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AAO Foundation Final Report Form
(a/o 2/9/2021)

Please prepare a report that addresses the following:

1. Type of Award (Check One):
   ___ Orthodontic Faculty Development Fellowship Award
   _x_ Postdoctoral Fellowship Award
   ___ Biomedical Research Award
   ___ Center Award
   ___ Educational Innovation Award
   ___ Program Award
   ___ Research Aid Award

2. Name(s) of Principal Investigator(s): Laura Jacox
3. Institution: UNC Chapel Hill
4. Title of Project: Roles of Autophagy in Orthodontic Tooth Movement
5. Period of AAOF support (e.g. 07-01-20 to 6-30-21): 07-01-2019 to 012-20-21
6. Amount of AAOF Funding: $50k per year for 2 years, $100k total

Summary/Abstract

Detailed results and inferences:

1. If the work has been published please attach a pdf of manuscript

Yes, the work has been published. Three associated manuscripts have been attached. The review article was published before this award began, and the two research articles are a direct result of AAOF support. One is fully published (PDF attached) and one is accepted and in press currently (submission proof attached.)

Respond to the following questions:

1. Were the original, specific aims of the proposal realized?

Yes, as summarized below and in the attached manuscripts.

Original Specific Aims

Orthodontic tooth movement (OTM) is dependent on efficient remodeling of alveolar bone. Mechanical force-induced aseptic inflammation is essential to the remodeling during OTM.[1–4] While acute inflammation promotes tissue adaptation to strain, chronic inflammation can cause unwanted damage. Inflammation during OTM is well-controlled in healthy patients, yet mechanisms responsible for such regulation remain elusive. Autophagy, an intracellular catabolic pathway, functions to maintain cellular homeostasis by recycling proteins and substrates, whose accumulation could be toxic. In disease states, autophagy protects cells from excessive inflammation, either directly by suppressing proinflammatory complexes or indirectly by allowing efficient clearance of damaged organelles.[5–8] Our preliminary data are the first to demonstrate a correlation between autophagy, inflammation and osteoclasts during OTM. Exploring these correlations may reveal mechanisms to target pharmacologically for accelerating tooth movement or reversibly halting it for retention or anchorage reinforcement. Understanding autophagy's inflammatory roles may provide targets for treating oral diseases due to excess inflammation such as periodontitis and orthodontic-induced apical root resorption.[9] *We propose the following specific aims to test the hypothesis that autophagy is activated by orthodontic force in peri-dental tissues (PDL & alveolar bone) and regulates the inflammatory response and bone turnover critical for OTM.*

Aim 1. Determine if orthodontic loading is correlated with cell-specific autophagic activity using GFP-LC3 reporter mice.

We evaluated autophagic activity and flux using confocal microscopy, Western Blot, and immunohistochemistry for cell-specific markers to identify cell types exhibiting autophagic activity in peri-dental tissues. Our results are summarized below and in the associated manuscripts.

Results Summary: Upon loading, we found that LC3-GFP signal was activated as early as day 1 in the compression (mesial) side, located more apically (Li/Jacox et al-Fig 2F-J). Compression side GFP+ puncta increased from day 3 to day 7, peaking at day 7 with activity extending more coronally (Fig 2F-K, F''-J"). By contrast, there was minimal change in autophagic puncta on the tension (distal) side (Fig 2F-J, F'-J', L) and on both sides of the control molar (Fig 2A-E'', K-L). An increase of LC3-autophagosome puncta indicates autophagy induction or downstream suppression. To confirm autophagic flux, molar PDL and surrounding alveolar bone were isolated for Western blot analysis of autophagy markers p-ULK1 and p62. With autophagy induction, p-ULK1 increases while p62 decreases or stabilizes as the pathway proceeds, as observed in our samples, consistent with autophagy activation occurring from day 1 onwards (Fig 2M). Taken together, the increase in GFP-LC3+ puncta and p-ULK1 protein indicate autophagy is induced upon mechanical loading *in vivo*.

In our subsequent study (Jacox et al), we determined that orthodontic loading activates autophagy but varies by force level (Fig 2-3). Sufficient but not excessive force is needed for appropriate autophagy activation, downstream signaling, osteoclast recruitment and tooth movement (Fig 1-4). Cell-type specific autophagy activation is found in macrophages and osteoclasts, which demonstrate colocalization of autophagy and cell type specific markers (Fig 5-6). Data indicate orthodontic loading activates autophagy in a force-dependent and cell type-specific manner. Autophagy is induced in peri-dental osteoclasts and macrophages by orthodontic loading (Fig 5-6). Autophagic activity, gene expression and osteoclast recruitment were correlated with load (Fig 2-4). Therefore, autophagy may play a role in regulating bone turnover needed for orthodontic tooth movement.

Aim 2. Assess effect of autophagy gain (GOF) and loss of function (LOF) on OTM.

Pharmacological effects of rapamycin were evaluated, with results summarized below. Significant adverse effects of Spautin-1 were observed in our mice even with new tritrations, preventing use. Results Summary: We injected rapamycin, an autophagy inducer, to provide a pharmacological gain of function (GOF). Rapamycin causes a statistically significant reduction in tooth movement compared to control groups (Li/Jacox et al- Fig 3J-O). There is no statistically significant difference between vehicle and no injection groups for tooth movement amount (Fig 3O). Data suggest that excess autophagy inhibits OTM, possibly through inhibition of the inflammatory cascade. Injection of rapamycin was associated with a significant decrease in osteoclasts relative to the vehicle. Lack of osteoclasts precludes bone resorption under compression, limiting orthodontic tooth movement, as observed in rapamycin-treated mice (Fig 3N-O).

Aim 3. Identify mechanisms by which autophagy impacts OTM.

We have conducted quantitative PCR (qPCR) on peri-dental tissues of extracted molars of control and rapamycin GOF mice after loading to quantify expression of autophagy (ATG5, Beclin-1, LC3), bone turnover (RANK Ligand, OPG, MMP9) and inflammatory markers (NFATC-1, TNFα, interleukins). We also looked at these markers as a function of time after loading and varying force level. We performed Osx IHC and TRAP staining to visualize osteoblasts and osteoclasts in control and rapamycin GOF molar sections. Finally, we conducted IHC for cell specific markers including F4/80 for macrophages, Cathepsin K for osteoclasts, and OSX for osteoblasts to explore which cells were activating autophagy in the periodontal ligament using confocal imaging and 3D cellular reconstructions.

Results Summary: We examined mRNA expression of inflammatory cytokines (NFATC1, TNFα, Il-6, and Il-1β), bone turnover markers (RANKL, OPG and MMP9) and autophagy pathway genes (Becn1, LC3 and Atg5) in peri-dental tissues after force loading by qRT-PCR (in Li/Jacox et al- Fig 4). Autophagy markers LC3 and ATG5 increased over time after loading, while BECN1 and inflammatory markers NFATC1, TNFα and IL-1β peaked early and then decreased (Fig 4A-C, G-I). Bone turnover markers RANKL and MMP9 were upregulated after force loading, though MMP9 decreased following day 3 (Fig 4D-F). However, Il-6 was not transcriptionally altered over time, consistent with published reports; translational or post-translational variations may be occurring (Fig 4J). Our data (Fig 4) demonstrate a correlated increase in autophagy, inflammatory and bone turnover markers secondary to orthodontic loading.

In our subsequent study (Jacox et al), we evaluate mRNA expression of autophagy, inflammatory and bone turnover markers as a function of time post-loading and loading force. The time course of expression indicates that autophagy activation, inflammatory and bone resorptive genes are upregulated shortly after loading by day 1, while a week later, bone formation genes are upregulated (Jacox et al- Fig 3). There appears to be an ordered sequence of events where shortly after force application, autophagy activation, inflammation and bone breakdown predominate, and by day 7, bone formation takes over with inflammation and autophagic activity subsiding. This sequence appears to function to the greatest degree at a moderate force level of 30g. A light force (15g) may be insufficient to elicit a response, and 45g may cause tissue damage altering the normal course of gene expression and autophagy's regulation of inflammation and bone turnover.

To determine which cell types exhibit autophagic activity in peri-dental tissues, sections of control and orthodontically-loaded molars from LC3-GFP mice were antibody labeled for cell-specific markers including macrophages (F4/80), osteoclasts (Cathepsin K), and osteoblasts (OSX), (Jacox et al- Fig 5, 6, Sup Fig 1). Confocal imaging allowed for evaluation of colocalization of red fluorescent cell markers with elevated GFP puncta indicative of autophagy induction. We observed autophagy activity in osteoclasts and macrophages in peri-dental tissues of orthodontically-loaded molars (Figs 5B-B'''', D-D'''', 6, Sup Fig 1). Osteoclasts with autophagic puncta were primarily located on the mesial/compression side of the PDL and in boney lacunae adjacent to the mesial root, likely at sites of resorption (Fig 5B-B'''', Sup Fig 1); these locations are consistent with the areas displaying TRAP

staining (Fig 4G-I, M) and autophagy active cells (Fig2G-I,M). Macrophages were concentrated around the root apex (Fig 5D-D’’’’, Sup Fig 1). To further confirm cellular colocalization of LC3-GFP puncta (GFP) and red fluorescent cell-specific labels (RFP), we assembled three-dimensional reconstructions of osteoclasts (Fig 6A-A’) and macrophages (Fig 6B-B’). White regions represent areas of overlap between the cell-type (in red) and LC3 green fluorescent autophagic puncta, confirming macrophages and osteoclasts activate autophagy. No autophagy induction was found in osteoblasts (data not shown). Autophagy active osteoclasts and macrophages were missing from the peri-dental tissues of control molars, suggesting that force application is required for autophagy induction (Fig 5A-A’’’’, C-C’’’’, Sup Fig 1).

2. Were the results published?
    a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers

Li Y*, **Jacox L***, Holder S, Kwon J, Ko C. (2021). Roles of Autophagy in Orthodontic Tooth Movement. Am J Orthod Dentofacial Orthop. 159(5):582-593. doi: 10.1016/j.ajodo.2020.01.027. Epub 2021 Mar 23. **Equal contribution*

Jacox L*, Tang N*, Yina L, Bocklage C, Coats S, Graves C, Miao M, Glesener T, Kwon J, Giduz N, Feng-Chang L, Martinez J, Ko C. (2021). Orthodontic loading activates cellspecific autophagy in a force-dependent manner. Accepted by AJODO. *In press in December 2021*. March 2022 in print. **Equal contribution*

Review article: Li Y*, **Jacox L***, Holder S, Ko C. (2018). Orthodontic tooth movement: The Biology and Clinical Implications. Kaohsiung Journal of Medicine Sciences (KJMS), 34(4), 207-214. DOI: 10.1016/j.kjms.2018.01.007 **Equal contribution* (Published shortly before AAOF support began). ***Listed on the American Board of Orthodontics Exam Reading List*

<u>Other papers published by the Jacox lab while I was supported by AAOF Junior Faculty Fellowship:</u>
Danze A*, **Jacox L***, Bocklage C, Moss K, Hardigan P, Godoy C, Jackson T (2020). Influence of childhood obesity on craniofacial morphology and development. EJO. https://doi.org/10.1093/ejo/cjaa056 **Equal contribution*, Corresponding author

Panchalingam K, **Jacox L**, Cappiello B, Sheley J (2020). Non-random sister chromatid segregation in human tissue stem cells. Symmetry. https://doi.org/10.3390/sym12111868

Lui D, Gallo G, Babikow E, Wiesen C, Jackson T, **Jacox L.** (2021). Identifying the Pandemic’s Impact on General Dentists’ Workforce Confidence and Workflow. Accepted by JADA. *In press.* Corresponding author. DOI: 10.1016/j.adaj.2021.11.011

Jacox L, Bocklage C, Edwards T, Mihas P, Lin F, Ko C. (2021). Technology Adoption by Orthodontists: A national survey. Am J Orthod Dentofacial Orthop. DOI: <u>https://doi.org/10.1016/j.ajodo.2020.08.024</u> Epub ahead of print 2021 Oct 21.

Lathrop H, Keyser MM, Jhingree S, Giduz N, Bocklage C, Couldwell S, Edwards H, Glesener T, Moss K, Frazier-Bowers S, Phillips C, Turvey T, Blakey G, White R, Mielke J, Zajac D, **Jacox L.** (2021). Orthognathic speech pathology: Impact of Class III malocclusion on speech. Euro J Orthod. *In press.* Epub Sept 25. DOI: 10.1093/ejo/cjab067. Corresponding author.

Lee D, Sulkowski T, Bocklage C, Frazier-Bowers SA, Wiesen C, Mihas P, **Jacox L.** (2021). Identifying factors that impact general dentists’ referrals to orthodontists. Am J Orthod Dentofacial Orthop. https://doi.org/10.1016/j.ajodo.2021.07.010. Epub 2021 Sept 16. Corresponding author.

Worthington CC*, Mihas P, Bocklage C, Frazier-Bowers SA, Lin FC, Ko CC, **Jacox LA**.*^ (2021) Educational debt and the gender gap: Understanding factors influencing orthodontists' career decisions. Am J Orthod Dentofacial Orthop. Sep 6:S08895406(21)00371-1. doi: 10.1016/j.ajodo.2020.10.027. Epub ahead of print. PMID: 34503861. **Equal contribution*, ^Corresponding author.
Jacox L, Mihas P, Lin F, Ko C. (2019). Technology Adoption by Orthodontists: A qualitative study. AJODO. 155(3):432-442. doi: 10.1016/j.ajodo.2018.08.018.

a. Was AAOF support acknowledged?

Yes, AAOF support was acknowledged on publications. Publications are listed above.

b. If not, are there plans to publish? If not, why not?

N/A

3. Have the results of this proposal been presented?
   a. If so, list titles, author or co-authors of these presentation/s, year and locations

All lab presentations given during the period of AAOF support have been included below with autophagy presentations underlined.

National:

2021 E-poster presentation: Identifying COVID-19's Impact on Dentists' Workforce Confidence and Workflow Changes. Academy of General Dentistry Annual Meeting. Virtual due to pandemic/Austin, Texas. Mentees: Gallo G, Liu D,

2021 Oral Presentation: Orthognathic Speech Pathology: Understanding How Dentofacial Disharmonies Effect Speech. Oral Presentation, American Association of Orthodontics, Virtual due to pandemic/Boston, MA

2021 E-poster presentation: Orthognathic speech pathology: Understanding how anterior open bites effect speech. American Association of Orthodontics Virtual Meeting/Boston, MA (virtual due to COVID-19 pandemic) Mentee: Keyser MM

2021 E-poster presentation: Perceptions of Patients and Caregivers on Therapy Dogs in Orthodontic Offices. American Association of Orthodontics Virtual Meeting/Boston, MA (virtual due to COVID-19 pandemic) Mentee: Cass K

2020 E-poster presentation: Orthognathic speech pathology: Understanding how dentofacial disharmonies effect speech. American Association of Orthodontics Virtual Meeting, Atlanta, Georgia (virtual due to COVID-19 pandemic)

2020 E-poster presentation: Understanding factors influencing orthodontist career decision-making. American Association of Orthodontics Virtual Meeting, Atlanta, Georgia (virtual due to COVID-19 pandemic) Mentee: Campbell C

2019 Oral presentation: Roles of Autophagy in Orthodontic Tooth Movement. American Association of Orthodontics, Los Angeles, CA Collaborator/Mentee: Li-

Thomas Graber Award of Special Merit Presentation

2019 Poster presentation: Orthognathic speech pathologies: Understanding how class III jaw disharmonies influence speech. Annual meeting of the American Association of Orthodontics (AAO), Los Angeles, CA Mentee: Lathrop H

2019 Poster presentation: Technology Adoption by Orthodontists: A qualitative and quantitative study. Annual meeting of the American Association of Orthodontics (AAO), Los Angeles, CA

2018 Poster presentation: Roles of Autophagy in Orthodontic Tooth Movement. American Association of Orthodontics, Washington, DC Collaborator/Mentee: Yina Li - Presentation at the Charley Schultz Resident Research Competition

International

2021 Oral Presentation: Orthognathic Speech Pathology: Understanding How Dentofacial Disharmonies Effect Speech. International Association of Dental Research (IADR) Meeting, Virtual due to pandemic/Boston, MA, USA
Growth and Development Research Award Finalist- Certificate of Excellence

2020 E-poster presentation: Orthognathic speech pathology: Understanding how Class III dentofacial disharmonies effect speech. International Orthodontic Conference Virtual Meeting, Tokyo, Japan (virtual due to COVID-19 pandemic)

2019 Poster presentation: Fixed orthodontic treatment induces time-varying dysbiosis in both composition and function of supragingival oral biofilm. European Orthodontic Society Congress, Nice, France

2019 Poster presentation: Effects of Class III malocclusion on acoustic characteristics of voiceless consonants. American Speech-Language-Hearing Association Convention, Orlando, FL

Invited Oral Presentations and Unpublished Abstracts

Local

2021 Orthognathic speech pathology: Understanding how dentofacial disproportions impact speech. University of North Carolina Linguistics Colloquium, Chapel Hill, NC. Friday lecture series with Dr. David Zajac and Dr. Jeff Mielke.

2021 Animal Assisted Therapy for Management of Dental Anxiety. North Carolina Public Health Association, Asheville, NC

2021 E-poster presentation: Identifying COVID-19's Impact on Dentists' Workforce Confidence and Workflow Changes. Adams School of Dentistry Dental Research and Review Day, Chapel Hill, NC. Mentees: Gallo G, Liu D, Babikow E
Award: James Bawden Student Research Award

2021 E-poster presentation: Roles of Autophagy in Orthodontic Tooth Movement. Adams School of Dentistry Dental Research and Review Day, Chapel Hill, NC. Mentee: Bocklage C
Award: 1st place NC-AADR Turner Award, Staff/Postdoctoral Category, ASOD-

UNC

2021 E-poster presentation: Validating Spectral Moment Analysis for Evaluation of Speech Distortions in Dentofacial Disharmony Patients
Adams School of Dentistry Dental Research and Review Day, Chapel Hill, NC.
Mentee: Jhingree S

2021 E-poster presentation: Orthognathic speech pathology: Understanding how anterior open bites effect speech.
Adams School of Dentistry Dental Research and Review Day, Chapel Hill, NC.
(virtual due to COVID-19 pandemic) Mentee: Keyser MM

2021 E-poster presentation: Perceptions of Patients and Caregivers on Therapy Animals in Orthodontic Offices.
Adams School of Dentistry Dental Research and Review Day, Chapel Hill, NC.
(virtual due to COVID-19 pandemic) Mentee: Cass K

2021 Oral presentation: Phase I Orthodontics: Early Dentofacial Interventions for Children and Appropriate Orthodontic Referrals. UNC Chapel Hill, Chapel Hill, NC.

2020 Oral presentation: Understanding factors influencing young orthodontist career decisions. Adams School of Dentistry Research Day, Chapel Hill, NC. 02/12/20
Mentee: Campbell C

2020 Oral presentation: Phase I Orthodontics: Early Dentofacial Interventions for Children. UNC Chapel Hill, Chapel Hill, NC

2019 Oral presentation: Current trends in Orthodontic technology adoption: A qualitative and quantitative study.
Adams School of Dentistry Dental Research and Review Day, Chapel Hill, NC.

2019 Oral presentation: Current trends in orthodontic technology adoption. UNC Chapel Hill and Align Technology, Chapel Hill, NC

2019 Oral presentation: Current trends in orthodontic technology adoption: A qualitative and quantitative study. Master's Thesis Defense, UNC Chapel Hill, Chapel Hill, NC

b. Was AAOF support acknowledged?
Yes, in all presentations listed above.

c. If not, are there plans to do so? If not, why not?
N/A

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

It is no exaggeration to say that the AAOF funding has been critical for my academic career and

I may not have succeeded as a junior investigator without the Martin 'Bud' Schulman Junior Faculty Award. When I was hired, our dental school was encountering serious budgetary shortfalls and stopped offering start-up support to new faculty. This AAOF funding provided necessary support for my new lab, to allow us to pursue these studies, publish papers, generate preliminary data and recruit additional support. Having an award from the AAOF also indicated that other clinician-scientists believed in and supported our work, which allowed me to renegotiate terms for my position and lab, resulting in startup funds beginning this year. Through the help of AAOF support, I was also promoted to our Director of Graduate Orthodontic Research, to help support the research mission of our program.

I am incredibly grateful for the AAOF and their support of junior orthodontic faculty like myself. Funding from the AAOF provided the support to execute this study and explore these impactful questions. Working with the AAOF has been a wonderful experience, and your continued support of our other research endeavors and for our residents is deeply appreciated. I plan to apply for future AAOF resources as my research program develops and I will encourage other faculty and residents to do the same.

Accounting for Project; (i.e.), any leftover funds, etc.
All funds were utilized for project expenses. Account balance is $0.00.

5. Illustrations, addendum - *Selected figures from two accepted manuscripts in AJODO. All figures are included in the attached manuscript pdfs.*

Selected Figures from Li/Jacox et al: Li Y*, **Jacox L***, Holder S, Kwon J, Ko C. (2021). Roles of Autophagy in Orthodontic Tooth Movement. Am J Orthod Dentofacial Orthop. 159(5):582-593. doi: 10.1016/j.ajodo.2020.01.027. Epub 2021 Mar 23. **Equal contribution*

Figure 2. Orthodontic loading activates autophagy. [A-J''] Zeiss LSM710 confocal imaged sections of first molar distal roots from GFP-LC3 mice post-loading. [A-J] Mag 40x, scale bar: 150 μm; [A'- J''] Mag 60x, scale bar: 100 μm; [A'-J'] Mesial PDL zoom-in (left yellow box in A). [A''-J''] Distal PDL zoom-in (right yellow box in A). [F-J''] Experimental: Mesial, compression- left. Distal, tension- right. [F] Large yellow arrow indicates direction of force application with compression/mesial on the right and tension/distal on the left. [F', F''] Arrowheads: zoomed-in cells with puncta. Green: GFP-LC3; Blue: DAPI nuclei. [K] Quantification of autophagosome puncta versus days post-loading in PDL of control and loaded molars on the mesial side of the molars. Fluorescent puncta quantified in a uniform (100 μm x 150 μm) area using Image J. [L] Quantification of autophagosome puncta versus days post-loading in PDL of control and loaded molars on the distal side of the molars. [M] Western blot analysis of p-Ulk1 and p62.

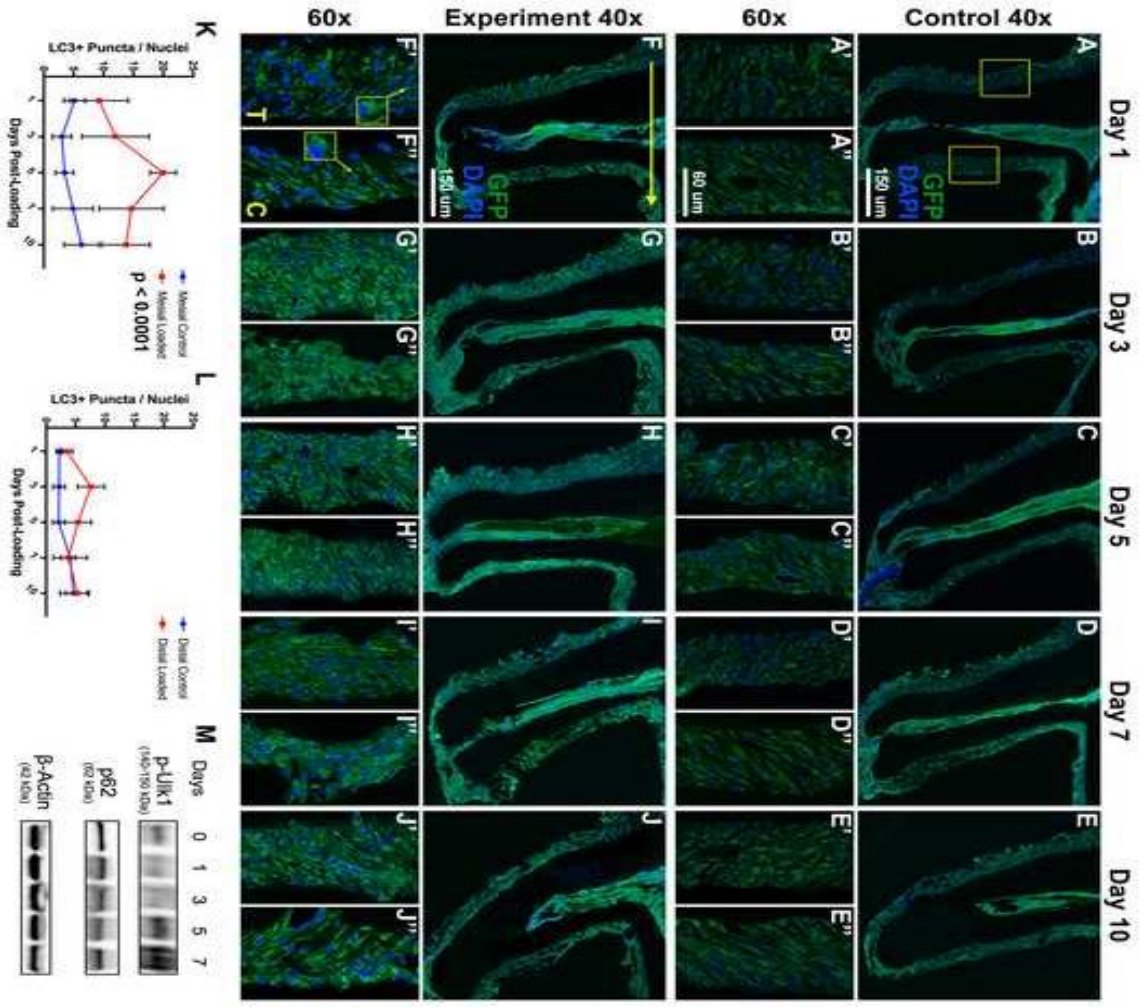


Figure 3. Orthodontic tooth movement (OTM) is drastically reduced by autophagy GOF with rapamycin. [A-C] Schematic of orthodontic force application in mice with a split-mouth design. Occlusal view of the maxilla before [A] and after [C] placement of a NiTi coil spring on

the experimental (E) side. The control (C) side has no spring placed and the molar remains unloaded. [B] 30 g of force measured by a force gauge prior to cementing the spring with light-cured composite resin. [D-I] 2D CT radiographs of control [D-F] and experimental [G-I] side molars. Left: mesial, compression side. Right: distal, tension side. Scale bar: 2 mm. [J-M] 2D CT radiographs of control [J-K] and experimental [L-M] side molars of mice injected with either saline vehicle or rapamycin. [N] OTM quantification at days post-loading in mice injected with saline vehicle or rapamycin. [O] Graph comparing OTM measured in μm of uninjected mice, saline vehicle injected mice and rapamycin injected mice.

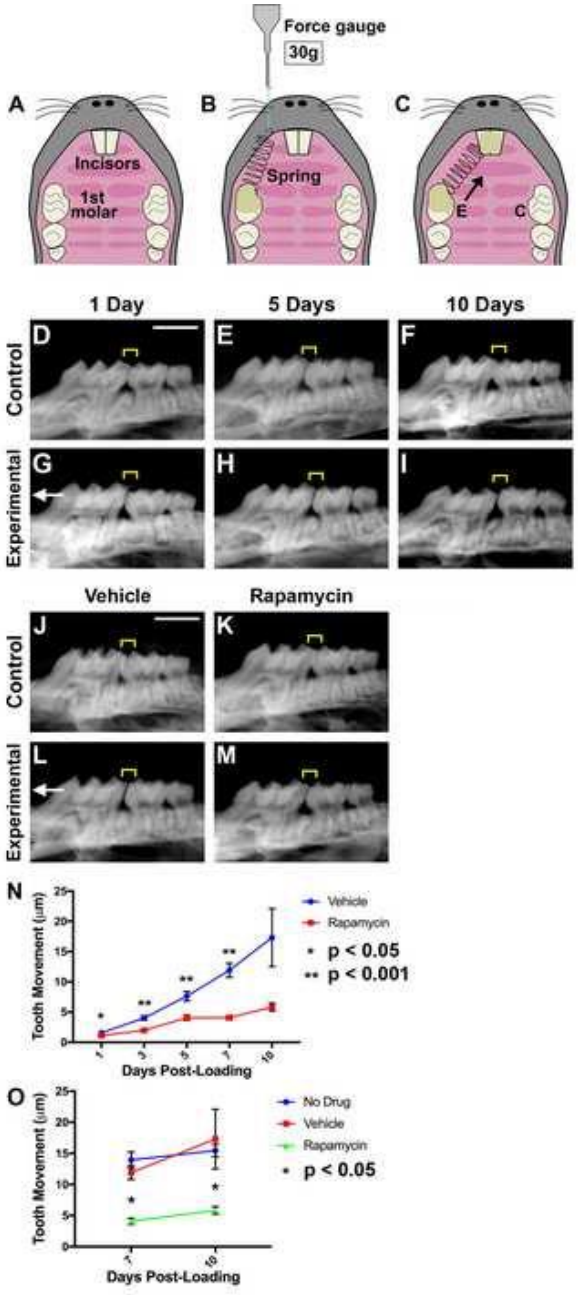
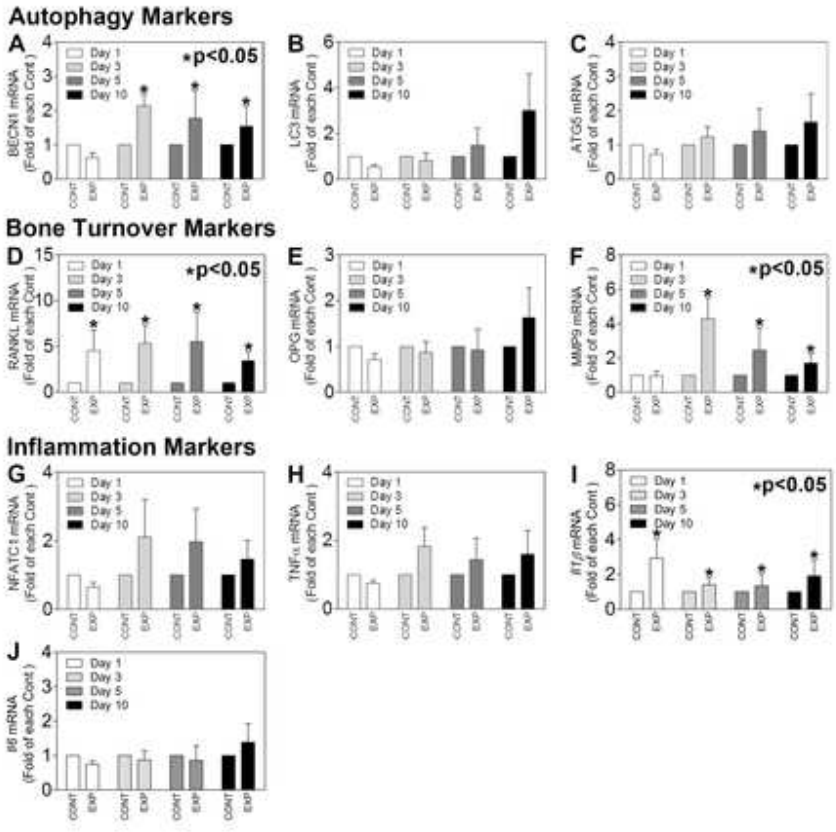


Figure 4. Expression qPCR analysis of autophagy, inflammatory and bone turnover markers. Expression of autophagy [A-C, BECN1, LC3, ATG5], bone turnover [D-F, RANKL, OPG, MMP9], and inflammatory markers [G-H, NFATC1, TFNα, IL-1β] increases in peri-dental tissues after orthodontic loading at time points days 1, 3, 5, and 10 after loading. Inflammatory marker *Il-6* was not transcriptionally altered in peri-dental tissues after loading.



Selected Figures from Jacox et al: **Jacox L***, Tang N*, Yina L, Bocklage C, Coats S, Graves C, Miao M, Glesener T, Kwon J, Giduz N, Feng-Chang L, Martinez J, Ko C. (2021). Orthodontic loading activates cellspecific autophagy in a force-dependent manner. Accepted by AJODO. *In press in December 2021.* March 2022 in print. **Equal contribution*

Figure 2. Orthodontic loading activates autophagy but varies by force level. [A-L'] Nikon fluorescent microscope imaged sections of first molar distal roots from GFP-LC3 mice post-loading. [A-L] Green: GFP-LC3, Scale = 100 μm; [A'- L'] Green: GFP-LC3; Blue: DAPI nuclei, Scale = 100 μm. [D-L'] Experimental: Mesial, compression- right. Distal, tension- left. [D, G, J] Large yellow arrow indicates direction of force application with compression/mesial on the right and tension/distal on the left. PDL: Periodontal ligament. RC: root canal. Bone: alveolar bone. Yellow dashed lines- the outer lines the edge of the alveolar bone and the inner lines the root canal. [M-N] Quantification of autophagosome puncta / DAPI nuclei versus days post-loading in PDL of control (0g) and loaded (15g, 30g, 45g) molars on the mesial (M) and distal (N) sides of the root. Fluorescent puncta were quantified in a uniform (300l x 300 ul) area. Statistical significance ($p < 0.05$) is indicated by an asterisk next to the timepoint, when there were

significant differences between all groups at that time point, and by an asterisk next to the force level, when there were significant trends over time within a force group. Graphs display the mean and SEM. Convention: * p<0.05. [O] Quantification of PDL dimension mesial / distal ratio versus days post-loading of control (0g) and loaded (15g, 30g, 45g) molars.

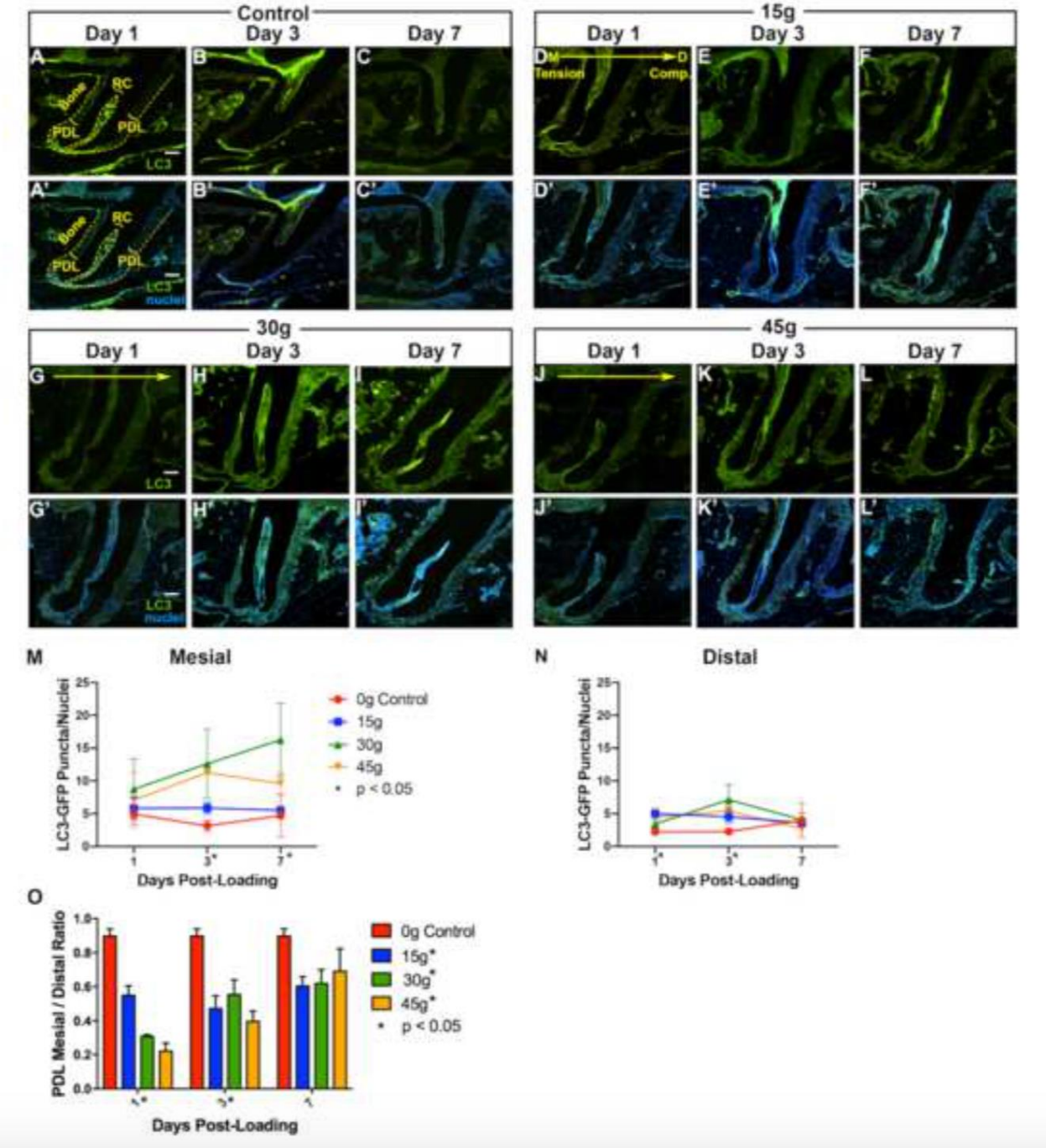


Figure 3. Expression qPCR analysis of autophagy, inflammatory and bone turnover markers. Expression of autophagy [A-D; BECN1, LC3, ATG5, ATG7], inflammatory [E,F; NFATC1, IL-1β], and bone turnover markers [G-M; RANKL, RANKL/OPG, OPG, OSX, RUNX2, MMP9, OCN] increases in peri-dental tissues after orthodontic loading at time points 1, 3, and 7 days after loading but fold change varies with force level applied (n=3 each at each time point and force level, 0g control, 15g, 30g and 45g). Y axis displays fold change in mRNA level. Statistical significance ($p < 0.05$) is indicated by an asterisk next to the timepoint, when there were significant differences between all groups at that time point. Significant pairwise comparisons ($p < 0.05$) are noted with an asterisk and a bracket. Convention: * $p<0.05$.

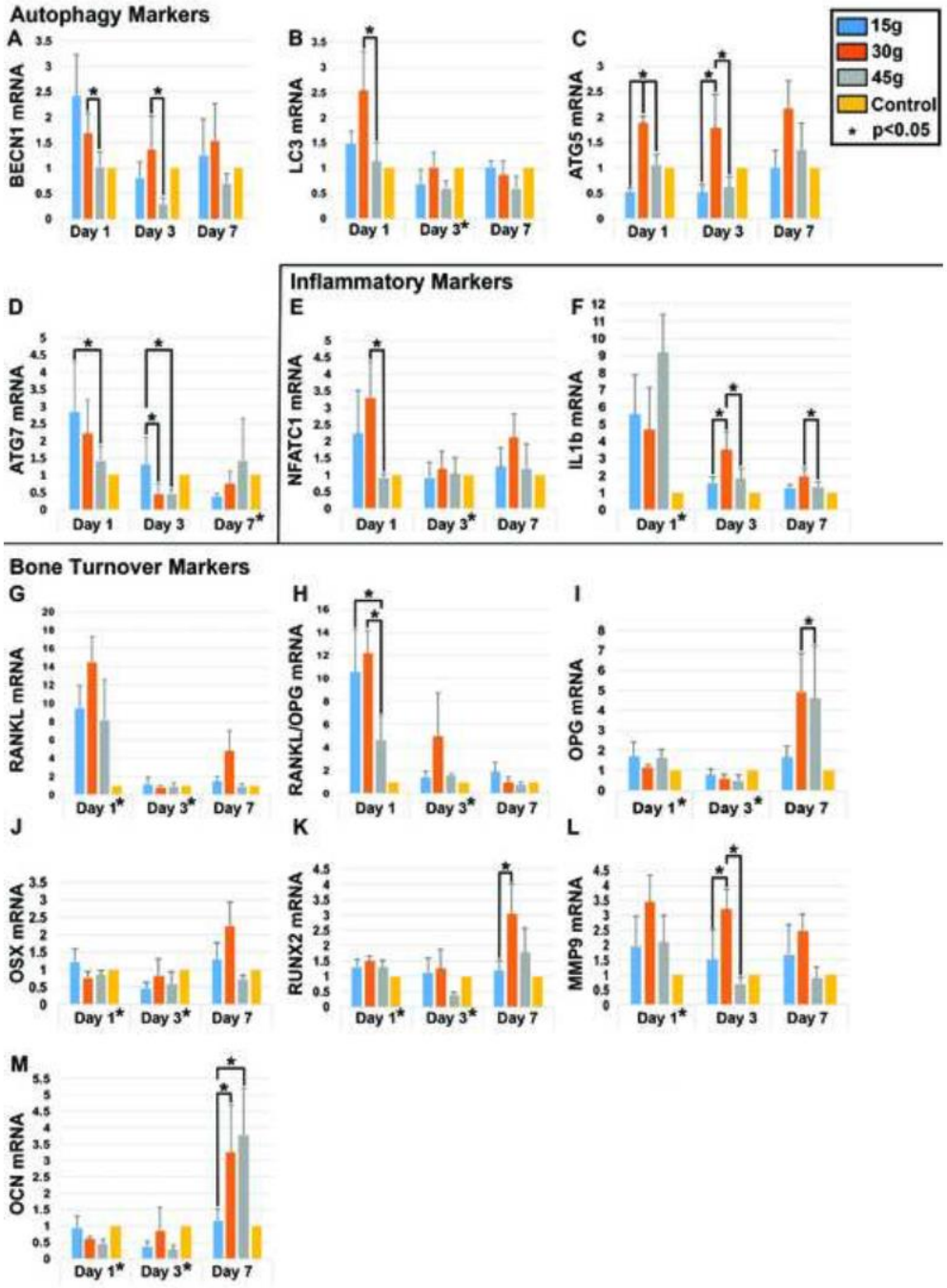


Figure 5: Cell type-specific autophagy activation is found in macrophages and osteoclasts after orthodontic loading. Sections of the first molar distal root with GFP [A-D’’’], Cathepsin K [A-B’’’] and F4/80 [C-D’’’] immunolabeling, to visualize LC3-GFP, osteoclasts and macrophages, respectively. Anti-GFP: green. Anti-Cathepsin K (Cath K) and F4/80: red with nuclear DAPI: blue. PDL: Periodontal ligament. RC: root canal. Bone: alveolar bone. Yellow dashed lines- the outer lines the edge of the alveolar bone and the inner lines the root canal. [A, C] Control: no loading, n= 3 mice with 4-8 sections each. [B, D] Experimental: loaded at time 0 with 30g, sacrificed at day 3 and imaged, n=3 mice with 4-8 sections each. Mesial, compression: right. Distal, tension: left. Yellow arrow indicates direction of force application. Yellow box indicates area that is enlarged below in the zoomed in images to visualize individual cells. Scale = 100 μm. [A’-A’’’, C’-C’’’] Control: no loading. Pairs of fluorescent channels (A’-A’’’, C’-C’’’) followed by all fluorescent channels (A’’’, C’’’’). [B’-B’’’, D’-D’’’] Experimental: loaded with 30g. Pairs of fluorescent channels (B’-B’’’, D’-D’’’) followed by all fluorescent channels (B’’’, D’’’), demonstrating co-localization of osteoclast and macrophage red fluorescence with green LC3 fluorescence. Scale = 10 μm.

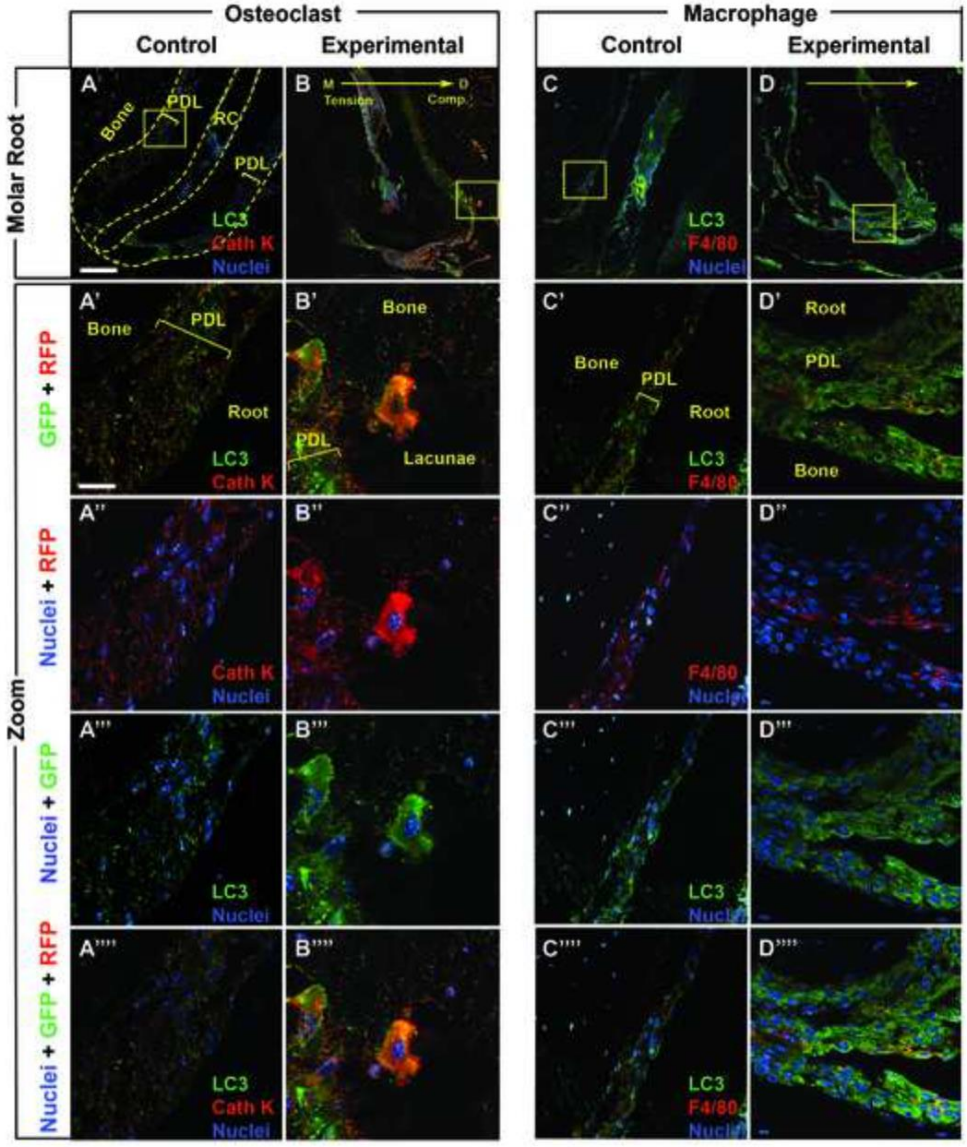


Figure 6: 3D reconstruction demonstrates co-localization of autophagy, macrophage and osteoclasts markers. 3D reconstructions of confocal *Z*-stacks of peri-dental cells from the first molar distal root. GFP [A-B'], Cathepsin K [A, A'] and F4/80 [B, B'] immunolabeling were used to visualize LC3-GFP puncta, osteoclasts and macrophages, respectively. Experimental molars of LC3-GFP mice were loaded at time 0 with 30g of force and sacrificed at day 3, n=3 mice. LC3-GFP: green. Cathepsin K (Cath K) and F4/80: red with nuclear DAPI: blue. [A, A'] 3D reconstruction of an osteoclast demonstrating co-localization of red fluorescent signal (Cathepsin K of osteoclasts) and LC3-GFP puncta (autophagic activity). [A'] Confocal *Z*-stack illustrative projection for visualization of percent volume of fluorescence cellular co-localization. White regions demonstrate colocalization of Cathepsin K with LC3-GFP puncta. [B, B'] 3D reconstruction of a macrophage demonstrating co-localization of red fluorescence (F4/80 of macrophages) and LC3-GFP puncta (autophagic activity). [B'] Confocal *Z*-stack illustrative projection for visualization of percent volume of green co-localized with red fluorescence. White regions demonstrate colocalization of F4/80 with LC3-GFP puncta. Scale = 5 μm.