Osteocyte-Derived CaMKK2 Regulates Osteoclasts and Bone Mass in a Sex-Dependent Manner Through Secreted Calpastatin

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Running Title: Female Osteocyte-Derived CaMKK2-Extracellular Calpastatin Mechanism Regulates Osteoclasts

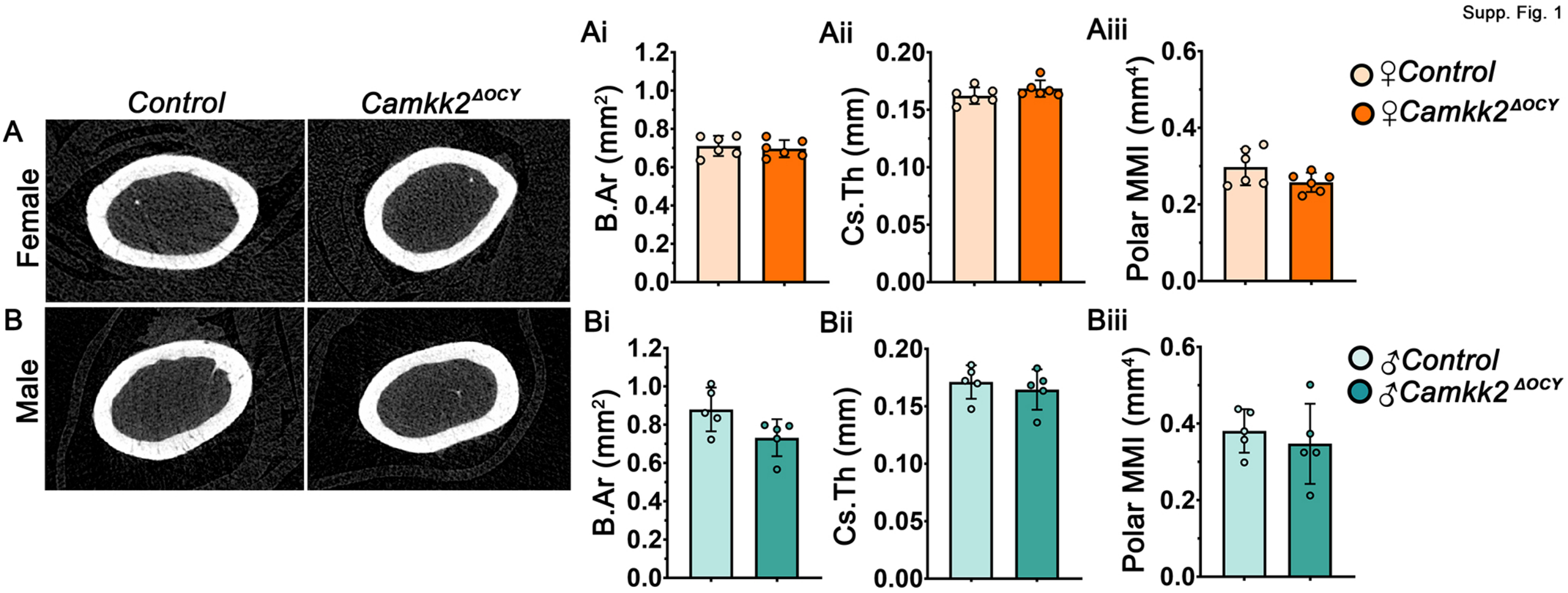
Supplementary Figure 1: Conditional deletion of CaMKK2 from osteocytes does not impact cortical bone geometry in female or male mice. (A) Representative crosssections of the femoral midshaft of female and male control and *Camkk2*$^{\Delta OCY}$ mice. Cortical bone parameters for female and male Control and *Camkk2*$^{\Delta OCY}$ femurs including **(Ai, Bi)** cortical bone area, **(Aii, Bii)** cross-sectional thickness and **(Aiii, Biii)** polar moment of inertia. Error bars represent SD.

Supplementary Figure 2: Expression of classical bone remodeling factors remain unaltered in CaMKK2-deficient osteocytes. **(A-B)** Expression of *Rankl* and *Opg* mRNA, as well as the *Rankl*/*Opg* ratio in osteocytes isolated from individual female and male control and *Camkk2*$^{\Delta OCY}$ mice (n=10-12 mice/group). *Rankl* and *Opg* mRNA levels were normalized to those of *β-Actin*.

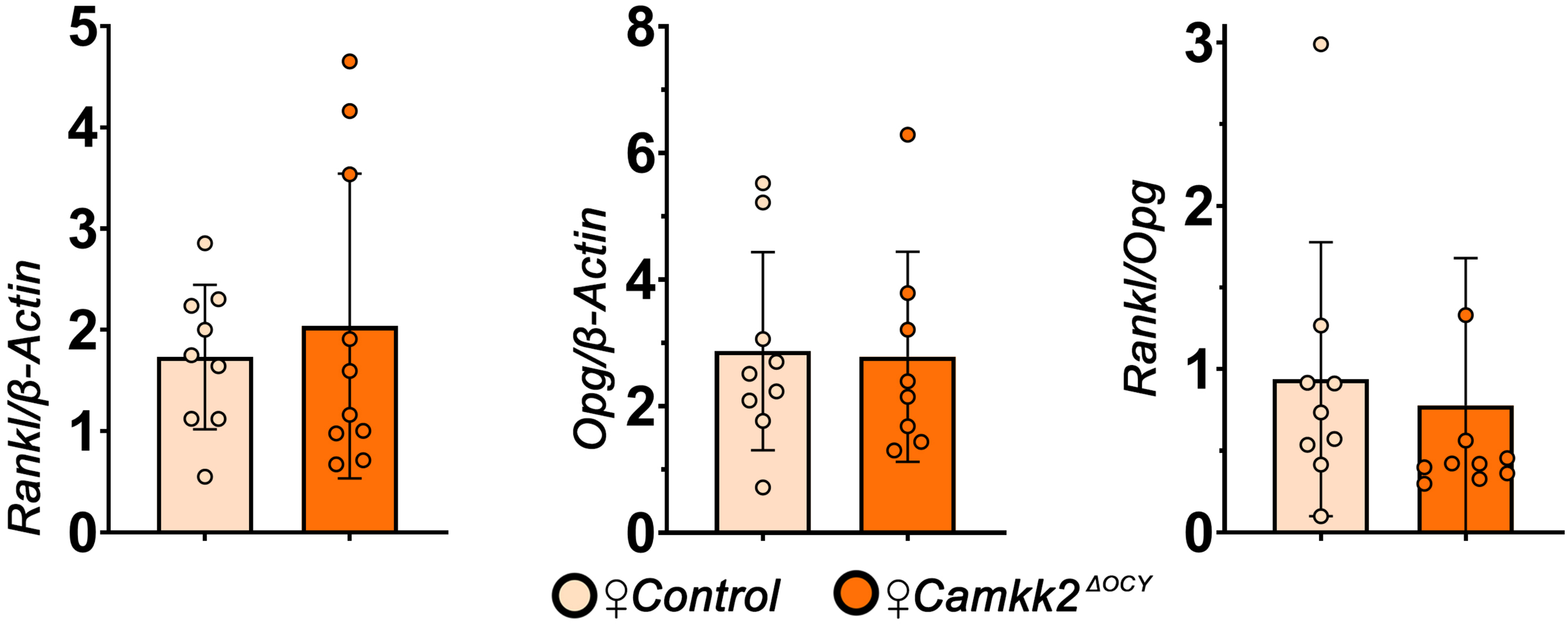
Supplementary Figure 3: Treatment with non-cell permeable recombinant calpastatin results in a dose-dependent inhibition of OCs in a sex-divergent manner. (A) Representative images (magnification – 40X) showing hydroxyapatite resorption pits generated by female and male WT OCs that were treated with 0, 0.1 μM, 0.5 μM, 1.0 μM and 5.0 μM NCP human calpastatin domain I. BM-derived monocytes were treated with the indicated dose of recombinant calpastatin in OC media containing RANKL and M-CSF1 for 6 days. **(B, E)** total number of pits measured; **(C, F)** percent area resorbed; and **(D, G)** number of TRAP-positive osteoclasts with ≥ 3 nuclei, in male and female cohorts, respectively. Error bars represent SD. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

Supplementary Figure 4: Conditioned media from CaMKK2-deficient osteocytes does not affect osteoclast precursor migration or intracellular levels of Cast in OCs. **(A-B)** Representative phase contrast images (magnification 100X) of WT BM-derived OC precursors at **(A)** 0 hours (immediately after scratch) and **(B)** 18 hours post-scratch. **(C)** Percent of area within the scratch generated gap that was covered by OC precursors at 18 hours post-scratch

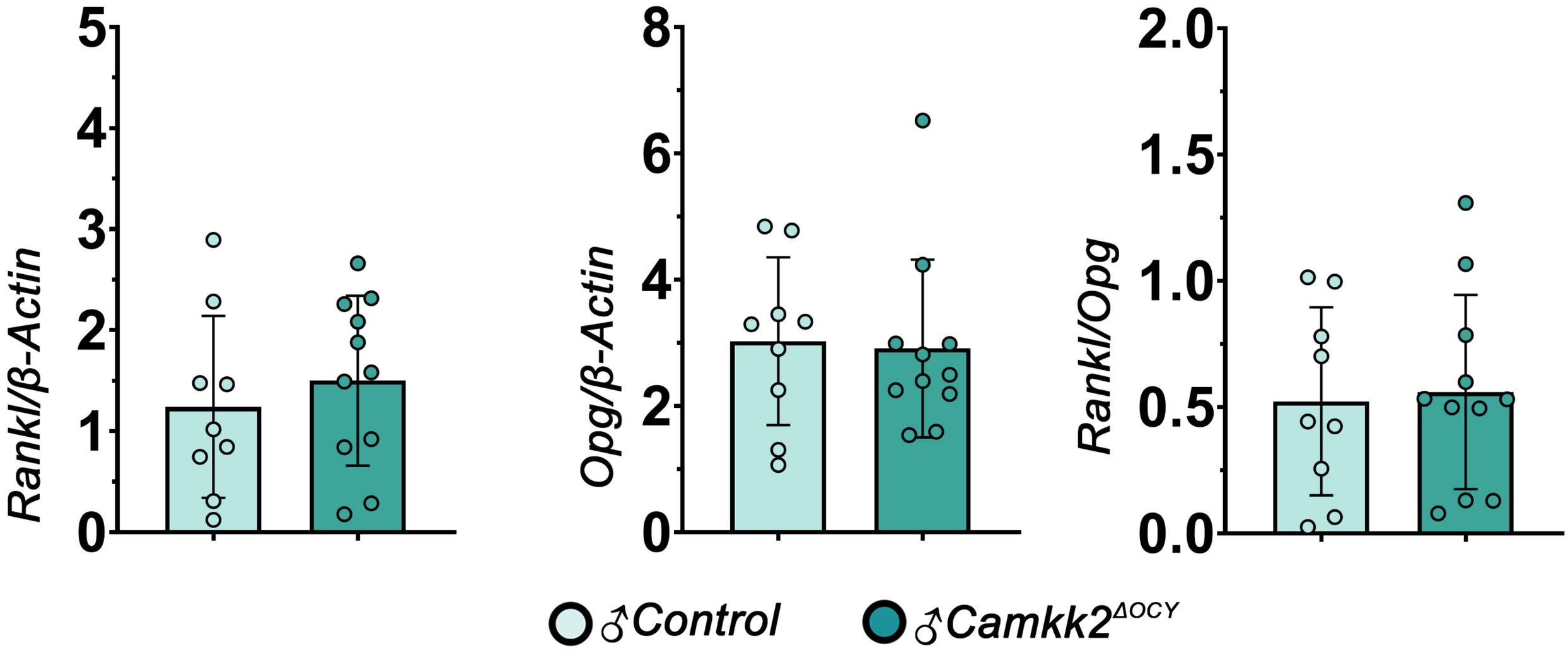
as calculated using ImageJ software. **(D)** Representative immunoblots showing levels of intracellular talin (calpain substrate), calpastatin, phosphorylated PKA-C and total PKA-C (upstream activator of calpains) relative to b-Actin in WT OCs, following treatment with female control osteocyte CM or *Camkk2*$^{\Delta OCY}$osteocyte CM depleted of calpastatin or treated with control IgG.


Control
Camkk2ΔOCY
A
Female
B
Male
Ai
Aii
Aiii
B.Ar (mm²)
Cs.Th (mm)
Polar MMI (mm⁴)
♀Control
♀Camkk2ΔOCY
Bi
Bii
Biii
♂Control
♂Camkk2 ΔOCY

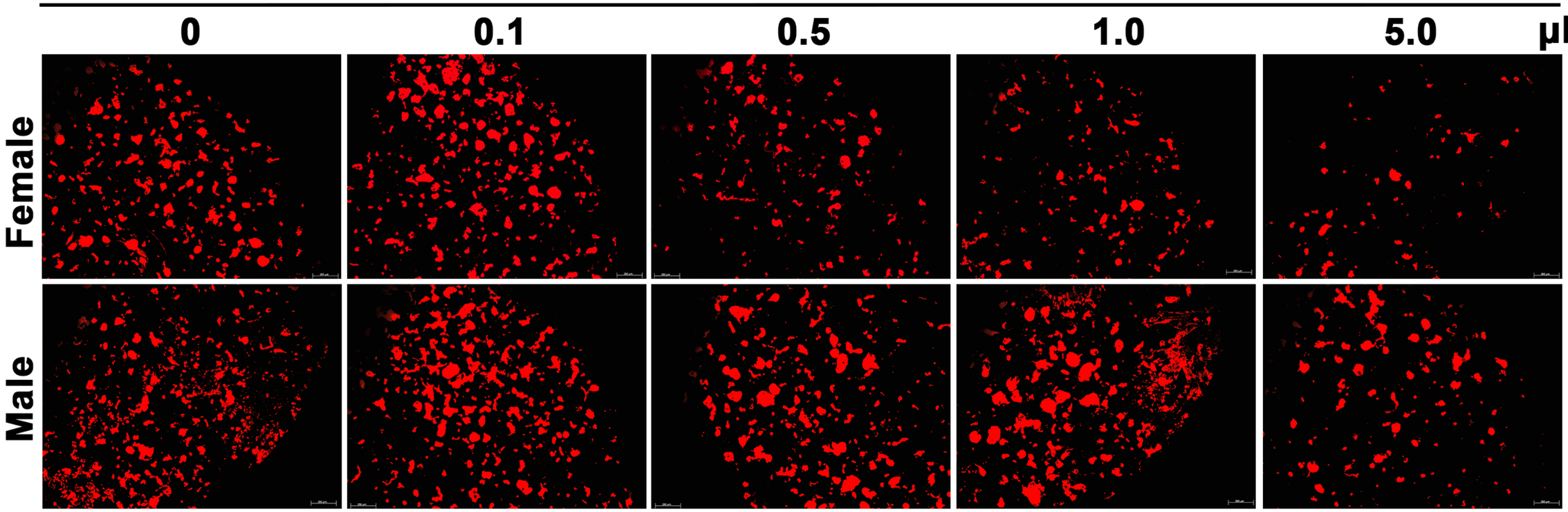
A


Rankl/β-Actin
0
1
2
3
4
5
Opg/β-Actin
0
2
4
6
8
Rankl/Opg
0
1
2
3
♀Control
♀Camkk2ΔOCY

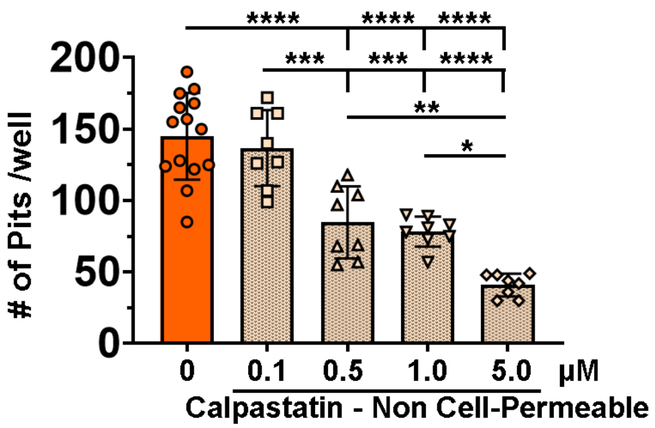
B


Rankl/β-Actin
0
1
2
3
4
5
Opg/β-Actin
0
2
4
6
8
Rankl/Opg
0.0
0.5
1.0
1.5
2.0
♂Control
♂Camkk2ΔOCY

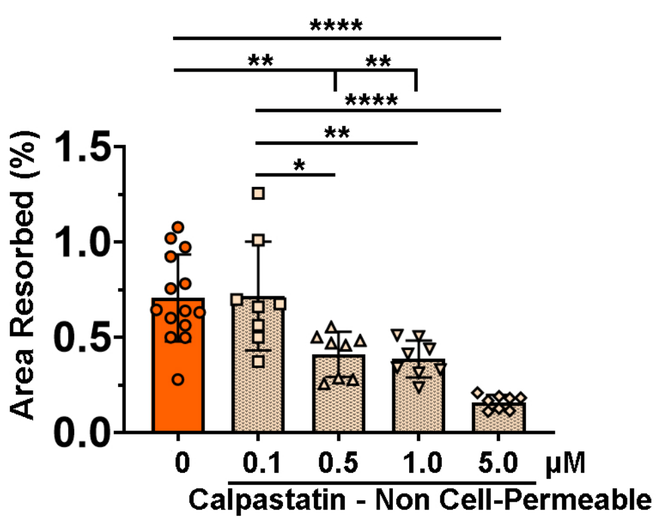
A


Recombinant Calpastatin Domain I - Non Cell Permeable
0
0.1
0.5
1.0
5.0
µM
Female
Male

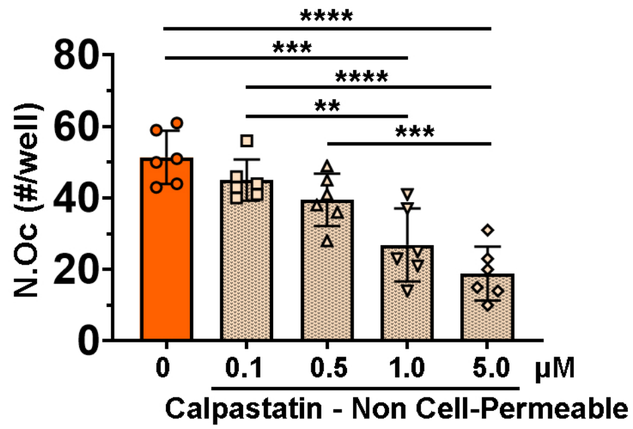
B


# of Pits /well
Calpastatin - Non Cell-Permeable
µM

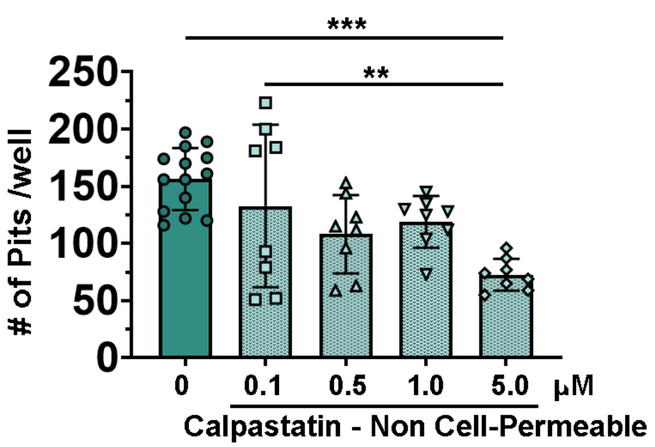
C


Area Resorbed (%)
Calpastatin - Non Cell-Permeable
µM

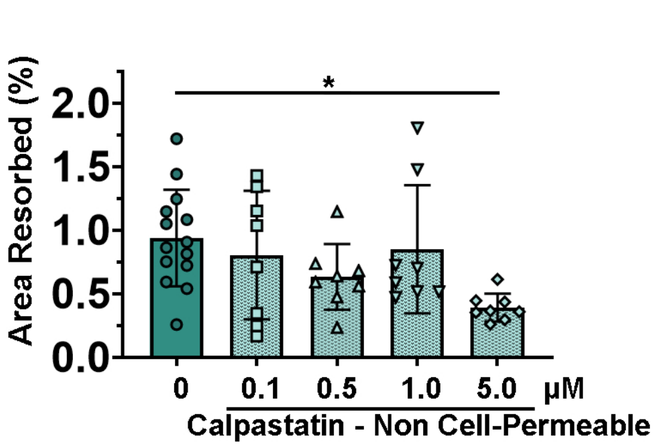
D


N.Oc (#/well)
Calpastatin - Non Cell-Permeable
µM

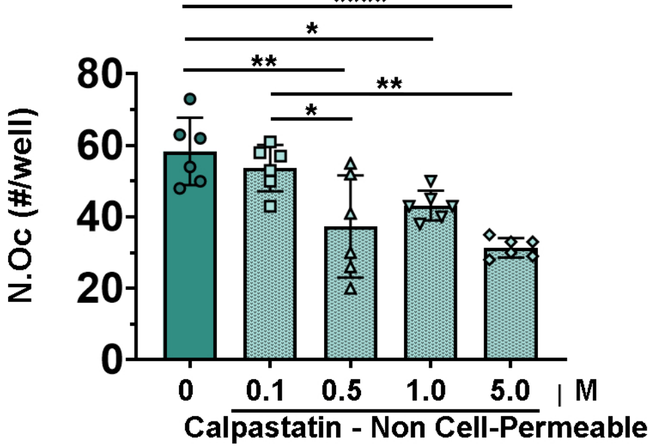
E


# of Pits /well
Calpastatin - Non Cell-Permeable
µM

F


Area Resorbed (%)
Calpastatin - Non Cell-Permeable
µM

G

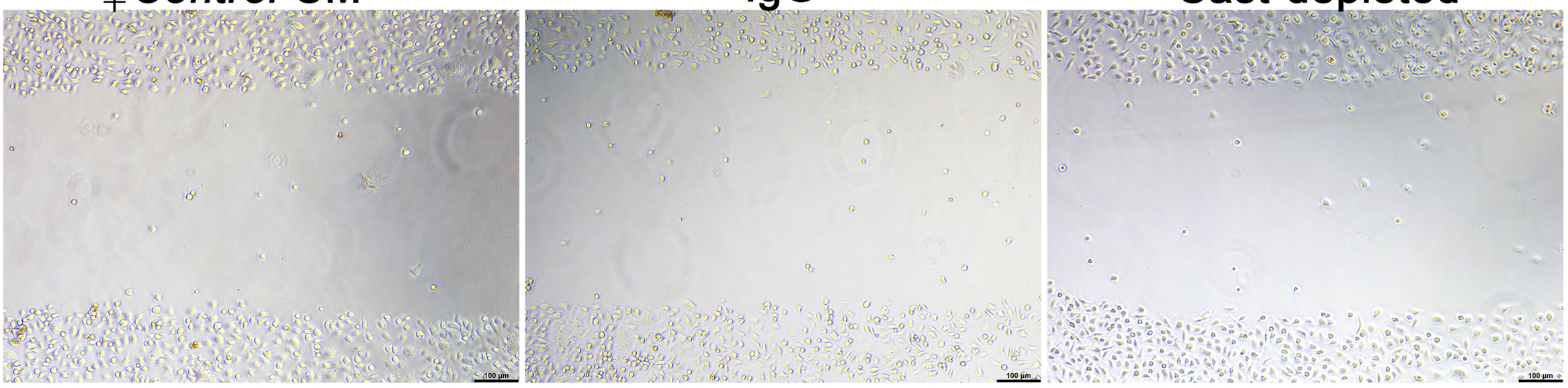

N.Oc (#/well)
Calpastatin - Non Cell-Permeable
µM

♀*Camkk2*$^{\Delta OCY}$

A

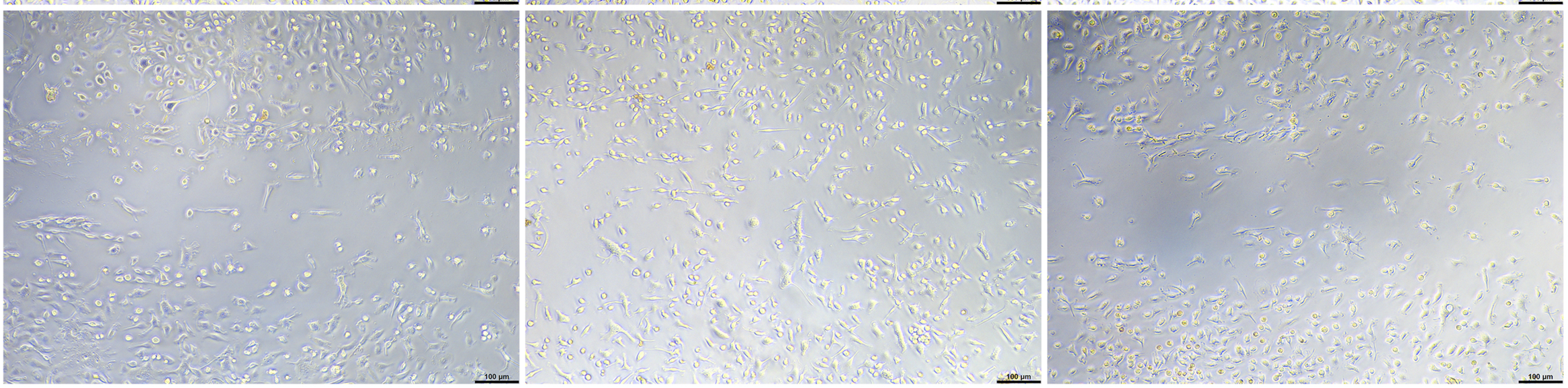
♀*Control* CM | + IgG | Cast-depleted

0 hr

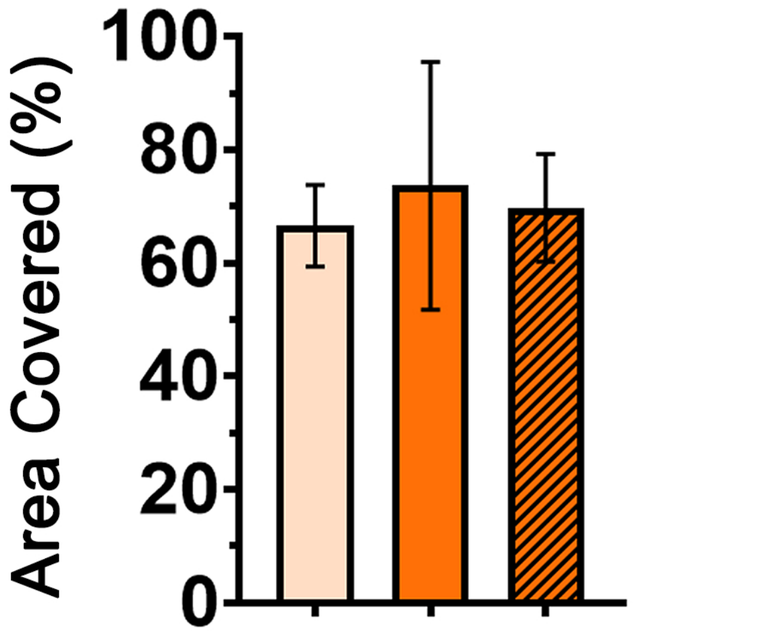


B

18 hr



C



♀*Control*

♀*Camkk2*$^{\Delta OCY}$+IgG

♀*Camkk2*$^{\Delta OCY}$-Cast

D

WT Osteoclast Lysate

♀*Control* CM | ♀*Camkk2*$^{\Delta OCY}$ CM: + IgG | -Cast