze the relevance of Lon protease in these colon carcinoma cells, we used HCT116 cells to perform in vitro and in vivo studies of loss- and gain-of-function. We reduced Lon protease levels using lentiviral-based shRNAs against LONP1, and ectopically induced LONP1 using a retroviral system that expresses a Flag-tagged LONP1 cDNA construct. The expression of LONP1 was confirmed with qPCR and western blot analysis (Figures 3C and S2A). Knockdown of LONP1 (shLon) significantly reduced cell proliferation in vitro compared to control cells (pLKO1; Figures 3D and S2B). However, ectopic expression of LONP1 (LON) did not increase proliferation when compared to control cells (pMX; Figure 3D). Likewise, overexpression of LONP1 in DLD-1, a colon cancer cell line with low levels of LONP1 expression, did not increase the proliferation rates (Figure S2C). We then examined the in vivo relevance of LONP1 expression on tumor growth, using a xenograft model. As shown in Figure 3E, tumor growth of shLon cells was significantly reduced when compared to pLKO1 control cells. On the other hand, ectopic expression of LONP1 increased growth of tumors in nude mice compared to those generated by pMX control cells (Figure 3F). These results indicate that LONP1 is necessary to maintain the proliferative rates and tumor growth of this colorectal cancer cell line.

To further evaluate the relevance of LONP1 expression in colon carcinoma, we performed an immunohistochemical analysis of human samples from normal and tumor colorectal sections. In agreement with previous data, LONP1 is expressed in normal colon samples and highly upregulated in colon cancer mucosa (Figure 3G). Moreover, analysis of transcriptional data from colorectal adenomas and normal mucosa showed a significant increase in LONP1 expression in adenomas compared to their matched normal mucosa (Figure 3H). Detailed analysis of reported data on survival of patients with Duke’s stages A-C colorectal carcinoma showed a positive correlation between high LONP1 expression and lower survival (Figure 3I). Collectively, these results suggest that LONP1 upregulation is a marker of poor prognosis in human melanoma.

LONP1 is Necessary for Proliferation and Metastasis of Melanoma Cells
As described above, LONP1-deficient cells.

LONP1 Induces a Metabolic Switch and Regulates Tumor Bioenergetics by Remodeling OXPHOS Complexes
To study the role of LONP1 in the regulation of mitochondrial function in cancer, we used the above described B16F10 melanoma cells with knockdown or overexpression of Lon protease. These melanoma cells are wild-type for BRAF, indicating that the mitochondrial activity and respiration are important and the energy production is not shifted to glycolysis (Castle et al., 2012; Haq et al., 2013). Knockdown of LONP1 decreased cellular ATP content, whereas ectopic expression of LONP1 increased ATP content (Figures 5A and S4A). Furthermore, analysis of mitochondrial respiration showed a decrease in basal oxygen consumption in LONP1 ablated cells when compared to controls (Figures 5B and S4B). Interestingly, overexpression of Lon protease also showed a decrease in mitochondrial respiration compared to control cells (Figure 5B). Stimulation with the chemical uncoupler trifluorocarbonyl cyanide phenylhydrazide increased oxygen consumption to maximal respiration rate in both pLKO1 and pMX control cells. Nevertheless, neither knockdown nor overexpression of Lon protease reached similar oxygen consumptions than control cells (Figures 5B and S4B). In addition, both knockdown and ectopic expression of LONP1 increased the glucose consumption and lactate production compared to controls (Figures 5C, 5D, S4C, and S4D). These results were puzzling because both conditions, i.e., ablation and overexpression of LONP1, induce the downregulation of respiration and the upregulation of the glycolytic pathway. To try to understand this phenomenon, we examined mitochondrial respiratory complexes and detected a significant decrease in complex I (Figures 5E and S4E) and a concomitant decrease in complex III-containing supercomplexes (Figures 5F and S4E) in shLon cells. Similarly, we observed a significant decrease in complex I, II, and IV in cells overexpressing LONP1 (Figures 5G and S4F), as well as a reduction in complex III-containing supercomplexes (Figures 5H and S4F). We also noticed that the relative amount of complex V in dimers was reduced in both models (Figures 5F and 5H). The observed decrease in mitochondrial complexes in both models likely derives from enhanced degradation or from a reduction in their assembly and could underlie the decrease in mitochondrial respiration in both LONP1-overexpressing and LONP1-deficient cells.
Figure 2. Lonp1+/+ Mice Display Less Susceptibility to Azoxymethane-Induced Colon Tumors and Skin Chemical Carcinogenesis Induced by DMBA/TPA

(A) Scheme of azoxymethane-induced colon tumor protocol.
(B) Percentage of animals with tumors (p < 0.05).
(C) Average of tumors observed in Lonp1+/+ and Lonp1+/−/C0 mice.
(D) Lonp1+/+
(E) Lonp1−/
(F) Clinical score

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We next evaluated the individual activities of mitochondrial complexes by spectrophotometric analysis of mitochondria isolated from both cells. We observed that shLon cells showed significantly lower activities of complex I (CI) and combined complex II+complex III (CI+II/III) activity (Table S1), consistent with the low levels of CI and assembled CI+III measured by BNGE (Figures 5E and 5F). In contrast, overexpression of LONP1 decreased almost all individual complex activities. Thus, we observed a reduction in CI and complex IV (CIV), as well as combined CI+III and CI+II+III activities (Table S1), consistent with the general decrease in almost all complexes observed by BNGE (Figures 5G and 5H). Moreover, knockdown of Lon protease specifically induced an increased activity of CI+III relative to CI (CI+III/CI) and decreased activity of CI+III relative to CI (CI+III/CI) (Figure 5I), suggesting that complex III is more dedicated to complex I than to complex II, favoring the use NADH equivalents and the respiration through complex I. However, overexpression of Lon protease showed a decrease in the activity of CI+III relative to CI (CI+III/CI), which indicated a reduction in the respiration through complex I while respiration efficiency through complex II (CI+II/CI) remained unaltered (Figure 5J). Collectively, the changes observed in the LONP1-overexpressing cells are consistent with a metabolic reprogramming of the OXPHOS system by primarily reducing the flux from NADH electrons (more catabolic), but maintaining the flux through the FAD-dependent enzymes, that are also involved in anabolic (nucleotide and amino acid metabolism) and detoxifying metabolic pathways. Nevertheless, the changes in respiration flux observed in shLon cells are more closely related to a catabolic metabolism.

To further clarify the differences observed in the regulation of OXPHOS complexes in LONP1-overexpressing and LONP1-knockdown cells, we performed a quantitative proteomic analysis, using iTRAQ labeling and subsequent LC-MS/MS analysis. As an internal control of our analysis, we found a significant increase in LONP1 protein levels in the overexpression model, whereas this protease was undetectable in the downregulation model (Figure 5K). Consistent with the above findings in OXPHOS complex levels, we detected a significant alteration in the amount of complex I proteins in both LONP1-overexpressing and LONP1-knockdown cells (Figure 5K). However, the affected complex I protein components differed between both cells, suggesting a distinct mechanism of regulation in each condition. Thus, all CI proteins were downregulated in LONP1-knockdown cells, while they presented variability in LONP1-overexpressing cells, some of them being upregulated and other downregulated. Furthermore, CI structural proteins, such as NDUF6B, 8, 10 and 11, were the more downregulated proteins in LONP1-knockdown cells, while they were upregulated in LONP1-overexpressing cells. Importantly, these proteins are associated with the assembly and stability of the CI membrane domains and are required for their activity (Andrews et al., 2013; Perales-Clemente et al., 2010). Conversely, among the most decreased proteins in the LONP1-overexpressing cells, there were four subunits of the NADH dehydrogenase and hydrogenase catalytic modules of CI, such as NDUFV1, NDUFV2, NDUFV3, and NDUFV7. Interestingly, it has been described that these proteins can be removed and replaced in the complex I without degrading the whole complex (Dieteren et al., 2012; Lazarou et al., 2007). These results indicate that the observed complex I decrease in shLon cells was a consequence of loss of stability of this complex, whereas in LONP1-overexpressing cells the decrease was functional, maintaining the structural domains. Moreover, complex V subunits were clearly downregulated in shLon cells (Figure 5K), which is in line with the observed decrease in complex V dimers (Figure 5F). Interestingly, LONP1-overexpressing cells showed an increase in complex V subunits (Figure 5K), despite the observed decrease in dimers (Figure 5H), which indicates that these cells have a different complex V regulation.

Overall, these data strongly suggest that the apparent convergence in the decrease in aerobic respiration and the subsequent glycolysis activation, by either knockdown or overexpression of LONP1, takes place through different mechanisms. The loss of mitochondrial complexes would operate in shLon cells, whereas OXPHOS complexes remodeling would underlie the alterations found in LONP1-overexpressing cells.

**LONP1 Keeps Mitochondrial Function and Induces a Global Metabolic Reprogramming**

To further clarify the observed alterations in LONP1-overexpressing and LONP1-knockdown cells, we analyzed other parameters related to mitochondrial function in these cells. We first found that LONP1-knockdown, but not its overexpression, induced a decrease in both mitochondrial membrane potential (Figure 6A) and mtDNA content (Figure 6B). shLon cells also displayed an increase in mitochondrial fragmentation (Figures 6C and 5S) and ROS production (Figure 6D), which caused the proteolytic processing of OPA1 (Figures 6E and S5B). These findings are indicative of a clear mitochondrial dysfunction, which is not observed in LONP1-overexpressing cells. Moreover, knockdown of Lon protease activated the AMPK pathway (Figure 6E), which is in line with the low ATP levels and the mitochondrial stress scenario observed in these cells. In contrast, overexpression of LONP1 decreased AMPK activation (Figure 6E). In addition, and consistent with the enhancement of the glycolytic pathway detected in both LONP1-overexpressing and LONP1-knockdown cells, there was an increase in the expression of glycolytic genes in both cases (Figure S5C).

(D) Representative images of hematoxylin and eosin staining of colon sections from Lonp1+/+ and Lonp1+/- mice at the end of the experiment. Scale bar represents 200 μm.

(E) Measurement of the length of the colon at the end of the experiment.

(F) Average number of papillomas per mouse grouped by size (diameter of the lesion in millimeters), and represented for each genotype (n = 10).

(G) Inverse Kaplan-Meier analysis of the percentage of mice with papillomas. Log-rank test p = 0.0439.

(H) Representative pictures of papilloma lesions of Lonp1+/- and Lonp1-/- at week 18. Scale bar represents 200 μm.
However, analysis of lipid synthesis showed a significant decrease in shLon cells, which agrees with the AMPK activation in these cells, whereas LONP1-overexpressing cells displayed similar rates than control cells (Figures 6F and SSD). Interestingly, and despite we did not detect differences in apoptosis (data not shown), we found increased levels of the antiapoptotic protein Bcl-2 in LONP1-overexpressing cells and decreased levels in shLon cells (Figure 6E), which suggests a possible increase in apoptosis sensitivity in LONP1-deficient cells. We next examined in both LONP1-overexpressing and LONP1-knockdown cells the levels and activity of aconitase (ACO2), a well-established substrate for Lon protease. We found a decrease in ACO2 activity in LONP1-overexpressing cells and an increased activity of this enzyme in shLon cells (Figure 6E). The low expression levels of ACO2 in these cells precluded the detection of clear differences in enzyme activities. However, after transfection of a cDNA encoding ACO2, we detected increased levels and activity of this enzyme in control cells, but not in LONP1-overexpressing cells (Figures 6F and S5G), indicating that LONP1 processes ACO2 under conditions where this substrate is highly expressed.

Taken together, these results indicate that the absence of LONP1 induces a generalized mitochondrial dysfunction, whereas its overexpression does not alter the mitochondrial homeostasis. To further characterize the cellular alterations induced by the depletion and overexpression of LONP1, we performed quantitative proteomic analysis of the corresponding cells and found that LONP1 overexpression increased the levels of proteins related with protein synthesis, such as eukaryotic translation initiation factor complex 3 and ribosomal proteins (Figure 6G). We also found increased levels of proteins related to the spliceosome, proteasome, and chaperone-containing T complex (Figure 6H), which is consistent with the increased tumorigenesis observed in LONP1-overexpressing cells. Conversely, LONP1-deficient cells showed reduced levels of all of these proteins (Figures 6G and 6H), which agrees well with the low proliferation rate and tumorigenic potential of these cells. These data illustrate the occurrence of marked differences in the process of metabolic adaptation after modulation of LONP1 levels, which leads to a profound mitochondrial dysfunction when cells are depleted of Lon protease and to a global metabolic remodeling when this mitochondrial enzyme is overexpressed.

LONP1 Contributes to Bypass Oncogene-Induced Senescence

LONP1 knockdown induces growth arrest and mitochondrial dysfunction in melanoma cells, impairing mitochondrial respiration and causing loss of mitochondrial integrity. Interestingly, we also observed that shLon cells displayed an increase in size compared with control pLKO1 or LONP1-overexpressing cells, suggesting the occurrence of a cellular senescence phenotype (Figure 7A). Moreover, β-galactosidase staining (a marker of senescent cells) showed an increased positivity in cells lacking Lonp1, whereas overexpression of LONP1 decreased the number of β-galactosidase positive cells (Figure 7B). The induction of cellular senescence is mainly mediated by signals derived from a permanent DNA damage response. Accordingly, we found that the loss of mitochondrial integrity caused by LONP1 deficiency induced a DNA damage response likely mediated by p53, as assessed by the increased levels of direct transcriptional targets of this tumor suppressor (Figure 7C). Interestingly, knockdown of LONP1 in p53-deficient cells resulted in lower proliferation defects when compared to wild-type cells, indicating that p53 contributes to the observed alterations (Figure 7D). Overexpression of LONP1 in B16F10 cells reduced the levels of these markers and β-galactosidase activity (Figures 7B and 7D), indicating a protection against senescence by Lon protease, which was consistent with the tumorigenic increase of these melanoma cells.

On the other hand, it is well established that mutations in HRAS or BRAF also induce a senescence phenotype, known as onco-gene-induced senescence (OIS), as part of a cell response against tumor transformation (Michaloglou et al., 2005; Serrano et al., 1997). Based on this information, we explored available cancer transcriptome databases looking for putative differences in LONP1 expression levels during OIS processes. Thus, by analyzing data from previous studies involving transformation of fibroblasts with mutant MEK, we found that induced OIS did not promote changes in LONP1 expression. However, bypassing senescence in these fibroblasts using viral oncoproteins significantly increased LONP1 expression (Figure 7E), further supporting the idea that upregulation of Lon protease may favor oncogenic transformation. These results demonstrate that LONP1 is essential to protect against senescence, and reinforce the proposal that levels of this serine protease contribute to modulate the tumorigenic properties of cancer cells.
A

Relative Lorp1 expression

Normal  Benign nevi  Melanoma

P-value < 0.001

B

Survival probability

Days

Low Lorp1  High Lorp1

C

pLKO1  shLon  pMX  LON
LONP1
FLAG
ACTB

D

Lorp1 expression (RQ)

pLKO1  shLon  pMX  LON

E

Absorbance (570 nm)

Days

pLKO1  shLon  pMX  LON

F

Number of metastasis

pLKO1  shLon

G

Number of metastasis

pMX  LON

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We describe herein the generation and phenotypic characterization of mice deficient in the mitochondrial Lon protease, as well as the functional analysis of this ATP-dependent enzyme controlling tumor bioenergetics. We found that mice deficient in LONP1 show a lethal phenotype during gastrulation, a period in which a well-fitted mitochondrial function is required to induce

**DISCUSSION**

We describe herein the generation and phenotypic characterization of mice deficient in the mitochondrial Lon protease, as well as the functional analysis of this ATP-dependent enzyme controlling tumor bioenergetics. We found that mice deficient in LONP1 show a lethal phenotype during gastrulation, a period in which a well-fitted mitochondrial function is required to induce
profound changes in embryo development (Nagy et al., 2003). At this stage, Lonp1 null embryos display loss of mtDNA and growth arrest, which are features observed in mice deficient in OXPHOS complex subunits, like GRIM19 and SCO2 (Huang et al., 2004; Yang et al., 2010), or in mtDNA-associated proteins, such as POLG, POLG2, and TFAM (Hance et al., 2005; Humble et al., 2013; Larsson et al., 1998), that also exhibit embryonic lethality. In contrast, Lonp1-heterozygous mice develop normally, allowing us to evaluate in vivo the functional and pathological relevance of LONP1 haploinsufficiency. Interestingly, Lonp1−/− mice are protected against chemically induced colorectal and skin tumors, suggesting an oncogenic function for this mitochondrial protease. Consistent with this hypothesis, LONP1 is upregulated in human colorectal and skin carcinomas and their levels are associated with poor clinical outcome of the corresponding patients. Accordingly, LONP1–knockdown in colorectal cancer cells reduces in vitro cell proliferation and in vivo growth of tumors derived from these cells, whereas ectopic expression of LONP1 increases tumor growth. Moreover, overexpression of LONP1 in melanoma cells increases experimental metastasis formation, whereas knockdown of this protease decreases cell proliferation and lung metastasis. Notably, LONP1 upregulation does not stimulate cell proliferation, despite the observed increase in tumor growth and metastasis in vivo. The metabolic and stress adaptations undergone by cancer cells during the tumorigenesis process, which are not perfectly replicated in the in vitro experiments, may explain these differences, also observed in similar studies involving other metabolic genes with oncogenic function (Bhalla et al., 2011). Accordingly, we propose that the upregulation of LONP1 observed in many cancer cell lines and tumor samples (Bernstein et al., 2012; Hu et al., 2005), is probably the consequence of an adaptive metabolic response during malignant transformation.

Figure 6. LONP1 Maintains Mitochondrial Function and Promotes a Metabolic Remodeling Process

(A–H) B16F10 melanoma cells with knockdown (shLon) and overexpression of Lon protease (LON) and their respective controls pLKO1 and pMX were used to study mitochondrial function. (A) Mitochondrial membrane potential (ΔΨM) represented as a percentage of TMRM fluorescence, in normal cells and after carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treatment. (B) mtDNA levels represented as percentage relative to each control. (C) Percentage of fragmented mitochondria quantified after mDsrRed2 transfection. (D) ROS levels represented as relative levels of DCF-DA fluorescence. (E) Western blot analysis showing processing of OPA1 long isoforms (a and b) and increase in the short isoforms (c to e), AMPK activation, and Bcl2 decrease in shLon cells. Overexpression of LON causes reduced AMPK activity and increase in Bcl2 levels. (F) Fatty acid synthesis rate measured as 14C incorporation into cellular lipids represented as relative units (RU). All experiments were independently carried out at least three times, using triplicates for each condition; data are presented as mean ± SEM, *p < 0.05, ***p < 0.001. Heatmap showing abundance changes of proteins belonging to the eukaryotic translation initiation factor 3 (Eif3) and the ribosomal complexes (G), and to the spliceosome, the proteasome and the chaperone-containing T (CCT) complex (H), obtained with high-throughput quantitative proteomics of whole-cell lysates. The relative abundance changes in LON and shLon cells are expressed using the Z score in relation to each control.
Metabolic reprogramming of tumor cells has been recently established as a hallmark of cancer (Hanahan and Weinberg, 2011). Several enzymes have been proposed to act as protumorigenic factors and contribute to this metabolic reprogramming (Chae et al., 2012; Vazquez et al., 2013). Furthermore, mutations in genes encoding mitochondrial and metabolic enzymes have been widely reported in human malignancies (Rodríguez et al., 2013; Yan et al., 2009), indicating that tumor cells harbor genetic alterations that affect metabolic function and facilitate tumor formation. The main feature of this reprogramming process is the switch from oxidative to glycolytic metabolism (Vander Heiden et al., 2009). Curiously, we have found that both upregulation and knockdown of Lon protease in melanoma cells induce the same glycolytic switch, turning cells from oxidative to anaerobic metabolism. In this regard, it is well established that upregulation of the glycolytic pathway during tumor transformation is triggered by oncogenic signals, such as activating mutations in the oncogene \textit{BRAF} in melanoma, as well as by many physiological and pathological conditions which cause metabolic changes (Hu et al., 2012). This impairment of mitochondrial OXPHOS triggers glycolytic metabolism as a survival mechanism that often ends with cell collapse due to the inability of these damaged cells to reprogram their metabolism (Wallace, 2005). Accordingly, the glycolytic switch observed in cells lacking LONP1 may be an obligate consequence of their mitochondrial dysfunction, not being related to metabolic reprogramming of cancer cells. Conversely, the switch observed in \textit{LONP1}-overexpressing cells is a mitochondria-controlled mechanism, which forms part of a generalized metabolic reprogramming that contributes to the development and progression of cancer.

The metabolic adaptation of shLon cells induces a change in respiration and in the use of electron equivalents, favoring the utilization of NADH electron equivalents feeding CI, which suggests the occurrence of a catabolic phenotype (Lapuente-Brun et al., 2013). However, the decrease in supercomplexes due to loss of specific structural subunits implies that this is not a regulated mechanism, but a consequence of defective assembly or mitochondrial membrane destabilization. In addition, the subsequent loss of membrane potential and the alterations in complex V cause a decrease in ATP generation, whereas the increase in ROS levels exacerbates this mitochondrial dysfunction. These
stress conditions induce the OPA1 processing and mitochondrial fragmentation events that may contribute to the decrease in respiration and functional supercomplexes assembly (Cogliati et al., 2013). Moreover, the loss of mitochondrial membrane potential may inhibit the entry of pyruvate into mitochondria (Herzig et al., 2012), reducing the Krebs cycle function and the generation of essential metabolites for cell viability. Consequently, the decrease in ATP levels and the mitochondrial stress signals induce AMPK activation, which downregulates anabolic pathways, such as lipid synthesis, and upregulates catabolic reactions, such as glycolysis, to try to counteract this stress scenario. However, despite the activation of all these stress-response mechanisms, shLon cells are unable to avoid mitochondrial dysfunction due to the dramatic alterations present in them. Accordingly, the mitochondrial dysfunction in shLon cells activates a genetic damage response, likely mediated by p53, which finally triggers cell cycle arrest and senescence. This response mechanism induced by mitochondrial damage reinforces the role of LONP1 as a guardian against reduced cellular and organismal fitness by contributing to maintain mitochondrial integrity.

Conversely, the glycolytic switch observed in LONP1-overexpressing cells is triggered by a regulated metabolic reprogramming, maintaining other mitochondrial functions unaltered. The upregulation of LONP1 induces a remodeling of mitochondrial complexes and supercomplexes, decreasing the mitochondrial respiration and favoring the metabolic switch because, under these conditions, respiratory reactions shift toward the glycolytic pathway (Wallace, 2012). Moreover, Lon protease overexpression reduces the use of NADH equivalents and the respiration through CI, which is frequently mutated and inactivated in tumor cells (Dieteren et al., 2012), reducing the Krebs cycle function and the generation of essential metabolites for cell viability. Consequently, the decrease in ATP levels and the mitochondrial stress signals induce AMPK activation, which downregulates anabolic pathways, such as lipid synthesis, and upregulates catabolic reactions, such as glycolysis, to try to counteract this stress scenario. However, despite the activation of all these stress-response mechanisms, shLon cells are unable to avoid mitochondrial dysfunction due to the dramatic alterations present in them. Accordingly, the mitochondrial dysfunction in shLon cells activates a genetic damage response, likely mediated by p53, which finally triggers cell cycle arrest and senescence. This response mechanism induced by mitochondrial damage reinforces the role of LONP1 as a guardian against reduced cellular and organismal fitness by contributing to maintain mitochondrial integrity.

**EXPERIMENTAL PROCEDURES**

**Generation of Lonp1−/− Mice and Genotyping**

The Lonp1–heterozygous mice were provided by Texas Institute for Genomic Medicine (Figure S1A). We used genomic DNA from tail samples for PCR genotyping under the following conditions: denaturation at 94°C for 15 s, annealing at 62°C for 15 s, and extension at 72°C for 45 s, 30 cycles. We used the following primers for genotyping: wild-type-specific forward 5′-cctcagcatgcggatttgatg-3′, and common reverse 5′-tcagctgcagctccagattg-3′.

**Animal Studies and Carcinogenesis Protocols**

All animal procedures were approved in accordance with the guidelines of the Committee for Animal Experimentation of the Universidad de Oviedo. Xenograft studies were performed as described (Fraile et al., 2013). Colon and skin carcinogenesis, and lung metastasis were induced as reported (Balbin et al., 2003; Gutiérrez-Fernández et al., 2008; Neufert et al., 2007). Complete methods can be found in the Supplemental Experimental Procedures.

**Cell Culture**

Cancer cell lines 293T, HCT116, HCT15, HT29, SW480, SW620, DLD-1, RKO, and FHC were purchased from the American Type Culture Collection. The luciferase-expressing cell line B16F10 Luc2 was purchased from Caliper Life Sciences. Cells were routinely maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies).

**DNA Constructs**

Mouse cDNA from LONP1 was cloned tagged with a FLAG epitope in the carboxy terminus and subcloned into the pMX retroviral vector. The construct was verified by capillary sequencing. For RNA interference experiments, five shRNA vectors were purchased for human and mouse LONP1 (RHS4533-NM_004793 for human and RMM4534-NM_028782 for mouse; Open Biosystems, Thermo Scientific).

**Seahorse Analysis of Mitochondrial Function**

Oxygen consumption was measured in 2 × 10^6 intact cells using a Seahorse Bioscience XF96 extracellular flux analyzer, following the manufacturer’s instructions. Briefly, after a 12 min equilibration, three measurements of 3 min were performed, separated by 3 min of mixing. Maximal membrane potential was assessed with the addition of 1 μM oligomycin, and uncoupled mitochondrial respiration was induced with injection of 1 μM carbonyl cyanide m-chlorophenyl hydrazone. To stop the mitochondrial-dependent oxygen consumption, both 1 μM rotenone and antimycin were used.
Statistical Analysis
All experimental data are reported as means and the error bars represent SEM. Differences between mean values were analyzed with two-tailed Student’s t test, except in the cases that indicate use of another statistical test. A p < 0.05 was considered significant and statistically significant differences are shown with asterisks. All statistical analyses were done using LibreOffice or the statistical package R (http://www.r-project.org/) and the application Rstudio (http://www.rstudio.org).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.018.

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