Age-associated decline of MondoA drives cellular senescence through impaired autophagy and mitochondrial homeostasis

**Graphical abstract**

**Highlights**

- The transcription factor MondoA is identified as a regulator of cellular senescence.
- MondoA counteracts senescence by activating autophagy via the suppression of Rubicon.
- MondoA delays senescence by maintaining mitochondrial function through Prdx3.
- Decrease of MondoA in the nucleus is correlated with senescence in human aging.

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**In brief**

Yamamoto-Imoto et al. identify the transcription factor MondoA as a regulator of cellular senescence. MondoA delays cellular senescence through two independent pathways: by activating autophagy through the suppression of Rubicon and by maintaining mitochondrial function via Prdx3. Decline of MondoA triggers cellular senescence in aging and age-related diseases.
Age-associated decline of MondoA drives cellular senescence through impaired autophagy and mitochondrial homeostasis

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SUMMARY

Accumulation of senescent cells affects organismal aging and the prevalence of age-associated disease. Emerging evidence suggests that activation of autophagy protects against age-associated diseases and promotes longevity, but the roles and regulatory mechanisms of autophagy in cellular senescence are not well understood. Here, we identify the transcription factor, MondoA, as a regulator of cellular senescence, autophagy, and mitochondrial homeostasis. MondoA protects against cellular senescence by activating autophagy partly through the suppression of an autophagy-negative regulator, Rubicon. In addition, we identify peroxiredoxin 3 (Prdx3) as another downstream regulator of MondoA essential for mitochondrial homeostasis and autophagy. Rubicon and Prdx3 work independently to regulate senescence. Furthermore, we find that MondoA knockout mice have exacerbated senescence during ischemic acute kidney injury (AKI), and a decrease of MondoA in the nucleus is correlated with human aging and ischemic AKI. Our results suggest that decline of MondoA worsens senescence and age-associated disease.

INTRODUCTION

Autophagy is essential for cellular homeostasis and adaptation to stress, and dysregulation of autophagy affects tissue homeostasis and individual health (Leidal et al., 2018). While it has been shown that autophagic activity decreases with organismal aging, a growing body of evidence implicates the activation of autophagy in promoting longevity and preventing age-associated phenotypes (Fernández et al., 2018, Barzilai et al., 2016, Baur et al., 2006; Eisenberg et al., 2016; Lamming et al., 2013). For instance, overexpression of one of the autophagy-related genes (Atgs), Atg5, in mice suffices to extend lifespan (Pyo et al., 2013). Introduction of mutation in one of the other Atgs, Beclin1, which causes constitutive activation of autophagy, promotes longevity and prevents age-associated phenotypes in mice (Fernández et al., 2018). Moreover, our previous work reveals that the suppression of autophagy-negative regulator, Rubicon (Matsunaga et al., 2009), promotes longevity in C. elegans and Drosophila.
Figure 1. Decline of autophagy promotes cellular senescence

(A) Expression of senescence markers, including p16, p21, p53, phosphorylated NF-κB (p-NFκB), Lamin B1, phosphorylated Rb (p-Rb), and LAMP1 in hRPE cells treated with doxorubicin (DXR) (150 ng/mL) for 5 or 10 days. Representative western blots from three independent experiments.

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and prevents age-associated kidney fibrosis and neurodegenerative disease in mice (Nakamura et al., 2019). Importantly, the expression of Rubicon increases with age in C. elegans, *Drosophila*, and several mouse tissues including kidney and liver (Tanaka et al., 2016; Nakamura et al., 2019), suggesting that an increase of Rubicon could be one of the causes of age-dependent autophagic declines. However, how activation of autophagy promotes longevity and prevents aging phenotypes are unclear. In addition, how autophagy decreases during aging remains elusive.

Cells exposed to many stressors, such as dysfunctional telomeres, non-telomeric DNA damage, excessive mitogenic signals, and mitochondrial dysfunction, trigger changes in their phenotypes termed cellular senescence (Gorgoulis et al., 2019). Senescent cells show non-proliferative irreversible growth arrest, resistance to apoptosis, and drastic changes in gene expression, which include dysregulated metabolism and senescence-associated secretory phenotype (SASP), as well as upregulation of cyclin-dependent kinase (CDK) inhibitors such as CDK2 inhibitor p24<sup>Wart/Op1/Sad1</sup> (Noda et al., 1994) and Nf-κB (Ito et al., 2017), and downregulation of Lamin B1 (Freund et al., 2012). Increases of lysosome number and size in senescent cells elevates numbers of senescence-associated β-galactosidase (SA-β-gal) and LAMP1-positive cells. In addition, mitochondrial dysfunction modulates senescence, which lacks the IL-1-mediated SASP through AMP-activated protein kinase (AMPK) and p53 activation (Wiley et al., 2016). Although senescence cells accumulate with age and affect the progression of aging and age-associated disease (López-Otin et al., 2013), the role of autophagy and its regulation in senescence remains unclear (Leidal et al., 2018; Lee et al., 2021). The findings that lower autophagic activity inhibits GATA4 degradation and facilitates the production of SASP via NF-κB activation (Kang et al., 2015) suggest that autophagy is crucial for suppressing senescence. In support of this idea, autophagy reduces the activation of p53 and generation of mitochondrial reactive oxygen species (ROS), resulting in a decrease in senescent phenotypes (Kang et al., 2011). However, in the senescent cells induced by oncogenic insult, autophagy contributes to the establishment of the senescent state via rapid protein turnover of the SASP (Young et al., 2009; Narita et al., 2011) and degradation of nuclear lamina (Dou et al., 2015).

MondoA, a basic-helix-loop-helix-leucine zipper heterodimerization partner of Max-like protein X (Mlx) (Billin et al., 2000), senses cellular energy status at the mitochondrial outer membrane (Wilde et al., 2019) and activates transcription for metabolic genes including glycolytic enzymes (Sans et al., 2006; Stoltzman et al., 2008). Regulation of thiorexin-interacting protein (Txnip) by MondoA participates in cholesterol/lipid metabolism, muscle lipid accumulation and insulin resistance, and tumorigenesis (Stoltzman et al., 2008; Ahn et al., 2016, 2019; Weger et al., 2020; Zhang et al., 2018). Interestingly, MondoA complexes promote longevity in response to a variety of signals, such as germline removal and calorie restriction in C. elegans (Nakamura et al., 2016; Johnson et al., 2014). Increased autophagic activity in germline-less long-lived animals, *glp-1*, are abolished by either *mni-1* (homolog to MondoA/ChREBP) or *mxl-2* (homolog to MLX) depletion, indicating that the Mondo complex is required for activating autophagy in those animals. However, roles of MondoA in mammalian aging and autophagy remain elusive. Here, we demonstrate that mammalian MondoA is a key modulator of autophagy and mitochondrial homeostasis capable of protecting from cellular senescence and senescence-associated kidney injury, as well as organismal aging in worms, mice, and humans.

**RESULTS**

**Decline of autophagy accelerates cellular senescence**

Since roles of autophagy during cellular senescence are unclear (Leidal et al., 2018; Kang et al., 2015; Young et al., 2009; Narita et al., 2011; Dou et al., 2015), we first investigated autophagic activity using two types of senescent cells: human retinal pigment epithelial cell line (hrPCE cells) induced with a genotoxic agent, doxorubicin (DXR) (DNA damage-induced senescence), and TIG-3 cells induced by replication (replicative senescence). DXR treatment for 5 and 10 days induced senescence as seen in the increased expression of p16, p21, p53, phosphorilated NF-κB, and lysosome-associated membrane protein 1 (LAMP1), and decreased levels of Lamin B1 and phosphorylated Rib (p-Rb) (Figure 1A), as well as increased number of SA-β-gal-positive cells (Figure 1B). In addition, cells at day 10 after DXR treatment showed increased levels of SASP, including IL-6, IL-1α, and IL-1β, and mitochondrial dysfunction determined by decreased ATP levels and increased expression of phosphorylated AMPK (Figures S1A–S1C). Autophagic activity determined by LC3 autophagic flux (Mizushima et al., 2010) was decreased in both DNA damage-induced and replicative senescence (Figures 1C and S1D), suggesting that autophagy is involved in cellular senescence. Consistent with this idea, siRNA knockdown of Atg7 and Atg13, positive regulators of autophagy (Levine and Kroemer, 2019), enhanced the expression levels of senescence.
markers including p21, p53, and LAMP1 even in non-induced cells (Figure 1D). In addition, the numbers of SA-β-gal-positive cells in senescent cells were increased in siAtg7- or siAtg13-treated cells (Figure 1E). While levels of IL-6 and IL-1α were significantly elevated upon Atg13 depletion at day 10 after DXR treatment (Figure S1E), ATP levels, oxygen consumption rate (OCR), and expression levels of phosphorylated AMPK were unaltered (Figures S1F and S1G), suggesting that impaired autophagy contributes to cellular senescence, including SASP, independent of mitochondrial dysfunction. Remarkably, similar to organismal aging, autophagy suppressor Rubicon (Matsunaga et al., 2009) was increased during DNA damage-induced and replicative senescence (Figures 1F, 1G, and S1H). To determine whether activation of autophagy could suppress senescence characterized by SA-β-gal activity, we depleted Rubicon and found that the number of SA-β-gal-positive cells was significantly reduced in Rubicon knockdown cells (Figure 1H). Knockdown of Rubicon did not ameliorate cell-cycle arrest at day 10 after DXR treatment, determined by Edu incorporation and SA-β-gal positivity (Figure S1I). However, kidneys examined from Rubicon knockout mice showed that p21 levels detected by immunostaining were markedly decreased compared with age-matched wild-type mice (Figure 1I). These data suggest that autophagic activity decreases during cellular senescence and that impaired autophagy promotes senescence.

Retained MondoA delays cellular senescence through the autophagic pathway

Since Mondo was reported as a regulator required for many longevity pathways in C. elegans (Park et al., 2018; Johnson et al., 2014), we examined whether the Mondo complex contributes to suppression of cellular senescence in mammals. Although the carbohydrate response element binding protein (ChREBP) is a paralog of MondoA, it is less abundant in hRPE cells (Yamashita et al., 2001) (Figure S2A). We therefore focused our attention on MondoA. MondoA expression levels were not affected by the knockdown of Atg7 and Atg13 (Figure S2B). We confirmed knockdown efficiency of siRNA knockdown against MondoA and its dimerization partner, Mlx (Billin et al., 2000), although they were likely to be regulated by each other (Figure S2C). We found that MondoA but not Mlx knockdown significantly enhanced expression levels of p16, p21, and p53 even in non-induced cells (Figure 2A), and also increased the number of SA-β-gal-positive cells among senescent cells (Figure 2B). In addition, MondoA depletion increased signs of senescence even in the replicative senescence model (Figure S2D). Consistent with this, DXR-induced SASP led to significantly increased expression of IL-6, CXCL10, and IFN-β in siMondoA-treated cells (Figure 2C).

Since Mondo A is required for activation of autophagy in long-lived worms, we next examined whether MondoA also regulates autophagy during cellular senescence. LC3 autophagic flux was decreased by MondoA depletion in both non-induced and senescent cells (Figure 2D), suggesting that MondoA prevents senescence, including induction of SASP expression by activating autophagy, which may not be together with canonical heterodimer partner Mlx. In addition, when we measured Atg mRNA expression, Atg7 but not Atg5 mRNA were significantly decreased in siMondoA-treated cells (Figure 2E), suggesting that MondoA could regulate some Atg transcription to retain autophagy during senescence.

Loss of MondoA disrupts the regulation of Prdx3 and Rubicon

To further understand the mechanism by which MondoA prevents DNA damage-induced cellular senescence, we compared transcriptomes in MondoA or Mlx knockdown cells, with or without DXR treatment, using RNA sequencing (RNA-seq). We confirmed effective knockdown of MondoA and Mlx (p = 3.01E−321 and p = 7.70E−100, respectively). Differentially expressed gene (DEG) analysis (false discovery rate [FDR] < 0.05) revealed that, in MondoA knockdown cells treated with DXR, only 246 upregulated and 574 downregulated genes were commonly regulated by MondoA and Mlx (10.3% and 18.1%, respectively). Eight hundred and sixty-four upregulated and 1,068 downregulated genes were regulated by a MondoA-dependent mechanism (36.3% and 33.7%, respectively), and 1,267 upregulated and 1,530 downregulated genes were Mlx dependent (53.3% and 48.2%, respectively), indicating that MondoA participates in senescence through Mlx-dependent and -independent pathways (Figure 3A).

When we performed gene ontology enrichment analysis from DEGs (FDR < 0.05) (Zhou et al., 2019) in MondoA knockdown cells treated with DXR, it indicated that pathways enriched in upregulated genes were the response to IFN-γ, regulation of cytokine production, negative regulation of cell population proliferation, and positive regulation of IκB kinase/NF-κB signaling (Figure 3B). Downregulated genes were enriched in pathways associated with cell division, regulation of the cell-cycle process, cell-cycle G1/S phase transition, kinase binding, and regulation of cellular response to stress (Figure 3C), consistent with the idea that MondoA reduction promotes cellular senescence.

To identify a key molecule in the MondoA pathway regulating senescence, we selected 15 genes based on significant p values, which were all downregulated in MondoA knockdown cells treated with DXR in the RNA-seq experiment (Table S1). Among them, nine genes were specifically downregulated in MondoA but not Mlx knockdown cells, and six genes were downregulated in both MondoA and Mlx knockdown cells treated with DXR. In addition, five genes that exhibited less dramatic downregulation were also selected for our downstream analyses because these genes are less thoroughly studied but have previously been associated with either autophagy or senescence (Levine and Kroemer, 2019; Wiley et al., 2016; Gorgoulis et al., 2019; Ravussin et al., 2021; Lee et al., 2019; Zidi et al., 2019). Although Txnip, which is induced by MondoA at high glucose concentrations (Stoltzman et al., 2008), was also significantly downregulated in both MondoA and Mlx knockdown cells exposed to DXR, we excluded Txnip from analyses hereafter because it has already been investigated in detail (Stoltzman et al., 2008; Ahn et al., 2016, 2019; Weger et al., 2020; Zhang et al., 2018, 2019).

When we examined the expression of senescence markers, we found that knockdown of Prdx3 led to drastic changes of p21, p53, and p-Rb in the presence or absence of DXR, although other factors, such as Vps35 and Pink1, also affected...
Figure 2. Retained MondoA delays cellular senescence through the autophagic pathway

(A) Expression of senescence markers in either siMondoA- or siMix-treated senescent cells (+DXR, for 3 days) or non-induced cells (−DXR). Representative western blots (left) and quantification of non-induced cells (right) from three or four independent experiments.

(B) Representative staining images (upper) and quantification (lower) of SA-β-gal-positive cells in siMondoA- or siMix-treated senescent cells induced by DXR treatment for 8 days (n = 3). Scale bar, 100 μm.

(C) Relative mRNA expression of SASP, including IL-6, CXCL10, and IFN-β in siMondoA-, siMix-, siAtg7-, or siAtg13-treated senescent cells (+DXR, for 3 days) or non-induced cells (−DXR) (n = 3–6).

(D) Representative western blots (upper) and quantification (lower) of LC3 in the siMondoA- or siMix-treated senescent cells (+DXR, for 3 days, n = 4) or non-induced cells (−DXR) (n = 3) in the presence or absence of Baf. A1 (125 nM) for 6 or 24 h, respectively.

(E) Atg5 and Atg7 mRNA expression during senescence in siMondoA-, siMix-, siAtg7-, or siAtg13-treated senescent cells (+DXR, for 3 days) or non-induced cells (−DXR) (n = 3). Values are presented as the mean ± SD, and statistical significance was determined using one-way ANOVA with Dunnett’s posttest.
the expression of these markers (Figure 3D). Among the genes showing substantial changes in expression of senescence markers, downregulation of Prdx3 is the most dramatic in MondoA knockdown cells based on p values using RNA-seq analyses (Table S1). These data were confirmed by qPCR (Figure 3E). Therefore, we focused on Prdx3 as a critical player involved in senescence, especially in a MondoA-regulated pathway. Prdx1-6, enzymatic antioxidants with redox signaling, are involved in the proper cell-cycle progression (Heo et al., 2020; Rhee and Kil, 2017). Consistent with this role, there is a report that Prdx3 levels are reduced in intrahepatic cholestasis pregnancy placenta with mitochondrial dysfunction and senescence (Wu et al., 2016). In addition, Rubicon was markedly increased in siMondoA-treated cells compared with controls exposed to DXR (p = 0.001472 in RNA-seq), and some autophagic factors, such as Atg7 and Atg12, were decreased in siMondoA-treated cells relative to controls, even in the absence of DXR (p = 4.71E−09 and p = 6.44E−06, respectively, in
Figure 4. MondoA contributes to mitochondrial homeostasis by regulating Prdx3

(A) Representative OCR profile (upper) and spare respiratory capacity (lower) from three independent experiments. The metabolic inhibitors, oligomycin (1.5 μM), FCCP (2 μM), and rotenone/antimycin A (0.5 μM) were injected into siMondoA-, siMlx-, siPrdx3-, or DXR-treated cells at the indicated time points.

(B) Mitochondrial distribution in siMondoA-, siMlx-, or siPrdx3-treated cells without DXR treatment detected by immunostaining with translocase of outer mitochondrial membrane 20 (TOMM20) antibody. Representative images (left) and quantification (right) of tubular mitochondria from three independent experiments. Scale bar, 10 μm.

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RNA-seq), consistent with the data in Figure 2E. These findings suggest that MondoA participates in maintaining autophagy through Rubicon and some Atgs during senescence. Looking at chromatin immunoprecipitation sequencing (ChIP-seq) data in the public domain by ChIP-Atlas (Oki et al., 2018), MondoA in the human skeletal myotubes was shown to bind to promoters/enhancers for Prdx3 and Rubicon (Ahn et al., 2019), further supporting the interplay of these proteins in transcriptional regulation (Figure S3).

MondoA contributes to mitochondrial homeostasis via Prdx3
Continuous mitochondrial fusion and fission are necessary for mitochondrial health (Chan, 2012), and mitochondrial quality control such as mitochondria-targeted autophagy, mitophagy, results in mitochondrial protein turnover at the organelle level and the prevention of some diseases (Pickles et al., 2018). Because MondoA shuttles between mitochondria and the nucleus (Sans et al., 2006; Peterson et al., 2010), and Prdx3 is located on mitochondria, we investigated whether Prdx3 has a significant role in maintaining mitochondrial function in the MondoA pathway regulating senescence. Mitochondrial spare respiratory capacity was significantly reduced in siMondoA- or siPrdx3-treated cells as well as with DXR treatment (Figure 4A). In addition, consistent with a report that mitochondria form a giant tubular network at the G1-S phase and that mistimed hyperfused tubular mitochondria contribute to cell-cycle defect (Mitra et al., 2009; Herbig et al., 2004), either siMondoA- or siPrdx3-treated cells, as well as DXR-induced and replication-induced senescent cells, showed increased tubular mitochondria staining with translocase of outer mitochondrial membrane 20 (Figures 4B, S4A, and S4B). On the other hand, siMix-treated cells did not show any increase in tubular formation. These data suggest that MondoA and Prdx3 are required for retaining functional mitochondria.

Since mitochondrial dynamics are regulated by phosphorylation of dynamin-related protein 1 (Drp1) by specific kinases and phosphatases, and phosphorylation of Drp1, especially at Ser616 promotes fission (Taguchi et al., 2007; Archer, 2013), we next assessed Drp1 phosphorylation in MondoA or Prdx3 knockdown cells. Phosphorylated levels of Drp1 at Ser616 were significantly attenuated in MondoA or Prdx3 knockdown cells, as well as in DXR-treated senescent cells (Figure 4C), consistent with a previous report for the expression of this protein in malignant mesothelioma cells (Cunniff et al., 2014). When siPrdx3-treated cells were incubated with Actinonin, an inducer of mitochondrial fragmentation (Richter et al., 2013) and mitophagy (Sun et al., 2015), p21 levels were significantly suppressed compared with the cells without Actinonin (Figure 4D). This observation was associated with the fragmentation of mitochondria (Figure 4E). These data indicate that MondoA and Prdx3 participate in Drp1-mediated mitochondrial fission, leading to suppression of senescence.

Since uncoupler CCCP decreases membrane potential before parkin recruitment to the mitochondria for mitophagy (Matsuda et al., 2010), we measured mitochondrial membrane potential. Both MondoA and Prdx3 knockdown cells showed significantly higher membrane potential compared with controls (Figure S4C). This finding is in agreement with a previous report that Prdx3 controls membrane potential and alters cell-cycle kinetics in cancer cells (Cunniff et al., 2014). In addition, because levels of Pink1, Bnip3, and Bnip3l, central regulators for mitophagy and mitochondrial dynamics (Landes et al., 2010), detected by RNA-seq were markedly reduced in MondoA knockdown cells treated with DXR (p = 3.76E−05, p = 3.06E−10, and p = 5.02E−10, respectively), we examined whether knockdown of Pink1 or Bnip3 affects senescence. Knockdown of either Pink1 or Bnip3 enhanced senescence detected by p21 and Lamin B1 expression levels (Figure 4F), and Pink1 depletion led to mitochondrial dysfunction as assessed by OCR (Figure S4D), supporting the idea that MondoA deficiency drives impaired mitophagy, contributing to mitochondrial dysfunction and is capable of senescence induction. Moreover, LC3 autophagic flux was significantly decreased (Figure 4G). These data indicate that Prdx3 participates in bulk autophagy for cellular maintenance as well as mitophagy for mitochondrial homeostasis.

We next assessed the possibility that Prdx3 could participate in organismal aging. We found the expression of prdx-3 (worm homolog of Prdx3) decreased during aging in C. elegans (Figure 4H). Importantly, prdx-3 was downregulated in mml-1 (worm homolog of MondoA)-depleted worms compared with wild-type animals (Figure 4I), suggesting that MondoA has a crucial role in Prdx3 regulation in C. elegans. Since disrupting the thiol redox state enhances mitochondrial ROS production, which accelerates aging, we analyzed reactive ROS production with DHE staining and found that ROS levels were elevated in both mml-1 and prdx-3 RNAi knockdown worms (Figure 4J). In agreement with this finding, Prdx3 overexpression in mice has been reported to reduce the amount of H2O2 in mitochondria (Chen et al., 2008). These results indicate that the regulation of...
Prdx3 by MondoA (mm1-1) is conserved in C. elegans and that this regulation might be relevant for animal aging.

Rubicon and Prdx3 work independently downstream of the MondoA pathway
Our RNA-seq results indicated that a negative regulator of autophagy, Rubicon, was upregulated in MondoA knockdown cells treated with DXR. We confirmed the RNA-seq results by western blotting and qPCR analysis even in the absence of DXR (Figures 5A and 5B). Since Rubicon was increased during senescence (Figures 1F and 1G) and aging (Nakamura et al., 2019), we speculated that decline of MondoA activity is one of the reasons for the observed increase in Rubicon, which drives senescence by inhibiting autophagy. Importantly, the level of Rubicon in Prdx3 knockdown cells was not significantly affected, while Prdx3 levels were also unaltered in Rubicon knockdown cells (Figure 5A). These results suggested that both Prdx3 and Rubicon affect senescence independently downstream of MondoA.

Transcriptional activation of MondoA by 2DG treatment or retaining autophagy during MondoA and Rubicon depletion attenuates senescence
To test the possibility that MondoA transcriptional activation could delay senescence, we overexpressed MondoA in hRPE cells. However, MondoA overexpression did not affect expression levels of p21, p53, and LAMP1 (Figure S5A) in senescent and non-induced cells. Since MondoA is a glucose sensor (Stoltzman et al., 2008), we next used 2-deoxy-D-glucose (2DG) which drives MondoA activity by increasing glucose-6-phosphate levels (Stoltzman et al., 2008; Kaadige et al., 2015; Peterson et al., 2010). As seen in Figure 6A, 2DG induced significant accumulation of MondoA in the nucleus (Figure S5B), further supporting hypothesis that MondoA may regulate senescence independent of its canonical partner, Mix.

Furthermore, to prove the cause-effect relationship between MondoA reduction, impaired autophagy, mitochondrial dysfunction, and cellular senescence, we confirmed whether double knockdown of MondoA and Rubicon could rescue the effect of MondoA depletion through activating autophagy. MondoA and Rubicon depletion rescued autophagic activity, associated with the reduced expression of p21 and increased levels of Lamin B1 in the absence of DXR (Figures 6C and 6D), suggesting that MondoA depletion induces cellular senescence dependent on its effect on autophagy. However, activation of autophagy during MondoA and Rubicon depletion did not rescue mitochondrial dysfunction determined by spare respiratory capacity (Figure S5C), which is consistent with the data shown in Figures S1F and S1G. These observations suggest that retaining autophagy delays senescence, independent of mitochondrial function.

To address whether impaired autophagy and mitochondrial dysfunction are independently involved in regulating senescence downstream of the MondoA pathway, we measured SASP expression during double knockdown of Atg7/Atg13 and Prdx3. Expression levels of IL-6 in siAtg13- and siPrdx3-treated cells were additionally enhanced compared with siAtg13- or siPrdx3-treated cells (Figure 6E). These data indicate that impaired autophagy and mitochondrial dysfunction contribute to senescence independently downstream of the MondoA pathway.

MondoA counteracts cellular senescence in aging and ischemic acute kidney injury in both mice and humans
To investigate the association between cellular senescence and the MondoA pathway in mice, we focused on the kidney because it is well known that senescent cells are prone to accumulate in this organ during aging (Baker et al., 2016). We found that Prdx3 expression was decreased in the aged kidney, while levels of Rubicon were increased (Figure S6A) (Nakamura et al., 2019). These changes were associated with enhanced cellular senescence, as determined by p21 expression (Figure S6B).
Inspired by previous reports that cellular senescence in tubular epithelial cells (TECs) plays an important role in the progression of kidney fibrosis after acute kidney injury (AKI) (Docherty et al., 2019; Li et al., 2021), we next investigated the role of MondoA in cellular senescence in ischemic AKI using TEC-specific MondoA knockout (MondoAflox/flox; Kap-cre) and control (MondoAflox/flox) mice. To evaluate cellular senescence in ischemic AKI, mice were subjected to sham operation or unilateral ischemia-reperfusion (I/R) injury. Notably, expression of p21 was significantly exacerbated in MondoA knockout mice compared with control mice at 48 h post-I/R injury (Figure 7A), indicating that MondoA inhibits cellular senescence in TECs during ischemic AKI.

To determine if the findings described above apply to humans, we compared kidney biopsy specimens between young and aged individuals, and ischemic AKI patients (Table S2). Immunohistochemical analysis revealed that, in aged individuals, the
number of p21-positive TECs was significantly higher compared with the young individuals, and this was accompanied by decreased expression of MondoA in the nucleus (Figure 7B). Similar to the aged kidneys, in the kidneys from ischemic AKI patients (Table S2), p21-positive senescent TECs were significantly increased while decreased expression of nuclear MondoA was observed (Figure 7B). Although it is not clear at this moment whether the cells that show decreased nuclear MondoA signals are the ones that exhibit increased p21 signals in ischemic AKI sections, collectively, MondoA counteracts cellular senescence in aging and ischemic AKI in both mice and humans.

**DISCUSSION**

Our findings demonstrate that MondoA delays cellular senescence by activating autophagy through suppression of Rubicon, and maintaining mitochondrial homeostasis via Prdx3 (Figure 7C). Decline of MondoA in the nucleus during aging causes dysregulation of autophagy and mitochondrial quality control, triggering cellular senescence and exacerbation of AKI.

We revealed that MondoA and Mix independently contribute to attenuation of senescence, such as SASP expression, autophagic activity, and the regulation of Prdx3 and Rubicon. Because MondoA was identified as an additional binding partner of Mix (Billin et al., 2000), there is a possibility that MondoA also interacts with additional proteins other than Mix. This idea may be supported by recent work showing that MondoA and Mix independently bind to dynamic organelle lipid droplets, which are accumulated with aging and in age-related diseases (Mar-schallinger et al., 2020; Beas et al., 2020), to reduce their transcriptional activity (Mejhert et al., 2020). In addition, it is possible that MondoA-Mix heterodimerization could be inhibited by binding of MondoA to mechanistic target of rapamycin complex 1 (mTORC1) in the presence of ROS, where mTORC1 negatively regulates MondoA activity (Kaadige et al., 2015). Decreased autophagy by activation of the mTORC1 pathway (Leidal et al., 2018) might be involved in impaired MondoA activity leading to acceleration of cellular senescence.

MondoA depletion would have other mechanisms to induce senescence in addition to impaired autophagy because there is surely a time lag between the decrease of autophagy and the induction of senescence markers including p21. In MondoA-depleted cells, we detected LAMP1 upregulation as well as a significant decrease of sodium-coupled neutral amino acid transporter 7, a selective transporter for glutamine and asparagine on lysosomes (p = 3.93E−47 in non-induced cells, and p = 1.38E−49 in senescent cells) (Kandasamy et al., 2018; Verdon et al., 2017). This finding suggests that MondoA
contributes to lysosomal homeostasis (Wild et al., 2019; Chen et al., 2010). MondoA is required for the activation of transcription factor EB (TFEB), a central regulator of lysosome biogenesis and autophagy, in C. elegans (Nakamura et al., 2016). Interestingly, since TFEB localization is regulated by amino acid transporters (AATs) (Verdon et al., 2017), MondoA may participate in lysosomal homeostasis through AATs.

Recent reports have elucidated that cellular senescence is associated with various human disorders, and senolytics, which target senescent cells for elimination, have received considerable attention as a potential strategy to overcome age-associated diseases (Paez-Ribes et al., 2019). However, the side effects of senolytics should be carefully monitored because the specific population of senescent cells is structurally and functionally important and acute removal of senescent cells deteriorates organ function under specific situations (Grosse et al., 2020). In this regard, strategies to activate the molecular pathway to suppress cellular senescence seem to be safe and promising. Decreased autophagy and mitochondrial dysfunction are known to be common features of various human age-associated diseases (Partridge et al., 2018), although the molecular links among them have been largely unknown. In this respect, our results clarify the close relationship between decreased autophagy and mitochondrial dysfunction during aging: dysregulation of MondoA decreases autophagic activity via the upregulation of Rubicon concomitant with disturbing mitochondrial function via the downregulation of Prdx3 during kidney aging.

Recently, neuronal progenitor cells from ataxia-telangiectasia disease, which show senescence-like phenotype, were found to express higher levels of Rubicon (Sunderland et al., 2020). Although Rubicon would not work on mitophagy in adipocytes (Yamamuro et al., 2020), the role of Rubicon in Prdx3-regulating mitochondrial dynamics needs to be elucidated. Furthermore, although we observed that MondoA in the nucleus was reduced in TECs during senescence-associated AKI and aging in mice and humans, concentrations of MondoA and Mix in the nucleus were unaltered in DNA damage-induced and replicative senescence (data not shown). It has been reported that transcriptional activity of MondoA in response to glucose depends on not only nuclear accumulation but also promoter occupancy and coactivator recruitment to target promoters (Peterson et al., 2010). Clarification of how the important step of the transcriptional regulation of Prdx3 and Rubicon by MondoA is capable of improving senescence is essential to a deeper understanding of the mechanism of aging and age-associated disease.

Limitations of the study
We suggest that Rubicon and Prdx3 work independently downstream of MondoA pathway. Regarding MondoA levels, they remained unaltered at day 10 after DXR treatment, and after knocking down of Atg7 and Atg13. Although we found that MondoA in the nucleus was decreased in the human aged kidney compared with young individuals, we still need to clarify the changes of MondoA binding ability to promoters/enhancers of Rubicon/Prdx3 during aging. There are likely other factors that also contribute to this transcription that are currently unknown and further analyses are needed. In addition, we should confirm if cells with decreased MondoA also exhibited increased p21 and Rubicon, and decreased Prdx3 in mouse and human kidney sections, although these experiments are currently challenging due to both technical problems and limited samples.

STAR Methods
Detailed methods are provided in the online version of this paper and include the following:

- Key resources table
- Resource availability
  - Lead contact
  - Materials availability
  - Data and code availability
- Experimental model and subject details
  - Cell culture
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  - SA-β-gal positivity
  - Cellular oxygen consumption rate
  - ATP measurement
  - Mitochondrial membrane potential
  - Reactive oxygen species (ROS)
- Quantification and statistical analysis
  - Statistical analysis
  - Processing of RNA-sequencing data
  - Image analysis

Supplemental Information
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.110444.

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AUTHOR CONTRIBUTIONS

H.Y.-I., S. Minami, and S.N. wrote the manuscript. H.O. helped plan ChIP analyses. R.E. and Y.O. performed bioinformatics analyses. D.E.A. and E.H. kindly gave us the MondoA \textit{lox/lox} mice and cells to induce senescence, respectively. H.Y.-I., S. Minami, and S.N. wrote the manuscript.

DECLARATION OF INTERESTS

T.Y. is a founder of AutoPhagyGO.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**       |        |            |
| Rabbit polyclonal anti-MondoA | Bethyl Laboratories | Cat#A303-195A; RRID:AB_10950904 |
| Rabbit monoclonal anti-Rubicon (D9F7) | Cell Signaling Technology | Cat#8465; RRID:AB_10891617 |
| Rabbit polyclonal anti- Peroxiredoxin 3/ PRDX3 | Abcam | Cat#ab73349; RRID:AB_1860862 |
| Rabbit monoclonal anti-Atg7 (D12B11) | Cell Signaling Technology | Cat#8558; RRID:AB_10831194 |
| Rabbit monoclonal anti-Atg13 (E1Y9V) | Cell Signaling Technology | Cat#13468; RRID:AB_2797419 |
| Rabbit polyclonal anti-LC3 | MBL | Cat#PM036; RRID:AB_2274121 |
| Rabbit monoclonal anti-p62 (SQSTM1) | MBL | Cat#PM045; RRID:AB_1273901 |
| Mouse monoclonal anti-Mlx | Santa Cruz Biotechnology | Cat#sc-393086; RRID:AB_2801474 |
| Goat polyclonal anti-ChREBP (P-13) | Santa Cruz Biotechnology | Cat#sc-21189; RRID:AB_2146396 |
| Rabbit monoclonal anti-p21 [EPR3999] | Abcam | Cat#ab109199; RRID:AB_10861551 |
| Rabbit monoclonal anti-p21 Waf1/Cip1 (12D1) | Cell Signaling Technology | Cat#2947; RRID:AB_823586 |
| Mouse monoclonal anti-p16INK4a (1H4) | IBL | Cat#11104 |
| Mouse monoclonal anti-p53 (DO-1) | Santa Cruz Biotechnology | Cat#sc-126; RRID:AB_628082 |
| Goat polyclonal anti-LaminB (M-20) | Santa Cruz Biotechnology | Cat#sc-6217; RRID:AB_648158 |
| Rabbit polyclonal anti-Lamin B1 - Nuclear Envelope Marker | Abcam | Cat#ab16048; RRID:AB_10107828 |
| Mouse monoclonal anti-LAMP1 (H4A3) | Santa Cruz Biotechnology | Cat#sc-20011; RRID:AB_626853 |
| Rabbit monoclonal anti-Phospho-AMPKα (Thr172) (40H9) | Cell Signaling Technology | Cat#2535; RRID:AB_331250 |
| Rabbit monoclonal anti-AMPKα | Cell Signaling Technology | Cat#2532; RRID:AB_330331 |
| Rabbit monoclonal anti-Phospho-NFκB p65 (Ser536) (93H1) | Cell Signaling Technology | Cat#3033; RRID:AB_331284 |
| Mouse monoclonal anti- NFκB p65 (A-12) | Santa Cruz Biotechnology | Cat#sc-514451; RRID:AB_2891257 |
| Rabbit monoclonal anti-Phospho-Rb (Ser807/811) | Cell Signaling Technology | Cat#3455; RRID:AB_2083352 |
| Mouse monoclonal anti-Rb (IF8) | Santa Cruz Biotechnology | Cat#sc-102; RRID:AB_628209 |
| Mouse monoclonal anti-Tom20 (F-10) | Santa Cruz Biotechnology | Cat#sc-17764; RRID:AB_628381 |
| Rabbit monoclonal anti-Phospho-Drp1 (Ser616) | Cell Signaling Technology | Cat#8570; RRID:AB_10950498 |
| Mouse monoclonal anti- alpha Tubulin | Abcam | Cat#ab11304; RRID:AB_297909 |
| Rabbit polyclonal anti-α-Tubulin | MBL | Cat#PM054; AB_10598496 |
| Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) | Jackson ImmunoResearch | Cat#111-035-003; RRID:AB_2313567 |
| Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L) | Jackson ImmunoResearch | Cat#115-035-003; RRID:AB_10015289 |
| Peroxidase-AffiniPure F(ab')2 Fragment Rabbit Anti-Goat IgG (H+L) | Jackson ImmunoResearch | Cat#305-036-003; RRID:AB_2339406 |
| Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488) | Abcam | Cat#ab150073; RRID:AB_2636996 |
| Donkey Anti-Mouse IgG H&L (Alexa Fluor® 568) | Abcam | Cat#ab150072; RRID:AB_2636996 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Human kidney samples, See Table S2 | This paper | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Doxorubicin | FUJIFILM Wako Chemicals | 040-21521; CAS RN® : 25316-40-9 |
| 2-deoxy-D-glucose | Sigma | D8375; CAS: 154-17-6 |
| Actinonin | Enzo Life Sciences | ALX-260-128; CAS: 13434-13-4 |
| Bafilomycin A1 | Cayman Chemical | 11038; CAS RN® : 88899-55-2 |
| Valinomycin | Sigma-Aldrich | V0627; CAS: 2001-95-8 |
| Dihydroethidium | Thermo Fisher Scientific | D11347; CAS: 38483-26-0 |
| Lipofectamine RNAiMax | Invitrogen | 13778150 |
| TRIzol™ Reagent | Thermo Fisher Scientific | 15596018 |
| QIAzol Lysis Reagent | QIAGEN | 79306 |
| Power SYBR® Green PCR Master Mix | Thermo Fisher Scientific | 4367659 |
| VECTASHIELD® Antifade Mounting Medium with DAPI | Vector Laboratories | H-1200 |
| Dako Target Retrieval Solution, pH 9 | Agilent Technologies | S2368 |
| Histofine® simple stain DAB solution | Nichirei Biosciences | 415171; CAS: 7411-49-6 |
| **Critical commercial assays** | | |
| RNeasy® Plus Mini Kit | QIAGEN | 74134 |
| miRNeasy® Mini Kit | QIAGEN | 217004 |
| iScript™ cDNA Synthesis Kit | BIO-RAD | 1708891 |
| Pierce™ bicinchoninic acid Protein Assay Kit | Thermo Fisher Scientific | 23225 |
| TruSeq Stranded mRNA Library Prep Kit | Illumina | 20020594 |
| Click-IT® EdU Imaging Kit | Invitrogen | C10337 |
| Senescence Cells Histochemical Staining Kit | Sigma | CS0030 |
| Cellular Senescence Plate Assay Kit | Dojindo | SG05 |
| Cell Count Normalization Kit | Dojindo | CS44 |
| XF Cell Mito Stress Test Kit (includes Oligomycin, FCCP, and Rotenone/ Antimycin A) | Agilent Technologies | 103015-100 |
| Deproteinization Sample Preparation Kit | BioVision | K808 |
| ATP Colorimetric/Fluorometric Assay Kit | BioVision | K354-100 |
| JC-1 MitoMP detection Kit | Dojindo | MT09 |
| **Deposited data** | | |
| RNA-sequence data | This paper | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179016 |
| **Experimental models: Cell lines** | | |
| hRPE cells | Lonza Inc. | 00194987 |
| TIG-3 cells | JCRB | JCRB0506 |
| **Experimental models: Organisms/strains** | | |
| C. elegans: Strain AA3234; mrl-1 (ok849) III | Nakamura et al., 2016 | N/A |
| Mouse: Systemic Rubicon knockout | Nakamura et al., 2019 | N/A |
| Mouse: MondoA flox | Ahn et al., 2019 | N/A |
| Mouse: KAP-Cre transgenic | Kimura et al., 2011 | N/A |
| **Oligonucleotides** | | |
| sRNAs, see Table S3 | N/A |
| Primers for qPCR analysis, see Table S4 | N/A |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tamotsu Yoshimori (tamyoshi@fbs.osaka-u.ac.jp).

Materials availability
All materials generated in this study will be available upon request to the lead contact with a Material Transfer Agreement.

Data and code availability
- RNA-seq data used in this study have been deposited to the National Center for Biotechnology Information Gene Expression Omnibus with accession number GSE179016.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture
human retinal pigment epithelial (hRPE) cells and TIG-3 cells were obtained from Lonza Inc., and the Japanese Cancer Research Resources Bank (JCRB), respectively. Cells were cultured in Dulbecco’s Modified Eagle’s medium (D6046, Sigma) supplemented...
with 10% fetal bovine serum (10270106, Gibco) and penicillin-streptomycin (P4333, Sigma), hRPE cells were incubated with 150 ng/mL doxorubicin (040-21521, FUJIFILM Wako Chemicals) for 3 or 8 days to induce DNA damage-induced cellular senescence. In replicative senescence, late passage TIG-3 cells (>70 population doublings, PDL) were used as senescent cells and early passage TIG-3 cells (<46 PDL) were as controls. Senescent and/or non-induced cells were incubated in the presence or absence of 2-deoxy-D-glucose (2DG, D8375, Sigma), Actinonin (ALX-260-128, Enzo Life Sciences), or Bafilomycin A1 (Baf. A1; 11038, Cayman Chemical) as indicated in the figure legends.

C. elegans
All C. elegans strains were cultured at 20°C on standard NGM plates seeded with the Escherichia coli strain OP50. N2 and mml-1 (ok849) III were used as the wild-type and mml-1 mutant (Strain AA3234), respectively.

Mouse studies
Systemic Rubicon knockout mice on a C57BL/6 background were generated as described previously (Nakamura et al., 2019). Conditional TEC-specific MondoA knockout mice on a C57BL/6 background were generated by crossing mice bearing a MondoA\textsuperscript{ex} allele (Ahn et al., 2019) with KAP-Cre transgenic mice, in which Cre recombinase is expressed under control of the KAP promoter (Kimura et al., 2011). I/R injury was performed on 8- to 10-week-old male mice as described previously (Kimura et al., 2011). Mice were housed in box cages, maintained on a 12 hours light/12 hours dark cycle, and fed a normal fat chow diet (Oriental Yeast, MF). The ambient temperature and humidity was 23°C ± 1.5°C and 45% ± 15%, respectively. All animal experiments were approved by the institutional committee of the Research Committee of Osaka University and the Japanese Animal Protection and Management Law (No. 25). We have complied with all relevant ethical regulations.

Human studies
All human studies were approved by the Institutional Review Board of Osaka University Hospital (IRB number 17334 and 20504) and adhered to the Declaration of Istanbul. We have complied with all of the relevant ethical regulations and informed consent was obtained. All kidney specimens were obtained from living patients by fine-needle aspiration during the last 8 yr. Young specimens came from biopsy specimens of young people (under 40 years old) with no significant abnormal changes. Aged specimens were obtained from biopsy sections of elderly people (over 60 years old) diagnosed as no significant abnormal changes or benign nephrosclerosis, which is the representative pathological findings in the aged kidney (Rule et al., 2010; Sturmlechner et al., 2017). Ischemic AKI specimens were derived from zero-h implantation biopsy specimens of patients who developed ischemia-induced post-transplant delayed graft function, which is defined as the need for dialysis in the first week following transplantation. Details are in Table S2.

METHOD DETAILS

Antibodies
The following antibodies were used: anti-MondoA (A303-195A, Bethyl Laboratories), anti-Rubicon (8465, Cell Signaling Technology), anti-Prdx3 (ab73349, Abcam), anti-Atg7 (8558, Cell Signaling Technology), anti-Atg13 (13468, Cell Signaling Technology), anti-LC3 (PM036, MBL), anti-p62 (PM045, MBL), anti-Mix (sc-393086, Santa Cruz Biotechnology), anti-ChREBP (sc-21199, Santa Cruz Biotechnology), anti-p21 (ab109199, Abcam; 2947, Cell Signaling Technology), anti-p16 INK4a (11104, IBL), anti-p53 (sc-126, Santa Cruz Biotechnology), anti-Lamin B (sc-6217, Santa Cruz Biotechnology; ab16048, Abcam), anti-LAMP1 (sc-20011, Santa Cruz Biotechnology), anti-Phospho-AMPK\textsubscript{a} (Thr172) (2535, Cell Signaling Technology), anti-Phospho-AMPK\textsubscript{z} (Thr172) (2532, Cell Signaling Technology), anti-Phospho-NF\textsubscript{k}B (Ser536) (3033, Cell Signaling Technology), anti-Phospho-NF\textsubscript{k}B (Ser536) (3033, Cell Signaling Technology), anti-p21 (ab109199, Abcam), anti-p62 (PM045, MBL), Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) (111-035-003, Jackson ImmunoResearch), Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L) (111-035-003, Jackson ImmunoResearch), Peroxidase-AffiniPure F(ab')2 Fragment Rabbit Anti-Goat IgG (H+L) (305-036-003, Jackson ImmunoResearch), Donkey Anti-Rabbit IgG H&L (Alexa Fluor\textsuperscript{®} 488) (ab150073, Abcam), and Donkey Anti-Mouse IgG H&L (Alexa Fluor\textsuperscript{®} 568) (ab17542, Abcam).

Plasmid construction
Human MondoA cDNA fragments were amplified by using the following primers and the digested fragments were then cloned into Xhol/Sall site of pENTR vector: Sall MondoA f: Tca gtgacgccgccgcc gcc gcc gcc gtc tct at: Notl MondoA r: a gT cCG CGC Ccg gga gga ctc cca tct cct gc. pMRX mStrawberry-MondoA was then generated by the gateway system (Invitrogen).

Transfection of siRNA
siRNAs were transfected to cells using Lipofectamine RNAiMax (13778150, Invitrogen) as manufacturer’s instructions. siRNAs used in this study can be found in Table S3. Two days after the transfection, cells were incubated with DXR. For analyses in worms, RNAi was conducted by feeding HT115 (DE3) bacteria transformed with L4440 vector that produces double stranded
RNA against the targeted gene (Nakamura et al., 2016). Cultures (37°C, 13 hours) in LB medium supplemented with Ampicillin Sodium (014-23302, Fujifilm Wako Chemicals) and Tetracycline Hydrochloride (T2525, Tokyo Chemical Industry) were diluted 1/5 and seeded on NGM growth containing ampicillin and Isopropyl-β-D-thiogalactopyranoside (092-05863, Fujifilm Wako Chemicals). Adult day 1 worms synchronized by egg lay were put on the plates.

Isolation of RNA, RT, and qPCR
Isolation of total RNA and the following cDNA synthesis were performed using RNeasy® Plus Mini Kit (74134, QIAGEN) for cells or TRIzol™ Reagent (15596018, Thermo Fisher Scientific) for mouse tissues, and iScript™ cDNA Synthesis Kit (1708891, BIO-RAD), respectively. Regarding worms, 200 worms were harvested in QIAzol Lysis Reagent (79306, QIAGEN) at day 1 of adulthood. Total RNA was extracted using miRNeasy® Mini Kit (217004, QIAGEN). Quantitative PCR were performed using Power SYBR® Green PCR Master Mix (4367659, Thermo Fisher Scientific). Primer details are in Table S4. Targets were measured using QuantStudio™ Real-Time PCR Software version 1.3. (Thermo Fisher Scientific).

RNA sequencing
RNA sequencing was conducted at the Center of Medical Innovation and translational research in Osaka University. RNA-seq libraries were prepared with the TrueSeq Stranded mRNA Library Prep Kit (20020594, Illumina), and quality and quantity was assessed at all steps by capillary electrophoresis (Agilent Bioanalyzer and Agilent TapeStation). Libraries were quantified by qPCR, immobilized and processed onto a flow cell with a cBot (Illumina) followed by sequencing-by-synthesis with NovaSeq 6000 S4 chemistry on a NovaSeq 6000 at Macrogen-Japan.

Western blotting
Cells were lysed on ice in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (11873580001, Roche). After centrifugation, the protein concentrations of supernatant were established by Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (23225, Thermo Fisher Scientific), and dissolved with sample buffer (30% glycercol, 50 mM Tris-HCl, 10% SDS, 250 mM dithiothreitol, 10 mM EDTA, 0.1% bromophenol blue, pH 6.8) for western blot. Lysates were run on reducing SDS-PAGE gels, transferred to PVDF membrane (IPVH00010, Merck). Membrane was stained with Ponceau-S, and incubated overnight at 4°C in primary antibody, and then incubated with appropriate HRP-conjugated secondary antibody. Blots were developed with Immobilon forte western HRP substrate (WBLUF0500, Merck) or ImmunoStar LD (290-69904, Fujifilm Wako), and signals were detected in ChemiDoc™ Touch Imaging System (Biorad).

Immunofluorescence staining
After fixing with 4% paraformaldehyde (09154-85, Nacalai tesque) for 20 minutes at room temperature, cells were incubated with 50 μg/mL digitonin in phosphate-buffered saline (PBS) for 10 minutes at room temperature to detect mitochondrial structure, or treated with 0.1% TritonX-100 in PBS for 5 minutes to detect MondoA and Mix in the nucleus. Cells were incubated with 0.2% gelatin in PBS for 30 minutes, and then reacted with primary antibody for 60 minutes at room temperature. The following incubation with an appropriate Fluor-labeled secondary antibody for 60 minutes, cells were covered with VECTASHIELD® Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories) to analyze with FV3000 Confocal Laser Scanning Microscope (Olympus) operated by FV31S-SW (version 2.3.1.163).

For EdU staining, cells were cultured with EdU (10 μM) for 24 hours using Click-iT® EdU Imaging Kit (C10337, Invitrogen) according to the manufacturer’s instructions. Cells were fixed with 4% PFA for 10 min, permeabilized in 0.5% Triton X-100 for 30 minutes, and incubated with Click-iT reaction cocktail for 30 minutes. After staining using Hoechst 33342, cells were analyzed as described above.

Immunohistochemical staining
p21 staining was performed on paraffin-embedded sections. After antigen retrieval by autoclaving in Dako Target Retrieval Solution, pH 9 (S2368, Agilent Technologies) for 10 minutes at 120°C, the sections were blocked with 1% bovine serum albumin (BSA; A3059, Sigma-Aldrich) in PBS (pH 7.4) for 60 minutes. The blocked sections were incubated with anti-p21 antibody (1:200; ab109199, Abcam) at 4°C overnight, incubated with biotinylated anti-rabbit antibody (BA-1000, Vector Laboratories, 1:200) for 30 minutes at room temperature, followed by detection using a HRP-diaminobenzidine compound (Histofine® simple stain DAB solution, 415171, Nichirei Biosciences). Sections were counterstained with hematoxylin. To quantify p21-positive kidney proximal tubular epithelial cells, at least 10 high-power fields were reviewed for each tissue in a blinded manner. For human samples, it was performed on formalin fixed, paraffin-embedded sections after antigen retrieval via autoclaving in 0.01 mM citrate buffer (pH 6.0) for 10 minutes at 120°C and blocking with 1% BSA in PBS for 60 minutes. The primary antibodies and dilutions used for immunohistochemistry of the human kidney sections were as follows: p21 (1:200; 2947, Cell Signaling Technology), MondoA (1:100; A303-195A, Bethyl laboratories). Each specimen was scored on a 4-point scale (1, weakest; 4, strongest) based on the intensity of staining for each molecule in kidney TECs as described previously (Tran et al., 2016). For quantification, at least 10 high-power fields were reviewed for each tissue in a blinded manner.
SA-β-gal positivity
SA-β-gal positivity was measured using Senescence Cells Histochemical Staining Kit (CS0030, Sigma). Briefly, cells were incubated with Fixation buffer for 7 minutes at room temperature, stained with Staining mixture at 37°C for 24 hours without CO₂, and then observed using microscope (BX53, Olympus). Additionally, we measured SA-β-gal positivity using Cellular Senescence Plate Assay Kit (SG05, Dojindo) in Figure 1B. Cells were lysed with Lysis Buffer for 10 minutes at room temperature and then incubated with SPiDER-βGal working solution at 37°C for 30 minutes. After adding Stop Solution, excitation at 535 nm/emission at 580 nm were recorded in plate reader (SH-9000Lab, Hitachi). Cell number among wells were adjusted using Cell Count Normalization Kit (CS44, Dojindo).

Cellular oxygen consumption rate
Cellular oxygen consumption rate (OCR) was measured using a Seahorse XF extracellular flux analyzer (Agilent Technologies) according to the manufacturer’s instructions. Briefly, cells in Seahorse XF DMEM medium, pH 7.4 (103575-100, Agilent Technologies) supplemented with 10 mM glucose (103577-100, Agilent Technologies), 1 mM pyruvate (103578-100, Agilent Technologies), and 2 mM L-glutamine (103579-100, Agilent Technologies) were stimulated with Oligomycin (1.5 μM), FCCP (2 μM), and Rotenone/Anti-mycin A (0.5 μM) (103015-100, XF Cell Mito Stress Test Kit, Agilent Technologies) to determine mitochondrial activity. OCR was analyzed with Wave software v2.6.1 (Agilent Technologies).

ATP measurement
Cellular ATP levels were determined using Deproteinization Sample Preparation Kit (K808, BioVision) and ATP Colorimetric/Fluorometric Assay Kit (K354-100, BioVision) following the manufacturer’s instructions. Signals were detected in SH-9000Lab (HiTACHI), and normalized to protein concentration.

Mitochondrial membrane potential
Mitochondrial membrane potential was measured using JC-1 MitoMP detection Kit (MT09, Dojindo) according to the manufacturer’s instructions. Briefly, after the incubation with 4 μM JC-1 Working Solution for 90 minutes at 37°C and 5% CO₂, cells in Imaging Buffer were analyzed using CQ-1 Confocal Quantitative Image Cytometer (Yokogawa). Valinomycin (10 μg/mL, V0627, Sigma-Aldrich) was treated for 3 hours at 37°C and 5% CO₂ before the assay.

Reactive oxygen species (ROS)
The levels of ROS accumulation in C. elegans. were detected using the ROS-sensitive dye Dihydroethidium (DHE) (D11347, Thermo Fisher Scientific) as described (Wei and Kenyon, 2016). Briefly, after the incubation with M9 buffer containing 3 μM DHE for 30 minutes, worms were washed with M9 buffer and aligned on pre-cooled NGM plate to detect the signals using SZX16 (Olympus).

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis
All analyses were performed using GraphPad Prism 8 software. Statistical analyses were assessed as described in the text using either unpaired t-test or one-way ANOVA followed by Dunnett’s or Tukey’s multiple comparisons test. For nonparametric data, Kruskal-Wallis test followed by Dunn’s Multiple Comparison posttest were used. Data are denoted as means ± standard deviation (SD), or presented as vertical scatter plots with medians. p values ≤ 0.05 were considered significant (*), with values ≤ 0.01 designated **, values ≤ 0.001 designated ***. and values ≤ 0.0001 designated ****.

Processing of RNA-sequencing data
Reads were pre-processed by using Trimmomatic (v0.35) (Bolger et al., 2014). The trimmed reads were aligned to reference using TopHat (v2.1.1) (Kim et al., 2013). Read counts for each sample were obtained using FeatureCounts (v1.4.6) (Liao et al., 2014). Normalization and significance testing were done using the Bioconductor package, edgeR (Robinson et al., 2010). Differential expression was defined as with the FDR < 0.05. GO annotation was performed using metascape.org (Zhou et al., 2019).

Image analysis
Protein levels were quantified using densitometry with ImageJ (NIH). Accumulation of MondoA and Mix into the nucleus were determined by counting over 30 cells in each conditions from three independent experiments using Fiji (ImageJ version 2.1.0/1.53c) (Schneider et al., 2012). To detect membrane potential, more than 150 cells were quantified per condition using CQ-1 Confocal Quantitative Image Cytometer (Yokogawa, version 1.05.01.01).