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海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

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4. 受入機関名及び部局名
ジョンズ・ホプキンズ大学医学部細胞生物学科

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

Eliminating damaged mitochondria *in vivo*

Tatsuya Yamada, Ph.D. (Department of Cell Biology)

Mitophagy maintains mitochondrial health

Mitochondria are essential cellular organelles that participate in a variety of cellular processes, such as bioenergetics, metabolism, and cell death. A prominent feature of mitochondria is their dynamic nature in which they continuously divide and fuse to maintain their healthy structures within cells. Although the exact roles of mitochondrial division and fusion remain to be determined, these processes have been shown to play critical roles in mitochondrial function[1, 2]. Mitochondria employ multiple quality control systems to maintain their function and integrity against highly toxic reactive oxygen species that they themselves produce. Autophagic elimination of damaged mitochondria, termed mitophagy, makes an important contribution to mitochondrial turnover. However, its molecular underpinning is poorly understood and controversial especially in *in vivo* systems since previous studies have predominantly used cell culture systems. Mechanisms of mitophagy found in *in vitro* culture systems often fail to show the clear relevance of autophagy *in vivo*. I recently discovered a novel mechanism that drives mitophagy *in vivo* in addition to its significance for the pathogenesis and treatment of the non-alcoholic fatty liver disease (NAFLD), such as non-alcoholic steatohepatitis (NASH), as discussed by Yamada et al, Cell Metab. (2018)[3] and Yamada et al., Autophagy, (2019)[4].

Mitochondrial size controls mitophagy

Previous studies, including mine, have shown that in the absence of Drp1, a dynamin-related GTPase that mediates mitochondrial division, mitophagy is slowed in the brain and heart in tissue-specific Drp1 knockout (KO) mice[5,6]. As a consequence, mitophagy intermediates that contain ubiquitinated proteins, p62 and LC3, show a significant accumulation within cells. This Drp1 loss causes cell death in neurons and cardiomyocytes, thus making it difficult to further analyze the molecular mechanism of mitophagy in these cells. To overcome this issue, I decided to employ the hepatocyte-specific gene deletion model since the loss of Drp1 in hepatocytes does not cause cell death. I found that mitochondrial size in Drp1KO hepatocytes was significantly increased due to unopposed fusion in the absence of Drp1-mediated mitochondrial division. I also deleted a mitochondrial fusion gene, Opa1, and found that that in contrast to Drp1KO hepatocytes, the mitochondrial size in Opa1KO hepatocytes was significantly decreased due to unopposed division (Figures 1A-C). When both Drp1 and Opa1 were knocked out, mitochondria in Drp1Opa1KO hepatocytes returned to approximately their original size (Figures 1A-C). These data show that Drp1-mediated division and Opa1-mediated fusion act in an antagonistic manner to maintain mitochondrial size and that Drp1Opa1KO re-establishes mitochondrial size in the absence of these activities.

To directly visualize mitophagy *in vivo* in the liver, I developed a mitophagy biosensor (mitochondrial matrix targeted fusion protein of mCherry and GFP; Su9-mCherry-GFP) and introduced the plasmids to hepatocytes via hydrodynamic tail vein injection. Su9-mCherry-GFP is first imported into the mitochondria, in which both mCherry and GFP signals are observed[7, 8]. When mitochondria are subjected to mitophagy and transported to lysosomes, only the mCherry signal is detected because the GFP signal is lost due to the acidic pH of the lysosomes (Figure 1D). Using this mitophagy biosensor, I found that mitophagy is indeed decreased in Drp1KO hepatocytes. Furthermore, restoring normal mitochondrial size in Drp1Opa1KO hepatocytes caused restoration of mitophagy, demonstrating that mitochondrial size is critical for driving mitophagy (Figures 1E and F). In addition, in Drp1KO hepatocytes, I showed that mitophagy intermediates, which had accumulated in Drp1KO hepatocytes, were eliminated in Drp1Opa1KO hepatocytes (Figures 1G and H). This finding is consistent with the restoration of mitophagy in the double KO hepatocytes. These results show that Drp1 knockout significantly decreases mitophagic flux and that the delay of the mitophagy causes accumulation of mitophagy intermediates. Maintaining healthy mitochondrial size is important for mitophagy.

Discovering the enzyme complex that ubiquitinates mitochondria in mitophagy *in vivo*

Since the ubiquitination of damaged mitochondrial protein is the initial step to induce mitophagy, I examined how mitochondrial ubiquitination occurs *in vivo*. It has been proposed that the mitochondrial ubiquitination is mainly dependent on Parkin (a ubiquitin E3 ligase) and PINK1 (a protein kinase that phosphorylates Parkin and ubiquitin), both of which are defective in Parkinson's disease[9]. However, I found that further loss of Parkin or PINK1 in Drp1KO hepatocytes did not reduce mitochondrial ubiquitination (Figure 1I), suggesting Parkin or PINK1 are not required for the ubiquitination of mitochondrial proteins. Instead, I discovered that the ubiquitin ligase complex consisting of p62 (adaptor protein), Keap1 (scaffold protein that connects p62 to Rbx1), and Rbx1 (E3 ubiquitin ligase) ubiquitinates mitochondrial proteins *in vivo*. The ubiquitin ligase complex was recruited to mitochondria in Drp1KO hepatocytes but not in Drp1p62KO hepatocytes (Figures 1J and K). Mitochondrial recruitment of Keap1 and Rbx1 depends on the mitochondrial localization of p62. This was quite surprising because p62 has been considered to be downstream of ubiquitination; however, in contrast to this prevalent view, my data show that p62 functions upstream of mitochondrial ubiquitination by recruiting the E3 ligase to mitochondria.

What is the role of Parkin and PINK1? I demonstrated that Parkin and PINK1 prevent mitochondria from excessive fusion *in vivo*. Although previous studies have shown that mitofusin 1 and 2, the GTPases required for outer membrane fusion, are degraded in Drp1KO cells[10], it remains to be determined how mitofusins are degraded. My data show that mitofusin 1 and 2 are degraded by a proteasomal degradation process involving Parkin and PINK1. Mitofusin 1 and 2 protein levels decreased in Drp1KO hepatocytes while their levels were restored in Drp1ParkinKO hepatocytes. Interestingly, mitofusin 1 and 2 proteins levels were also restored in Drp1Opa1KO hepatocytes (Figure 2A). These data suggest that the defense mechanism that prevents mitochondria from excessive fusion when mitochondrial division is decreased requires Parkin and PINK1. My data also show that this safeguard mechanism is activated by mitochondrial enlargement but not loss of mitochondrial division.

Mitophagy-targeted treatment of NASH

Many clinical studies have reported extremely enlarged mitochondria, termed megamitochondria, in hepatocytes of human patients with NASH, the most common liver disease in Western countries[11]. However, it was unclear how megamitochondria are formed in NASH and what the role of megamitochondria in this liver disease. To address these questions, I examined mitochondria in a NASH mouse model and found that mitophagy intermediates were accumulated on megamitochondria similar to enlarged mitochondria in Drp1KO hepatocytes with an accumulation of p62, ubiquitin and Keap1 (Figures 2B and C). Remarkably, Opa1KO caused a significant decrease in mitochondrial size (Figures 2D) and eliminated accumulation of mitophagy intermediates (Figures 2B and C) in the NASH model, similar to Drp1Opa1KO hepatocytes. Furthermore, Opa1KO also rescued liver damage in this NASH model (Figure 2E). These data suggest that megamitochondria decrease mitophagy in NASH and that restoring mitochondrial size and thereby mitophagy may serve as a potential treatment for NAFLD.

Currently, I am translating my knockout approaches (which are not applicable to human patients) to gene knockdown approaches (which are applicable human patients) using antisense oligonucleotides against Opa1. My recent data show that liver-targeted Opa1 antisense oligonucleotides not only prevent the pathogenesis of NASH but also reverse NASH phenotypes in the mouse model. Therefore, my work reveals a new *in vivo* mechanism of mitophagy and translates the fundamental biology into a potential therapeutic treatment for this rapidly-growing metabolic disease for which there is currently no effective medical treatments.

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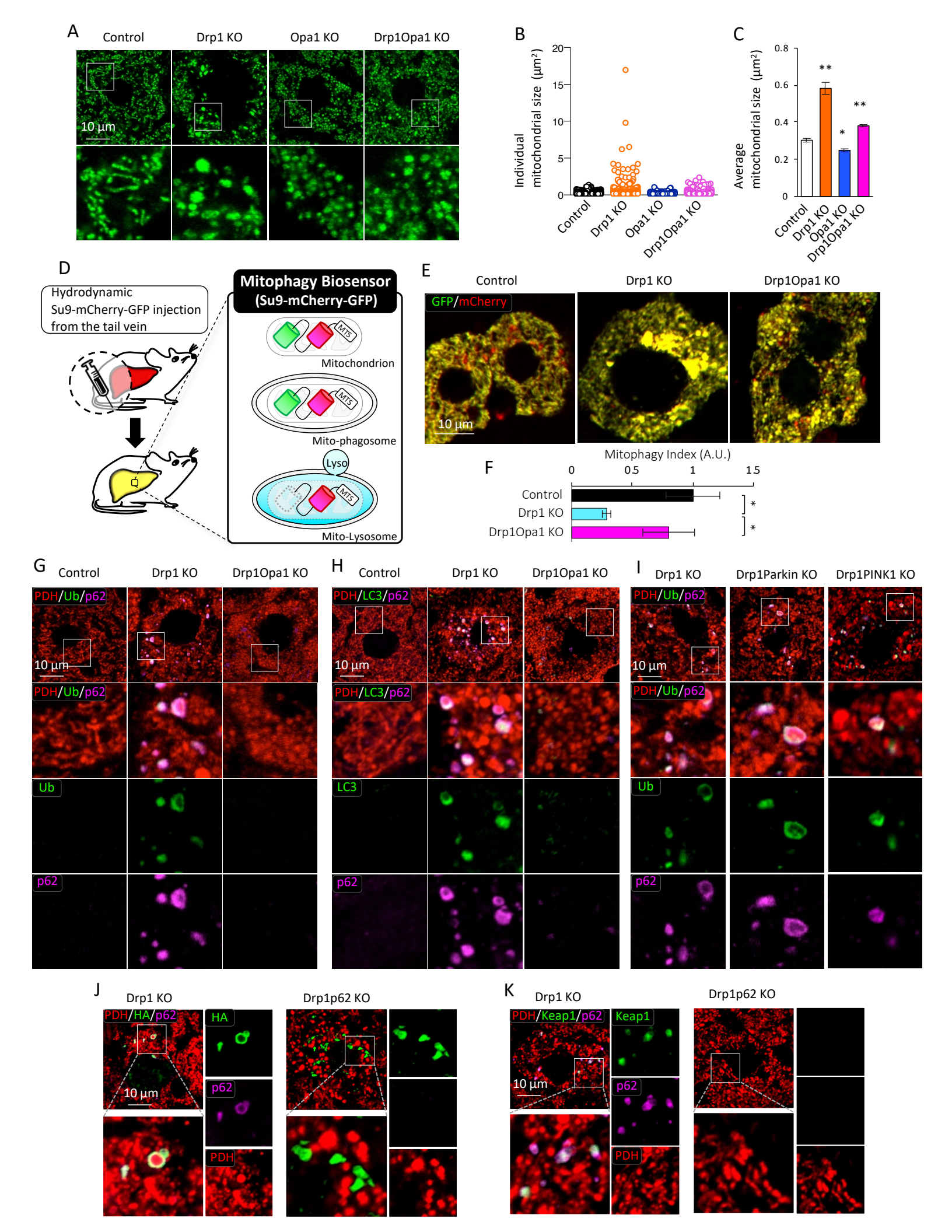
A
Control
Drp1 KO
Opa1 KO
Drp1Opa1 KO
10 μm
B
Individual mitochondrial size (μm²)
20
15
10
5
0
Control
Drp1 KO
Opa1 KO
Drp1Opa1 KO
C
Average mitochondrial size (μm²)
0.8
0.6
0.4
0.2
0
**
*
**
Control
Drp1 KO
Opa1 KO
Drp1Opa1 KO
D
Hydrodynamic
Su9-mCherry-GFP injection
from the tail vein
Mitophagy Biosensor
(Su9-mCherry-GFP)
MTS
Mitochondrion
Mito-phagosome
Lyso
Mito-Lysosome
E
Control
Drp1 KO
Drp1Opa1 KO
GFP/mCherry
10 μm
F
Mitophagy Index (A.U.)
0
0.5
1
1.5
Control
Drp1 KO
Drp1Opa1 KO
*
*
G
Control
Drp1 KO
Drp1Opa1 KO
PDH/Ub/p62
10 μm
PDH/Ub/p62
Ub
p62
H
Control
Drp1 KO
Drp1Opa1 KO
PDH/LC3/p62
10 μm
PDH/LC3/p62
LC3
p62
I
Drp1 KO
Drp1Parkin KO
Drp1PINK1 KO
PDH/Ub/p62
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PDH/Ub/p62
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p62
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Drp1 KO
Drp1p62 KO
PDH/HA/p62
10 μm
HA
p62
PDH
K
Drp1 KO
Drp1p62 KO
PDH/Keap1/p62
10 μm
Keap1
p62
PDH

Figure. 1 Mitochondrial size has crucial role in mitophagy, and mitochondrial ubiquitination is mediated by p62-Keap1-Rbx1 complex *in vivo*. (A) Liver mitochondria were analyzed by confocal immunofluorescence microscopy with antibody to PDH. Boxed regions are enlarged. (B and C) Individual (B) and average (C) mitochondrial size were quantified. (D) Work-flow of *in vivo* mitophagy biosensor assay. Su9-mCherry-GFP is imported to mitochondria in hepatocyte. Those mitochondria that undergo autophagic engulfment (Mito-phagosome) eventually fused with lysosome (Mito-Lysosome). GFP Fluorescence in Mito-Lysosome is quenched due to low pH. (E) Images of Su9-mCherry-GFP introduced hepatocytes of indicated mice. (F) Mitophagy index was determined by measuring the relative area of mCherry that did not overlap with GFP over the total area of mCherry in each cell. Values are average ± SEM (n=3-4 mice). (G-I) Confocal microscopy of liver sections using antibodies to p62 and PDH along with ubiquitin (G, I) or LC3 (H). (J) Plasmids carrying HA-Rbx1 were delivered to the livers of indicated mice. The livers were analyzed by confocal immunofluorescence microscopy with antibodies to PDH, HA, and p62. (K) Livers were analyzed using confocal microscopy using antibodies to PDH, Keap1, and p62. *p < 0.05, **p < 0.01.

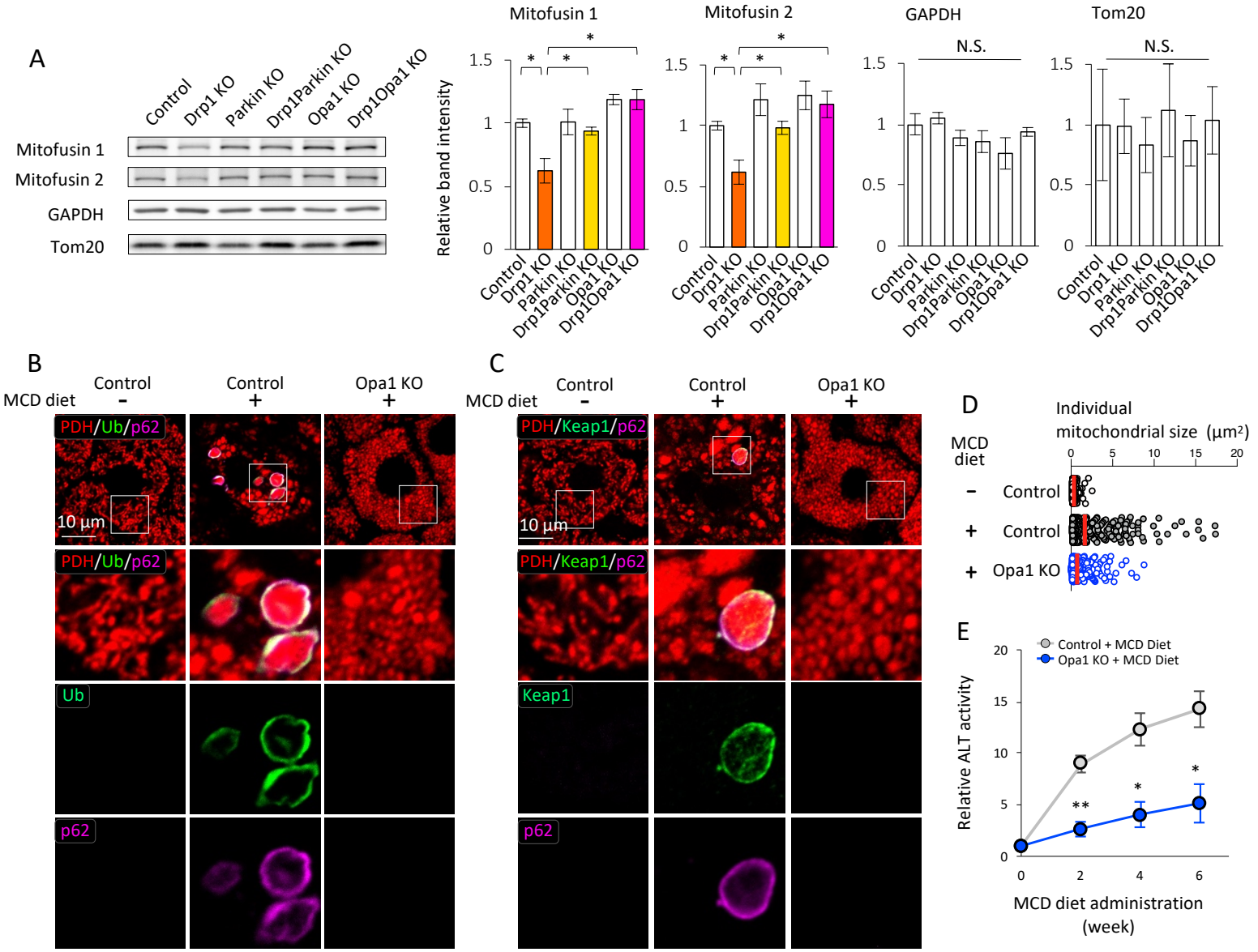

Figure 2. PINK1-Parkin degrade mitofusin 1 and 2, and Alb-Opa1KO mitigates NAFLD. (A) Western blotting of livers isolated from the indicated mice using antibodies to mitofusin 1 and 2, Opa1, GAPDH and Tom20. Quantification of band intensity is shown. Values are average ± SEM (n=3 mice). (B and C) Control Opa1$^{flox/flox}$ mice and Alb-Opa1KO mice were fed a methionine- and choline-deficient (MCD) diet for 6 weeks. Liver sections from these mice were analyzed by confocal microscopy with antibodies to ubiquitin (B), Keap1 (C), PDH and p62. (D) Individual mitochondrial sizes were quantified. Red lines represent average. (E) Serum ALT levels were measured before and during administration at 2, 4, and 6 weeks. ALT activity levels for each mouse were normalized to that obtained before MCD diet administration. Values are average ± SEM (n = 3–5 mice). (F) Summary of this study. p62-Keap1-Rbx1 mediated ubiquitination induces basal mitophagy in healthy liver. In fatty liver, the formation of megamitochondria reduces mitophagy flux and as a result, mitophagy intermediates accumulate on the surface of megamitochondria. *p < 0.05, **p < 0.01.