Defective lysosome reformation during autophagy causes skeletal muscle disease

Meagan J. McGrath,1 Matthew J. Eramo,1 Rajendra Gurung,1 Absorn Sriratana,1 Stefan M. Gehrig,2 Gordon S. Lynch,2 Sonia Raveena Lourdes,1 Frank Koentgen,3 Sandra J. Feeney,1 Michael Lazarou,4 Catriona A. McLean,5 and Christina A. Mitchell1

1Cancer Program and Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, Melbourne, Victoria, Australia. 2Centre for Muscle Research, School of Biomedical Sciences, The University of Melbourne, Melbourne, Victoria, Australia. 3Ozgene Pty Ltd, Bentley, Perth, Western Australia, Australia. 4Neuroscience Program and Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, Melbourne, Victoria, Australia. 5Department of Anatomical Pathology, Alfred Hospital, Prahran, Melbourne, Victoria, Australia.

The regulation of autophagy-dependent lysosome homeostasis in vivo is unclear. We showed that the inositol polyphosphate 5-phosphatase INPP5K regulates autophagic lysosome reformation (ALR), a lysosome recycling pathway, in muscle. INPP5K hydrolyzes phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] to phosphatidylinositol 4-phosphate [PI(4)P], and INPP5K mutations cause muscular dystrophy by unknown mechanisms. We report that loss of INPP5K in muscle caused severe disease, autophagy inhibition, and lysosome depletion. Reduced PI(4,5)P2 turnover on autolysosomes in Inpp5k−/− muscle suppressed autophagy and lysosome repopulation via ALR inhibition. Defective ALR in Inpp5k−/− myoblasts was characterized by enlarged autolysosomes and the persistence of hyperextended reformation tubules, structures that participate in membrane recycling to form lysosomes. Reduced disengagement of the PI(4,5)P2 effector clathrin was observed on reformation tubules, which we propose interfered with ALR completion. Inhibition of PI(4,5)P2 synthesis or expression of WT INPP5K but not INPP5K disease mutants in INPP5K-depleted myoblasts restored lysosomal homeostasis. Therefore, bidirectional interconversion of PI(4)P/PI(4,5)P2 on autolysosomes was integral to lysosome replenishment and autophagy function in muscle. Activation of TFE3-dependent de novo lysosome biogenesis did not compensate for loss of ALR in Inpp5k−/− muscle, revealing a dependence on this lysosome recycling pathway. Therefore, in muscle, ALR is indispensable for lysosome homeostasis during autophagy and when defective is associated with muscular dystrophy.

Introduction

Autophagy is a fundamental catabolic and cytoprotective process. During autophagy, multiple lysosomes fuse with autophagosomes to form autolysosomes in which cellular debris is degraded (1). Lysosomes are critical for the terminal degradative stages of the autophagy pathway, and the ability to repopulate lysosomes is essential because they are rapidly consumed during autophagy (2, 3). Skeletal muscle is heavily reliant on the cytoprotective functions of autophagy (4, 5) and has a high rate of basal autophagy (6). Skeletal muscle autophagy is further enhanced by fasting (7) or exercise (8) for the mobilization of amino acids and mitochondrial quality control, respectively. These conditions place a significant demand on autophagy-dependent lysosome repopulation in skeletal muscle, but this process is not well understood in this tissue.

The serine/threonine kinase mTOR couples autophagy activation with lysosome repopulation. mTOR inhibition during autophagy stimulates autophagosome formation (9), and concurrent suppression with lysosome repopulation. mTOR inhibition during autophagy stimulates autophagosome formation (9), and concurrent

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called reformation tubules, which undergo scission to generate protolysosomes that mature to functional lysosomes (2). However, the physiological role of ALR is yet to be fully determined.

Membrane-bound phosphoinositides, including phosphatidylinositol 4-phosphate [PI(4)P] and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], play significant roles at several stages of the autophagy pathway, including autophagosome formation (23–26), autophagosome-lysosome fusion (27–29), and ALR (30–32). The synthesis of PI(4)P and PI(4,5)P₂ on autolysosomes via the sequential actions of PI-4 and PI-4P-5 kinases, respectively, is required for the initiation and progression of ALR (30, 33). Localized generation of PI(4,5)P₂-enriched microdomains on autolysosomes leads to the recruitment of effector proteins, which drive changes to autolysosome membrane ultrastructure to form reformation tubules, structures utilized in the generation of new lysosomes (30–32). These PI(4,5)P₂-binding effectors include the AP-2/clathrin complex, which is required for membrane budding (30), and the microtubule-associated kinesin motor protein KIF5B (31) and the microtubule-associated protein responsible for binding to proteins within the base membrane tubules. To date, these ALR studies have been undertaken at the cellular level or through the use of purified membrane fractions, and the contribution of ALR to the regulation of tissue homeostasis is still emerging. Moreover, there is currently little evidence that PI(4)P/PI(4,5)P₂-dependent pathways contribute to lysosome or autophagy regulation in vivo (34).

Inpp5k is an inositol polyphosphate 5-phosphatase that dephosphorylates PI(4,5)P₂ to PI(4)P and, with reduced affinity, PI(3,4,5)P₃ to PI(3,4)P₂ (35, 36). Missense INPP5K mutations are causative for congenital muscular dystrophy overlapping with Marinosco-Sjögren syndrome (MSS), in which affected individuals exhibit a constellation of clinical manifestations, including muscular dystrophy, cataracts, and variable penetrance of brain abnormalities (37–39). The majority of these mutations map to the 5-phoshatase domain, reducing catalytic function toward PI(4,5)P₂ by approximately 50%–75% (37, 38). Muscular dystrophy caused by INPP5K mutations shows features suggestive of autophagy inhibition, including the accumulation of rimmed vacuoles, p62/SQSTM1, and αB-crystallin, but whether autophagy is impaired remains unresolved (37, 38).

Here, we investigated the role INPP5K plays in skeletal muscle homeostasis. INPP5K loss of function caused severe and progressive muscle disease, accompanied by marked lysosome depletion and autophagy inhibition. This occurred because of reduced conversion of PI(4,5)P₂ to PI(4)P on autolysosomes, which impaired ALR progression. Our study identified that functional ALR is essential for lysosome repopulation during autophagy in skeletal muscle and when defective is causative for muscular dystrophy.

Results

Skeletal muscle-specific Inpp5k deletion leads to an early-onset and progressive muscle disease. Global deletion of the Inpp5k gene in mice is embryonically lethal (40), so skeletal muscle–specific Inpp5k-KO mice (Inpp5k<sup>−/−</sup> MCK-Cre) were generated, which were viable up to 2 years and showed reduced muscle weight from 1 year (Supplemental Figure 1, A–C, and Supplemental Table 4; supplemental material available online with this article; https://doi.org/10.1172/JCI135124DS1). Inpp5k<sup>−/−</sup> MCK-Cre mice developed muscle disease resembling that caused by INPP5K mutations (37, 38). This included early signs of muscle disease from 6 weeks of age (in quadriceps, gastrocnemius, and tibialis anterior muscles), which progressively worsened, with degenerating and regenerating fibers (with centralized nuclei), infiltration of mononucleated cells, and muscle fiber size variability (Figure 1A and Supplemental Figure 1, D–H). By 12 weeks of age, muscle disease was severe. Elevated serum creatine kinase (CK), a clinical indicator of muscle damage, was observed at all ages (Figure 1B). By 1 year of age, muscle fibers were heavily vacuolated (Figure 1A; black arrowhead and Figure 1D; white arrows), and extensive fibrosis indicated advanced disease (Supplemental Figure 1I). Maximum absolute tetanic (Supplemental Figure 1J) and specific force (maximum force normalized to overall muscle cross-sectional area) (Figure 1C) were significantly reduced (~50%) in tibialis anterior muscles of Inpp5k<sup>−/−</sup> MCK-Cre mice compared with controls.

Hypoglycosylation of α-dystroglycan occurs in the muscle of some patients with muscular dystrophy caused by INPP5K mutations (38), but this is not a universal finding because some individuals exhibit no detectable reduction (37). α-Dystroglycan, an essential component of the dystrophin-glycoprotein complex, is a transmembrane protein responsible for binding to proteins within the basement membrane in the extracellular space (41). This interaction is essential for several processes, including the preservation of muscle fiber integrity. Mutations in α-dystroglycan (DAGI) cause muscular dystrophy (42–44), as do mutations in many proteins (at least 20) that function in the biochemical pathway responsible for α-dystroglycan glycosylation (41, 45, 46). These are called dystroglycanopathies and result from α-dystroglycan hypoglycosylation. Glycosylation of α-dystroglycan is critical for its interaction with extracellular proteins, including the α2 chain of laminin-2 (41). We utilized Inpp5k<sup>−/−</sup> MCK-Cre mice to explore the idea that hypoglycosylation of α-dystroglycan could be uncoupled from the primary cause of muscle disease due to INPP5K mutations, given that it is not a universal finding in all patients. Immunostaining (Supplemental Figure 2A) and immunoblot (Supplemental Figure 2C) analysis of muscle using the IIH6C4 antibody (Supplemental Figure 2A) revealed no differences in α-dystroglycan glycosylation in patients with INPP5K mutations (37, 38), and the onset of muscle disease was uncoupled from effects on α-dystroglycan glycosylation. Severe muscle disease caused by loss of INPP5K occurs with marked autophagy inhibition and lysosome depletion. Given that autophagy-related changes are a consistent histopathological feature of muscle disease in INPP5K muscular dystrophy (37, 38), and our data suggests that Inpp5k may be an autophagy-responsive gene that is induced by fasting (Supplemental Figure 3, A and B), we examined whether autophagy inhibition contributes to disease. Enlarged vacuoles occur in the muscle of patients with INPP5K...
Cre muscle, suggesting decreased lysosome homeostasis (Figure 1, E and F). Significant autophagy inhibition was also detected in Inpp5kfl/fl MCK-Cre muscle, as shown by marked accumulation of LC3-II, p62/SQSTM1, and ubiquitinated proteins as early as at 6 weeks, which progressively worsened with age (Figure 2, A and B).

Figure 1. Skeletal muscle-specific Inpp5k deletion leads to early-onset and progressive muscle disease. (A) H&E-stained muscle (quadriceps). Arrows: black = degenerating fibers; white = centralized nuclei; arrowhead = vacuolated fibers. n = 6 mice/genotype/age. Scale bar: 25 μm. (B) Serum creatine kinase, n = 5–6 mice/genotype (12 weeks), and n = 8 mice/genotype (1 year). ***P = 0.0008, **P = 0.0065, †††P = 0.0005. (C) Specific (normalized) force: 12-week-old Inpp5kfl/fl (n = 5) and Inpp5kfl/fl MCK-Cre (n = 7) mice. Unpaired 2-tailed Student’s t test, ***P < 0.0001. (D) Transmission electron microscopy images of vacuoles in Inpp5kfl/fl MCK-Cre muscle (white arrows), n = 3 mice/genotype. Scale bar: 0.5 μm. White boxed region shown at high magnification in panels on right. (E) Muscle sections costained for LC3 and LAMP1. Arrows: LC3+/LAMP1+ autolysosomes, n = 3 mice/genotype. Scale bar: 12.5 μm. Yellow boxed region shown at high magnification below. Used for (F) quantification of lysosomes (LC3−/LAMP1+) versus autolysosomes (LC3+/LAMP1+), n = 3 mice/genotype. ***P < 0.0001, ###P < 0.0001. (G) Myoblasts were cultured in nutrient-free EBSS to activate starvation-induced autophagy and immunostained for autolysosomes (LC3+/LAMP1+) which are enlarged in INPP5K-KO (Inpp5kfl/fl Cre) but not control (Inpp5kfl/fl LacZ) cells (arrows). Yellow boxed region shown at high magnification in the panels on right. Scale bars: 20 μm. Unless otherwise stated, data presented in all graphs are the mean ± SEM, with a 2-way ANOVA followed by Bonferroni’s post hoc multiple-comparisons test to determine statistical significance.
The elevated LC3-II observed in \textit{Inpp5k}^{fl/fl} \textit{MCK-Cre} muscle (Figure 2B) was insensitive to colchicine treatment (48), confirming that LC3-II was increased because of inhibition of autophagic flux (Figure 2, C and D). Therefore, pronounced lysosome depletion and autophagy inhibition are features of muscle disease caused by \textit{INPP5K} ablation.

\textit{INPP5K} regulates lysosome homeostasis during autophagy. Loss of \textit{INPP5K} did not affect autophagosome formation (Supplemental Figure 3, C–E) or autophagosome-lysosome fusion (Supplemental Figure 3, F and G) during starvation-induced autophagy. Lysosomes were not reduced under growth conditions in \textit{Inpp5k}-knockdown (KD) C2C12 myoblasts (Supplemental Figure 3, H and I) or primary \textit{Inpp5k}-KO myoblasts (Figure 3, A–C), but were depleted under prolonged starvation-induced autophagy by culturing cells for 8 hours in Earle’s balanced salt solution (EBSS).

In control myoblasts, LAMP1-stained lysosomes were depleted (4 hours EBSS), but recovered to basal levels within 8 hours of autophagy activation (8 hours EBSS); however, in cells with loss of \textit{INPP5K}, lysosomes remained depleted within this time frame (Figure 3, B and C). Lysosomal protein (Figure 3, E–H, and Supplemental Figure 3, K–N) but not mRNA expression levels (Supplemental Figure 3, O–R) were reduced in \textit{INPP5K}-depleted cells, suggesting a posttranslational defect. Functional lysosomes were reduced during autophagy in \textit{Inpp5k}-KD myoblasts (Figure 3, I and J), but lysosomal pH was unaffected (Supplemental Figure 4, A and B). The starvation-induced depletion of lysosomes in myoblasts with loss of \textit{INPP5K} function was autophagy-dependent because this was rescued by suppression of autophagy induction.
INPP5K does not regulate autophagy via AKT signaling. Sustained AKT/mTOR activation causes muscle disease by suppressing autophagosome formation and inhibiting autophagy; however, changes to lysosomal homeostasis were not reported (7, 51, 52). mTOR activation also suppresses TFEB/TFE3 lysosomal biogenes.
esis (10–12). It is established from multiple studies that INPP5K (also called SKIP) degrades PI(3,4,5)P₃ to suppress AKT/mTOR signaling (40, 53–58); therefore, we questioned whether INPP5K regulation of autophagy was AKT dependent. Consistent with previous reports, enhanced AKT/mTOR activation was observed in Inpp5kfl/fl MCK-Cre muscle, with increased phosphorylated AKT (Ser-473 and Thr-308) and the mTOR target, ribosomal S6 kinase (S6, Ser-235 & 236) (Supplemental Figure 6, A and B). This was further supported by increased expression of 2 downstream AKT targets, PRAS40 (59) and TSC2 (60) (Supplemental Figure 6, C and D). An increase in total AKT protein expression was observed in Inpp5kfl/fl MCK-Cre mouse muscle (Supplemental Figure 6, A and B), and this has also been observed in mice with kidney-specific ablation of the related inositol polyphosphate 5-phosphatase Inpp5e via an undefined mechanism (61, 62).

Interestingly, despite evidence of increased AKT/mTOR activation in Inpp5kfl/fl MCK-Cre muscle, the formation of autophagosomes (shown by LC3-II detection) was maintained under
basal-fed conditions (Supplemental Figure 6, E and F). The capacity to increase autophagosome production in response to fasting induced–autophagy (7) was also retained in Inpp5kfl/fl MCK-Cre muscle, shown by increased LC3-II relative to fed Inpp5kfl/fl MCK-Cre mice (Supplemental Figure 6, E and F). This is consistent with the absence of an autophagosome formation defect in Inpp5k-KD cells (Supplemental Figure 3, D and E). Administration of the AKT inhibitor MK-2206 reduced AKT activation in Inpp5k fl/fl MCK-Cre muscle (Supplemental Figure 6, G and H), but did not alleviate autophagy inhibition (Supplemental Figure 6, I and J) or muscle disease (Supplemental Figure 6, K-N). Therefore, despite published evidence from our laboratory (this study and ref. 58) and many others (40, 53–57) that loss of INPP5K causes hyperactivation of AKT signaling, this is unlikely to be the mechanism by which INPP5K ablation suppresses autophagy.

INPP5K does not regulate autophagy or lysosome homeostasis via an mTOR/TFEB-dependent pathway. mTOR inhibition during autophagy promotes TFEB translocation from lysosomes to the nucleus to induce expression of genes required for de novo lysosome biogenesis (11, 12). We observed mTOR hyperactivation on lysosomes/autolysosomes in Inpp5kfl/fl MCK-Cre muscle (Supplemental Figure 7A), which could inhibit TFEB-dependent lysosome homeostasis. To explore this as a mechanism for the lysosome depletion and autophagy inhibition in Inpp5kfl/fl MCK-Cre muscle, we characterized TFEB nuclear localization and lysosomal gene transcription. Under basal-fed conditions, TFEB was detected at the nucleus in control muscle (Supplemental Figure 7B and C). However, TFEB nuclear localization was decreased in Inpp5kfl/fl MCK-Cre muscle and instead, TFEB was abundant on LAMP1+ lysosomes/autolysosomes, a localization consistent with enhanced mTOR activation (11). Inpp5kfl/fl MCK-Cre muscle also showed reduced activation of some TFEB-target lysosomal genes under basal-fed conditions, but no change or increased transcription of others (Supplemental Figure 7D). Expression analysis of skeletal muscle from TFEB-overexpressing mice versus TFEB-KO mice revealed that under basal-fed conditions, the most prominent effect was on genes responsible for regulating metabolism and mitochondrial function (18). Fasting suppresses mTOR activation, and thereby enhances TFEB nuclear localization and activation of lysosomal genes (63, 64). Indeed, a previous study in muscle

Figure 5. INPP5K regulates PI(4,5)P₂ to PI(4)P conversion on autolysosomes. (A) CFP- INPP5K localization in myoblasts under growth or autophagy conditions. Costaining for autophagosomes (LC3+/LAMP1–), lysosomes (LC3–/LAMP1+), or autolysosomes (LC3+/LAMP1+) (arrows). Yellow boxed region shown at high magnification on right. Representative of n = 3 experiments. Scale bars: 2.5 μm. (B) Primary myoblasts incubated in EBSS (2 hours) to activate autophagy followed by FCS treatment (10%, 30 minutes) to stimulate ALR. Assessment of PI(4,5)P₂ staining at LAMP1+ autolysosomes/lysosomes (arrow heads). Scale bar: 5 μm. Yellow boxed region shown at high magnification below. n = 3 experiments used to quantify (C) the percentage of LAMP1 puncta positive for PI(4,5)P₂ staining (n = 100 cells/cell line). Graph is the mean ± SEM, and an unpaired 2-tailed Student’s t test was used to determine statistical significance; *P = 0.011. (D) Muscle sections immunostained for PI(4,5)P₂ or PI(4)P; dystrophin staining defines muscle fibers. Scale bars: 30 μm.
detected more consistent effects on the activation of TFEB-targeted lysosomal genes under fasted conditions compared with those observed basally (65). Collectively, these published observations may explain why we observed reduced expression of only some TFEB-target lysosomal genes in the muscle of Inpp5k<sup>fl/fl</sup> MCK-Cre mice under basal-fed conditions, despite increased mTOR activation and reduced TFEB nuclear localization. In agreement with this, muscle from fasted control mice showed increased activation of TFEB-targeted lysosomal genes compared with fed mice, and this TFEB-dependent transcriptional response was consistently blunted in Inpp5k<sup>fl/fl</sup> MCK-Cre muscle for all lysosomal genes examined (Supplemental Figure 7E).

Our data raise the possibility that mTOR suppression of TFEB function could be responsible for the defect in lysosome homeostasis and autophagy inhibition that occurred in Inpp5k<sup>fl/fl</sup> MCK-Cre muscle. The mTOR inhibitor rapamycin can activate TFEB-dependent transcription in muscle (66). Therefore, we evaluated whether rapamycin treatment of Inpp5k<sup>fl/fl</sup> MCK-Cre mice could restore lysosome biogenesis and autophagy function by alleviating mTOR-mediated suppression of TFEB function. Phospho-immunoblot and immunostaining experiments confirmed rapamycin treatment of Inpp5k<sup>fl/fl</sup> MCK-Cre mice reduced mTOR activation in muscle (Supplemental Figure 8, A and B), including on lysosomes/autolysosomes (Supplemental Figure 8C). Rapamycin treatment also restored TFEB nuclear localization (Supplemental Figure 8, D and E) and activation of lysosomal genes (Supplemental Figure 8F) in Inpp5k<sup>fl/fl</sup> MCK-Cre mice, but did not reduce muscle disease (Supplemental Figure 6, K–N) in Inpp5k<sup>fl/fl</sup> MCK-Cre mice. Therefore, defects in lysosome homeostasis and autophagy in Inpp5k<sup>fl/fl</sup> MCK-Cre muscle were not due to increased AKT/mTOR activation or suppressed TFEB function. In addition, activation of TFEB was unable to reverse lysosomal and autophagy defects due to loss of INPP5K.
If the mTOR/TFEB pathway was responsible for regulating lysosome repopulation in skeletal muscle, then it would be anticipated that the treatment of Inpp5kfl/fl MCK-Cre mice with the mTOR inhibitor rapamycin would enhance TFEB activation and thereby increase lysosome production. However, no compensatory increase in lysosome biogenesis in rapamycin-treated Inpp5kfl/fl MCK-Cre mice was observed (Supplemental Figure 9, D and E). Interestingly, cellular studies have revealed that ALR, the other major autophagy-dependent lysosome repopulation pathway, is suppressed by mTOR inhibition using rapamycin (2). This is because the ini-
INPP5K regulates lysosome homeostasis via autophagic lysosome reformation. ALR inhibition arrests autophagy at the autolysosome stage, causing enlarged LC3+/LAMP1+ autolysosomes, as we observed in Inpp5k-KD myoblasts during prolonged starvation-induced autophagy (Figure 1, D–F) (2, 30, 68).

The initiating signal for ALR is the amino acid–dependent reactivation of mTOR on autolysosomes during prolonged starvation-induced autophagy (2). Our data therefore raise the possibility that INPP5K may regulate lysosome homeostasis via ALR. In this context, rapamycin treatment of Inpp5kfl/fl MCK-Cre mice would be predicted to inhibit ALR-dependent lysosome generation, a pathway that may already be inherently suppressed because of the loss of INPP5K function. Because of this, the net effect of rapamycin treatment on lysosome content in Inpp5k fl/fl MCK-Cre muscle may be negligible, as we observed (Supplemental Figure 9, D and E). To further investigate, we compared lysosome homeostasis in control and Inpp5k-KD myoblasts under growth conditions or after prolonged rapamycin-induced autophagy (8 hours), which inhibits ALR (2). Rapamycin treatment reduced lysosome content in control and Inpp5k-KD cells to the same extent (Supplemental Figure 10, A and B), a different response compared with starvation-induced autophagy (Figure 3, B and C, and Supplemental Figure 3, H and I). Therefore, INPP5K effects on lysosome homeostasis were detected in cells that have the capacity to reactivate mTOR during autophagy, which is a requirement for ALR (2). This may also explain why a previous study identified no autophagy abnormalities in patient fibroblasts with INPP5K mutations under conditions of rapamycin-induced autophagy (37).
serum stimulation (8 hours EBSS + FCS) (Figure 4, A and B). We also developed a fixation method to preserve reformation tubules in intact cells and this enabled precise morphometric analysis. In both live-cell (Figure 4, A and B) and fixed-cell assays (Figure 4, C and D), comparable results were obtained. INPP5K-KD cells exhibited no defects in tubule initiation (8 hours EBSS), but showed a marked persistence of tubules (8 hours EBSS + 30 minutes FCS) (Figure 4, A–D), and reformation tubules were hyper-extended (Figure 4, C and E). Localization studies confirmed that INPP5K was recruited to lysosomes (LAMP1+/LC3–) and autolysosomes (LAMP1+/LC3+) during autophagy, the site at which ALR occurs (Figure 5A) (2, 30), contrasting with its localization to the ER under growth conditions (35, 72). Collectively, these data suggest that the turnover of autolysosome reformation tubules is compromised with loss of the PI(4,5)P$_2$ 5-phosphatase INPP5K, thereby reducing the generation of lysosomes during ALR.

**INPP5K regulates PI(4,5)P$_2$ to PI(4)P conversion on autolysosomes and clathrin association with reformation tubules during ALR.** Mechanistic understanding of how PI(4)P and PI(4,5)P$_2$ regulate ALR is still emerging. During ALR, PI is converted to PI(4)P by the PI-4 kinase PI4kIIIß (33), and in turn PI(4,5)P$_2$ is generated on the main autolysosome body and reformation tubules by the PI(4)P-5 kinases, Pip5k1b and Pip5k1a, respectively (30). In cell-based studies, Pip5k1b KD results in the absence of autolysosome tubules, suggesting that PI(4,5)P$_2$ generation from PI(4)P is an initiation signal for ALR (30). However, Pip5k1a depletion in cells causes reformation tubule persistence and hyperextension, suggesting that PI(4)P to PI(4,5)P$_2$ conversion also contributes to the latter stages of ALR, including membrane scission and lysosome generation (30). PI(4)P depletion on autolysosomes via PI4kIIIß KD also results in autolysosome/lysosome tubule hyperextension, suggesting a functional role for PI(4)P in suppressing tubulation by promoting cargo sorting and possibly the scission of membrane vesicles (33). Loss of INPP5K, which degrades PI(4,5)P$_2$ to form PI(4)P, resulted in a very similar hyperextended reformation tubule phenotype to Pip5k1a and PI4kIIIß-KD cells. We therefore examined PI(4,5)P$_2$ and PI(4)P during ALR in cells with loss of INPP5K. PI(4,5)P$_2$-positive vesicles were increased under ALR conditions in INPP5K-depleted myoblasts (Supplemental Figure 11, A–C), particularly on LAMP1-stained autolysosomes/lysosomes (Figure 5, B and C), concomitant with a reduction of PI(4)P vesicles (Supplemental Figure 11, D and E). PI(4,5)P$_2$ could not be detected on reformation tubules in intact cells (data not shown), as in other studies, perhaps because of the low level of this phosphoinositide on tubules and/or technical issues related to tubule visualization (31, 73). In Inpp5kfl/fl MCK-Cre muscle, marked accumulation of PI(4,5)P$_2$ was observed (Figure 5D) on LC3+/LAMP1+ autolysosomes (Figure 6A) and PI(4)P staining was reduced (Figure 5D). In control studies, PI(4)P, which promotes ALR (74) but is not regulated by INPP5K, remained unchanged during ALR in cells with loss of INPP5K (Supplemental Figure 11, F and G). Therefore, PI(4,5)P$_2$ was not degraded in the absence of INPP5K and this lipid accumulated on autolysosomes, while the product of INPP5K hydrolysis of PI(4,5)P$_2$, PI(4)P, was reduced.

Clathrin is a marker for PI(4,5)P$_2$, with which it associates via adaptor complex AP-2 (75). Both clathrin and AP-2 recruitment are required for membrane budding at autolysosomes to initiate reformation tubules and on reformation tubules to form lysosomes (30, 31). The recruitment of clathrin and AP-2 to autolysosomes is reduced in cells lacking PI(4)P-5 kinase function (30) but interestingly, the presence of clathrin on membrane tubules is enhanced under conditions of low PI(4)P, where it is predicted to interfere with the membrane scission machinery (33). Increased staining for the PI(4,5)P$_2$ effectors AP-2 and clathrin was observed during ALR in cells with loss of INPP5K (Supplemental Figure 11, H and I). Therefore, PI(4,5)P$_2$ is not degraded in the absence of INPP5K and this lipid accumulated on autolysosomes, while the product of INPP5K hydrolysis of PI(4,5)P$_2$, PI(4)P, was reduced. The Journal of Clinical Investigation
and colocalized with PI(4,5)P₂-enriched LAMP1+ autolysosomes/lysosomes in Inpp5k<sup>fl/fl</sup> MCK-Cre muscle (Figure 6, B and C). During ALR, increased association of clathrin with reformation tubules was also observed in myoblasts with depletion of INPP5K (Figure 6, D and E). Therefore, INPP5K-mediated hydrolysis of PI(4,5)P₂ on autolysosomes generated PI(4)P and regulated the association of AP-2/clathrin during ALR.

**PI(4,5)P₂ hydrolysis is required for the completion of ALR.** To explore whether regulation of the PI(4)P-PI(4,5)P₂ axis by INPP5K is critical for the progression of ALR, we investigated whether the ALR defect induced by INPP5K depletion could be counteracted by reducing PI(4,5)P₂ synthesis, which would also increase PI(4)P. To this end, lysosome homeostasis during autophagy was examined in *Inpp5k-KD* myoblasts with codepletion of either of the PI(4)P-5 kinases, Pip5k1a or Pip5k1b, that generate PI(4,5)P₂ on autolysosomes during ALR (30). There are 3 Pip5k1 isoforms, Pip5k1a, Pip5k1b, and Pip5k1c, and Pip5k1a is the most abundant in skeletal muscle (76). Immunoblot analysis of skeletal muscle confirmed that both of the PIP5K1 isoforms involved in ALR regulation, Pip5k1a and Pip5k1b, were expressed (Supplemental Figure 12A), but only Pip5k1b mRNA increased during autophagy in myoblasts (Supplemental Figure 12, B and C). In contrast to reports in nonmuscle cells (30), an ALR defect was only observed in myoblasts with depletion of Pip5k1b but not Pip5k1a, as shown by reduced LAMP1+ vesicles and the accumulation of abnormal, enlarged LPOs that are characteristic of ALR inhibition (Figure 7, A–C, and Supplemental Figure 12, D and F) (2, 22, 30, 31). KD of Pip5k1b but not Pip5k1a in *Inpp5k-KD* myoblasts (Supplemental Figure 12, G–I) rescued the ALR defect, whereby lysosome numbers returned to control levels and the number of enlarged LPOs was reduced (Figure 7, A–C). This result was confirmed by analyzing the effects of 2 independent and validated shRNAs specific for Pip5k1a or Pip5k1b in *Inpp5k-KD* cells, and only Pip5k1b KD restored PI(4)P regulation (Supplemental Figure 11, H and I) and lysosome homeostasis in cells with loss of INPP5K (Figure 7, A and B). Unfortunately, technical issues (as mentioned above) precluded analysis of PI(4,5)P₂ on reformation tubules in intact cells.

Our data suggest a functional interaction between INPP5K and Pip5k1b in the regulation of ALR, but Pip5k1a appears dispensable for this role in myoblasts. In further support of this conclusion, we observed no differences between control and Pip5k1a-KD cells in the proportion of myoblasts exhibiting reformation tubules or the length of these tubules formed during autophagy (Figure 7, D–F). This contrasts with previous studies that indicated loss of Pip5k1a results in hyperextended tubules in NRK cells (30) and suggests that Pip5k1a function in muscle cells is not required for ALR. Indeed, in muscle cells, Pip5k1a has other identified roles in regulating AKT-dependent myoblast differentiation and calcium release (76). In contrast, Pip5k1b depletion in myoblasts robustly suppressed the formation of reformation tubules, consistent with previous reports (30). Codepletion of Pip5k1b in *Inpp5k-KD* cells restored both the turnover and length of reformation tubules to levels seen in control cells (Figure 7, D–F). Altogether, these data are consistent with an interpretation that INPP5K hydrolyzes a pool of PI(4,5)P₂ generated by Pip5k1b for ALR regulation and lysosome homeostasis.

Lysosome dysfunction (77, 78), α-dystroglycan hypoglycosylation (45), and autophagy inhibition (4, 5, 79) are each known to cause muscle disease. However, we questioned whether there was any association between the ALR defect caused by INPP5K ablation and α-dystroglycan hypoglycosylation in *Inpp5k<sup>fl/fl</sup> MCK-Cre* mice with advanced disease. The rationale for assessing this association was our observation that in *Inpp5k<sup>fl/fl</sup> MCK-Cre* mice, the defect in lysosome homeostasis due to suppression of ALR caused autophagy inhibition at the onset of muscle disease, prior to evidence of α-dystroglycan hypoglycosylation. Protein hypoglycosylation disorders can arise because of defects in lysosomal function, which may also be associated with autophagy abnormalities (80–82). Lysosomal function may also regulate glycosylated α-dystroglycan (83) with links to muscular dystrophy (84). This may occur because the lysosomal-dependent catabolism of glycoproteins is part of their normal cellular turnover (85). Damaged or improperly folded glycoproteins are delivered to lysosomes for catabolism either by endocytosis from the outside of the cell or via autophagy within the cell. Once inside the lysosome, glycoproteins are broken down into their amino acid and glycan constituents (monosaccharides), which are then transported from the lysosome back into the cytosol for recycled use in the biosynthesis of new glycosylated proteins. As such, the maintenance of lysosome homeostasis is integral not only for the quality control of glycosylated proteins but also glycoprotein production by ensuring an efficient supply of glycan moieties. Interestingly, *Inpp5k-KD* cells exhibited a defect in lysosome reformation (Figure 7, A and B) caused by suppression of ALR completion (Figure 7, D and E), and in these cells, glycosylation of α-dystroglycan was reduced compared with control cells (Supplemental Figure 2F). In contrast, in *Inpp5k/Pip5k1b double-KD* cells, in which ALR (Figure 7, D and E) and lysosome homeostasis (Figure 7, A and B) were restored, α-dystroglycan glycosylation was also reconstituted (Supplemental Figure 2F). This indicates that if the ALR and lysosome homeostasis defects are corrected in INPP5K-depleted cells by manipulation of key phosphoinositides that regulate this pathway, i.e., PI(4,5)P₂/PI(4)P (30–32), the glycosylation of α-dystroglycan is also restored. This also suggests that the glycosylation of α-dystroglycan occurs secondary to a defect in ALR.

**ALR inhibition occurs with disease INPP5K mutations.** Finally, to investigate a causal link between ALR inhibition and muscular dystrophy caused by INPP5K mutations, we evaluated whether the ALR defect due to loss of INPP5K could be restored by expression of either WT INPP5K, a catalytically inactive INPP5K mutant (D310G) that cannot hydrolyze PI(4,5)P₂ (35), or INPP5K disease mutants (G140S, IS0T, or Y300C), which show reduced PI(4,5)P₂-5-phosphatase activity (~70%–85%) (37, 38) (Figure 8, A and B). Critically, characteristic features of ALR inhibition (i.e., autophagy-dependent depletion of lysosomes and accumulation of enlarged LPOs) were rescued in *Inpp5k-KD* myoblasts by expressing WT INPP5K, but not a catalytically inactive INPP5K D310G mutant, or the G140S, IS0T, or Y300C INPP5K disease mutants (Figure 8, C–E). Therefore, INPP5K regulation of ALR was dependent upon its 5-phosphatase catalytic hydrolysis of PI(4,5)P₂ to PI(4)P, and this function was lost for the disease mutants that cause muscular dystrophy.
Mechanistically, INPP5E regulates autophagosome-lysosome fusion by altering lysosomal PI(3,5)P$_2$ and actin filament stabilization (87). Collectively, these studies and our study suggest that 5-phosphatase enzymes play distinct roles at specific stages of the autophagy pathway.

We propose that loss of ALR progression in INPP5K-null cells results from the accumulation of PI(4,5)P$_2$ coupled with the depletion of PI(4)P on autolysosomes, which leads to the accumulation of AP-2/clathrin, hyperelongation, and persistence of reformation tubules, and ultimately reduces lysosome production. The interconversion between PI, PI(4)P, and PI(4,5)P$_2$ is mediated by the PI-4 kinase PI4KIIIβ (33) and the PI(4)P-5 kinase Pip5K1b (30) enzymes, respectively, which synthesize these phosphoinositides, and as we report here was directly opposed by the 5-phosphatase INPP5K. In this regard, our data support a hypothesis that the bidirectional interconversion between PI(4)P and PI(4,5)P$_2$ acts as a gatekeeper for the control of lysosome homeostasis in vivo, the preservation of autophagy, and protection from muscle disease (Figure 9). Furthermore, we revealed that termination of PI(4,5)P$_2$ signals on autolysosomes was an integral step in the completion of the ALR process to generate lysosomes.

The majority of INPP5K disease mutations are located within the catalytic 5-phosphatase domain, exhibit decreased hydrolysis of PI(4,5)P$_2$ (37, 38), and as shown here, were unable to restore ALR in myoblasts with loss of INPP5K function. Muscular dystrophy directly caused by a primary defect in lysosome function (17, 77, 78) or primary defects in the autophagy pathway (7, 79) is described. Notably, our study identified defective ALR as a potentially novel cause of autophagy inhibition in skeletal muscle that led to muscle disease. Muscular dystrophy caused by INPP5K mutations exhibits histopathological features consistent with an autophagy-related muscle disorder (37, 38), and here we showed that muscle-specific ablation of INPP5K in mice led to disease with autophagy inhibition caused by suppression of ALR. Of note, even within the scope of muscle diseases known to be caused by autophagy inhibition (7, 92), the autophagy suppression that occurred in our mouse model of INPP5K muscular dystrophy was very severe even under basal conditions. This highlights the fundamental importance of ALR to sustaining autophagy function in muscle, a process that is essential to protect against muscle disease. Understanding the contribution of ALR defects to disease is only beginning to emerge but is of significant clinical interest. Recent studies have linked ALR dysfunction with neurodegenerative diseases, such as hereditary spastic paraplegia (68, 70, 93–96) and Parkinson disease (97). The key pathogenic features of lysosome depletion accompanied by enlarged autolysosomes (LPOs) and autophagy inhibition that we observed in the muscle of Inpp5k$^{-/-}$ MCK-Cre mice are definitive and consistent features observed in ALR-related neurodegenerative disorders (68, 70, 93, 96). This further supports our interpretation of a causal relationship between ALR suppression, autophagy inhibition, and muscular dystrophy.

Mechanistic understanding of the processes responsible for regulating ALR, as we revealed here for INPP5K-related muscular dystrophy, may reveal unrecognized disease genes and disorders associated with defects in this pathway. Interestingly, INPP5K binds the protein ARL6IP1 (72), mutations in which occur in...
hereditary spastic paraplegia (98, 99). Additionally, recent proteomics analysis of purified autolysosome membranes has identified additional proteins with functional links to PI(4)P/PI(4,5)P₂ and associations with human disease, but with as yet undefined roles in ALR (30). Our study provides a rationale for screening of other ALR candidate genes for their involvement in disease, with particular emphasis on PI(4,5)P₂/PI(4)P regulation. Finally, ALR inhibition may be a pathogenic mechanism for other muscle diseases and autophagy-related disorders.

Methods

For detailed methods, refer to Supplemental Methods. See complete, unedited blots in the supplemental material.

Generation of muscle-specific Inpp5k-KO mice. The Inpp5k-floxed mouse line (Inpp5klox/lox) was generated by Ozgene Pty Ltd. by the insertion of loxP sites flanking exon 8 of the murine Inpp5k gene. The targeting construct was electroprotopated into a C57BL/6 embryonic stem (ES) cell line called Bruce4 (100). Homologous recombinant ES cell clones were identified by Southern hybridization and injected into goGerm-line blastocysts (101). Male chimeric mice were obtained and crossed to C57BL/6 females to establish heterozygous germline offspring on a C57BL/6 background. Inpp5klox/lox mice were then crossed with MCK-Cre mice to generate conditional muscle-specific Inpp5k-KO mice (Inpp5klox/lox MCK-Cre). Mice were housed in a temperature- and humidity-controlled room on a 12-hour light/12-hour dark cycle, with access to food and water ad libitum (Animal Research Laboratory, Monash University, Australia). For fasting experiments, mice were rehoused for 24 hours in a clean cage without food but with access to water ad libitum. For all studies, only male mice were used at 12 weeks of age, unless otherwise stated.

Visualization of autolysosome reformation tubules in fixed cells. The integrity of autolysosome reformation tubules is completely disrupted by conventional fixation methods, and as such it has been suggested that visualization and analysis of these structures is restricted only to live-cell experiments or using isolated membrane fractions (2, 31, 33, 73). However, imaging and accurate quantitative measurements of tubules under live-cell conditions is challenging because they are dynamic, form and recede, and oscillate back and forth across the x, y, and z imaging planes (2). We therefore developed a robust method for imaging ALR tubules in fixed cells, based on rapid fixation, strict temperature control, and microtubule stabilization, which consistently preserved intact LAMP1-positive tubules. Our approach was based on evidence that ALR tubules require scaffolding by an intact microtubule network (2). The day prior to treatment, 2.0 × 10⁴ C2C12 cells were seeded onto fibronectin-coated (5 μg/mL; Sigma-Aldrich, F1141) glass coverslips in a 12-well dish. Cells were treated with EBSS ± 10% FCS before rapid and immediate fixation at indicated time points under precise temperature-controlled conditions. Cells were fixed via the addition of an equal volume of prewarmed, freshly made 8% PFA in 2× microtubule stabilization buffer (MTSB; 160 mM PIPES pH 6.8, 10 mM EGTA, 2 mM MgCl₂) directly to the cell culture media (final concentration of 4% PFA in 1× MTSB) and returned to a 37°C incubator for 15 minutes to complete fixation. During all transportation, handling, and fixation of cells, culture dishes were placed on a stainless-steel block (2.5 cm thick) prewarmed to 37°C. Refer to Supplemental Methods for details on immunostaining reformation tubules and their morphometric analysis.

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Address correspondence to: Christina Mitchell, Cancer Program and Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, 23 Innovation Walk, Clayton 3800, Victoria, Australia. Phone: 61.3.9905.4318; Email: christina.mitchell@monash.edu.
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