Lysosomal TRPML1 promotes autophagic flux through increased lysosomal SNAREs recruitment and autophagosome-lysosome fusion

PhD thesis

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Introduction

Autophagy is a cellular degradation pathway and highly conserved in eukaryotes. It is defined as engulfing damaged or unwanted cellular cargo inside vesicles called autophagosomes, delivering them to lysosomes for degradation and recycling. This mechanism is essential for cell survival and homeostasis during stress conditions, including, hypoxia, nutrient deprivation, and reactive oxygen (Gómez-Virgilio et. al. Cells. 2022). Autophagy defects lead to diseases, including neurodegenerative disorders, such as Alzheimer's disease and parkinson's disease (Levine et. al. Cell. 2008). Autophagic degradation pathway ensures cellular energy production, degradation of damaged or toxic intracellular material, and reuse of organelles components (Anding et. al., Dev Cell. 2017). For proper autophagic degradation, two critical steps must properly function: first, autophagosome-lysosome fusion to form hybrid organelle called autolysosome, and second, autolysosomal degradation by lysosomal hydrolases. Defects in those two steps were associated with diseases including Vici syndrome and lysosomal storage disorders (LSD) causing neurodegeneration (Hori et. al. Sci Rep 2017; Lieberman et. al. Autophagy 2012).

Lysosomes are the secondary cellular Ca^{2+} stores after ER. Lysosomal Ca^{2+} uptake from ER Inositol trisphosphate receptor (InsP3R) through lysosomal Ca^{2+} uptake channel, possibly CAX to maintain lysosomal homeostasis (Garrity et. al. eLife 2016). Lysosomal Ca^{2+} content is essential for several cellular functions, including autophagy, exocytosis, and membrane repair (Medina Int Rev Cell Mol Biol 2021). Lysosome membranes are embedded with a number of ion channels and pumps. Among these channels and pumps, vacuolar-ATPase pump (v-ATPase) and transient receptor potential cation channel, mucolipin subfamily, member 1 (TRPML1), are critical for autophagy machinery (Abuammar et. al. Cells 2021). V-ATPase is composed of peripheral V1 domain and membrane-bound V0 domain, promoting luminal acidification by pumping protons into lysosomal lumen in ATP-dependent process. TRPML1 is Ca^{2+} efflux channel and active during starvation-induced autophagy, promoting autophagic flux through autophagosome formation and lysosome biogenesis (Abuammar et. al. Cells 2021).

Mucolipidosis type IV (MLIV), one type of lysosomal storage disorder (LSD), is an inherited neurodegenerative disease characterized by delayed development and vision impairment due to lysosomal degradation defect seen as massive accumulation of unfused autophagosomes (Wakabayashi et. al. Mol Genet Metab 2011). MLIV is caused by mutations in MCOLN1/TRPML1 gene, leading to lysosomal dysfunction (Wakabayashi et. al. Mol Genet

Metab 2011). TRPML1 is a lysosomal cation channel that releases Ca^{2+} from the lysosomes upon amino acid depletion (Wang et. al. Proc Natl Acad Sci U S A 2015). TRPML1 function is implicated in several pathways, including, lysosomal biogenesis, lysosome positioning and tabulation, TFEB activation, and exocytosis (Li et. al. Nat Cell Biol 2016; Scotto et. al. Nat Commun 2019; Kim et. al. Biochem Biophys Res Commun 2019). Here, we studied autophagic consequences of the short-term TRPML1 Ca^{2+}.

Aims

- To detect TRPML1 activity in response to different drug treatments
- To investigate the role of TRPML1 Ca^{2+} release in the regulation of autophagosome-lysosome fusion
- To investigate the role of TRPML1 Ca^{2+} release in the regulation of lysosomal acidification
- To study the connection between autophagosome-lysosome fusion and lysosome acidification

Techniques and methods applied

1. Cell culture
2. Live imaging and confocal microscopy
3. Immunoblotting
4. RNA extraction and qPCR
5. Drug treatments
6. Molecular cloning (GCaMP6m-TRPML1)
7. Inducible expression system of shRNA
8. Stable human cell line
9. DNA transfection
10. Lysosome immunopurification (Lyso-IP)

Summary

Autophagy is a cellular degradation pathway that is highly conserved in eukaryotes. Its main pathway is defined as engulfing damaged or unwanted cellular cargo inside vesicles called autophagosomes, delivering them to lysosomes for degradation and recycling. This mechanism is essential for cell survival and homeostasis during stress conditions, including hypoxia, nutrient deprivation, and reactive oxygen species [251]. Autophagy defects lead to diseases, including neurodegenerative disorders, such as AD and Parkinson's disease [252]. The autophagic degradation pathway ensures cellular energy production, degradation of damaged or toxic intracellular material, and reuse of organelles components [253]. For proper autophagic degradation, two critical steps must properly function after autophagosome formation: first, AP-LY fusion to form a hybrid organelle called autolysosome, and second, autolysosomal degradation by lysosomal hydrolases. Defects in those two steps were associated with diseases including Vici syndrome and LSDs causing neurodegeneration [254,255].

Lysosomes are the secondary cellular Ca^{2+} stores after ER. Lysosomal Ca^{2+} content is essential for several cellular functions, including autophagy, exocytosis, and membrane repair [160]. Lysosome membranes contain several ion channels and pumps. Among these channels and pumps, vacuolar-ATPase pump (v-ATPase) and transient receptor potential cation channel, mucolipin subfamily, member 1 (TRPML1), are critical for autophagy [256]. V-ATPase is composed of peripheral V1 and membrane-bound V0 domains, and it promotes luminal acidification by pumping protons into lysosomal lumen in an ATP-dependent manner. TRPML1 is a Ca^{2+} efflux channel that is active during starvation-induced autophagy, promoting autophagic flux through autophagosome formation and lysosome biogenesis [256].

Mucolipidosis type IV, one type of lysosomal storage disorder, is an inherited neurodegenerative disease characterized by delayed development and vision impairment due to lysosomal degradation defect that causes massive accumulation of autophagic vesicles containing undegraded cargo [257]. MLIV is caused by mutations in MCOLN1/TRPML1 gene, which is the cause of lysosomal dysfunction [257]. Since TRPML1 is a lysosomal cation channel that releases Ca^{2+} from the lysosomes upon amino acid depletion [176]. TRPML1 function is implicated in several pathways, including lysosomal biogenesis, lysosome positioning and tubulation, TFEB activation, and exocytosis [211,224,237].

Our study aimed to decipher the role of TRPML1-released Ca^{2+} in regulation of autophagosome (AP)-lysosome (LY) fusion, lysosomal acidification, and the connection between these two processes. The methods used in this study were: i) confocal microscopy, ii)

immunoblotting, iii) qPCR, iv) molecular cloning, v) inducible expression of shRNAs, and vi) Lyso-IP.

Our work finds an early-onset role of TRPML1 during autophagy, which is different from its well-established long-term functions that rely on transcription [213,237]. This early-onset role of TRPML1 was observed after pharmacological TRPML1 activation in the form of rapid lysosomal maturation accompanied by enhanced recruitment of lysosomal SNAREs, ultimately facilitating local AP-LY fusion, autolysosomal degradation, and post-fusion lysosomal SNARE recycling. Here, TRPML1 agonist treatment rapidly increased the perilysosomal Ca^{2+} levels detected as increased GCAMP-TRPML1 fluorescence, which was restored to its basal level within 20 seconds. This perilysosomal Ca^{2+} peak was blocked by intracellular Ca^{2+} chelation. Furthermore, TRPML1 activation greatly reduced intralysosomal pH within 15 minutes, which was also prevented by intracellular Ca^{2+} chelation. Although lysosomal acidification relies on lysosomal v-ATPase activity, assembly of v-ATPase holocomplexes only mildly increased during this period, simply more V1-V0 holocomplex-containing lysosomes became acidic. 15 minutes of TRPML1 agonist activation also clearly enhanced lysosomal cathepsin activity.

Additionally, 15 minutes of TRPML1 activation increased lysosomal fusion with autophagosomes, which was also prevented by intracellular Ca^{2+} chelation. Notably, this short period of TRPML1 activation showed a reduction in the number of unfused autophagosomes, indicating that new autophagosomes did not form during this short-term treatment, in contrast with its reported long-term role on autophagosome formation. This short period of TRPML1 activation did not significantly change lysosomal positioning, again in contrast with its reported long-term role on lysosomal migration. In support of this, microtubule inhibition surprisingly increased basal TRPML1 activity and AP-LY fusion. Microtubule inhibition also increased the accumulation of $PI(3,5)P_2$ on lysosomes concomitant with an enlargement of lysosomes, indicative of undegraded autolysosomes. This increased AP-LY fusion upon microtubule inhibitor treatment was abrogated during BafA1 co-treatment. These findings support an early role of TRPML1 in orchestrating local AP-LY fusion. Consistently, both AP-LY fusion and lysosome acidification were attenuated by expression of pore-forming mutant TRPML1 during starvation and in fed cells. In parallel, the synthesis of TRPML1 agonist $PI(3,5)P_2$ drastically increased in lysosomes of 2 hour starved cells. This likely triggers TRPML1-mediated Ca^{2+} release during starvation, as it is the only known cellular agonist for this channel. As a mechanism behind this increased local fusion, TRPML1 activity showed a marked increase in lysosomal fusion competence by promoting the association of SNAREs Stx7 and VAMP7 to

lysosomes on LAMP carrier vesicles. Crucially, our work shows that lysosomal fusion with autophagosomes is required for maintaining lysosomal Ca^{2+} effluxes and hydrolytic activity. Fusion-impaired lysosomes did not respond to TRPML1 agonist and starvation in the context of TRPML1 Ca^{2+} efflux, intralysosomal acidification, and cathepsin activity. $PI(3,5)P_2$ depletion by inhibiting PIKfyve resulted in abnormal sequestration of lysosomal SNAREs in the lumen. Given that TRPML1 has allosteric binding sites for $PI(3,5)P_2$ and the synthetic agonist ML-SA1, $PI(3,5)P_2$-depleted cells could be rescued by allosteric drug activation that abrogated the abnormal SNARE localization, including luminal sequestration of Stx7, and restored its recycling to the plasma membrane in a Calmodulin-dependent manner. Consequently, agonist activation of TRPML1 for 15 minutes was enough to rescue the AP-LY fusion defect arising from PIKfyve inhibition.

According to our working model, synthesis of lysosomal $PI(3,5)P_2$ is increased on autolysosomes formed after primary fusions between autophagosomes and naïve lysosomes during starvation-induced autophagy. Subsequently, this $PI(3,5)P_2$ (or pharmacological agonist activation by ML-SA1) leads to TRPML1 activation, promoting the enrichment of lysosomal SNARE proteins VAMP7 and Syntaxin 7 that are transported by LAMP carrier vesicles as well as v-ATPase-mediated lysosomal acidification. The resultant autolysosomes become acidic and fusion competent, facilitating more secondary autophagosome-autolysosome (AP-AL) fusion events for the proper degradation of intracellular cargo captured by autophagosomes. These are then followed by post-fusion SNARE recycling to the plasma membrane. PIKfyve inhibition disrupts this process, leading to: i) reduced TRPML1 activity, ii) abnormal sequestering of lysosomal SNARE proteins. Our study complements earlier efforts to understand TRPML1's complex role in regulating autophagy, which showed that it promotes activation of TFEB and biogenesis of autophagy-lysosome pathway genes.

While the role of TRPML1 in facilitating lysosomal cargo degradation has been confirmed by multiple reports [213,216,231,232], ours is the first study to show that TRPML1 promotes early steps: fusion of existing autophagosomes with lysosomes and lysosomal degradation of the first wave of incoming cargo. Our work shows that TRPML1 promotes AP-LY fusion by increased recruitment of lysosomal SNAREs. It is tempting to suggest further investigations on the role of TRPML1 in disease models because TRPML1 could be a potential therapeutic target to correct AP-LY fusion defects observed in autophagic-lysosomal degradation associated diseases such as Vici syndrome and vacuolar myopathy.

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Mandatory peer-reviewed international publications for the fulfilment of doctoral process and on which this thesis is based:

1. **Abuammar H**, Bhattacharjee A, Simon-Vecsei Z, Blastyák A, Csordás G, Páli T, Juhász G. Ion Channels and Pumps in Autophagy: A Reciprocal Relationship. Cells. 2021 Dec 14;10(12):3537. doi: 10.3390/cells10123537. PMID: 34944044; PMCID: PMC8700256. (**I.F: 6.7**)

2. Laczkó-Dobos H, Bhattacharjee A, Maddali AK, Kincses A, **Abuammar H**, Sebők-Nagy K, Páli T, Dér A, Hegedűs T, Csordás G, Juhász G. PtdIns4P is required for the autophagosomal recruitment of STX17 (syntaxin 17) to promote lysosomal fusion. Autophagy. 2024 Mar 8:1-12. doi: 10.1080/15548627.2024.2322493. Epub ahead of print. PMID: 38411137. (**I.F: 13.3**)

3. Arindam Bhattacharjee*, **Hussein Abuammar***, Gábor Juhász, "TRPML1-mediated Ca^{2+} release regulates lysosomal degradative activity through promoting autophagosomal fusions and SNARE recycling" (**Under review**)
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Poster presentations

1. CFATG11(Club Francophone de l'AuTophaGie) – Lyon, France, Nov 2023. Short-term activation of TRPML1 promotes autophagic flux. **Hussein Abuammar**, Arindam Bhattacharjee, Gábor Juhász

2. International FOR2625 Symposium on Lysosomes & Autophagia, Berlin, Germany, May 2022. Short-term effects of TRPML1 activation on autophagic flux. **Hussein Abuammar**, Arindam Bhattacharjee, Gábor Juhász

3. Straub-days in Biological Research Centre, Szeged, May 2022. Role of the lysosomal TRMPL1/mucolipin-1 calcium channel in starvation-induced autophagy.

4. Hungarian Molecular Life Sciences Conference, Eger, Hungary, Nov. 2021. Role of the lysosomal TRPMLl/mucolipin-1 calcium channel in starvation-induced autophagy, **Hussein Abuammar**, Arindam Bhattacharjee, Gábor Juhász

5. Hungarian Molecular Life Sciences Conference, Eger, Hungary, March 2019. Deciphering the role of the small GTPase Rab27 in Drosophila autophagy, Arindam Bhattacharjee, **Hussein Abuammar**, Gábor Juhász.

Fellowships

1. Junior Scientist Fellowship from Institute of Genetics, HUN-REN Biological Research Centre, Szeged, Hungary (2023-2024)

2. Full-time PhD study through Stipendium Hungaricum Scholarship from Tempus Public Foundation, Hungary. (2018- 2022)

3. Full-time MSc study through Stipendium Hungaricum Scholarship from Tempus Public Foundation, Hungary (2016-2018)

4. Teaching Assistant through Best Student program, Al-Azhar University, Gaza, Palestine. (2014-2015)

Co-author waiver

I declare that Hussein Abuammar's contributions to these papers were significant, and I approve his submission of a PhD thesis. His co-first author papers (number 1 and 3) will not be used to complete a different PhD by another student.

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