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Supplemental Information

Eomes partners with PU.1 and MITF to Regulate Transcription Factors Critical for osteoclast differentiation

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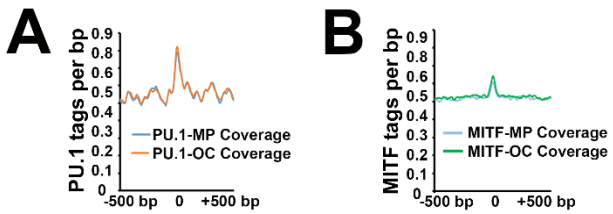
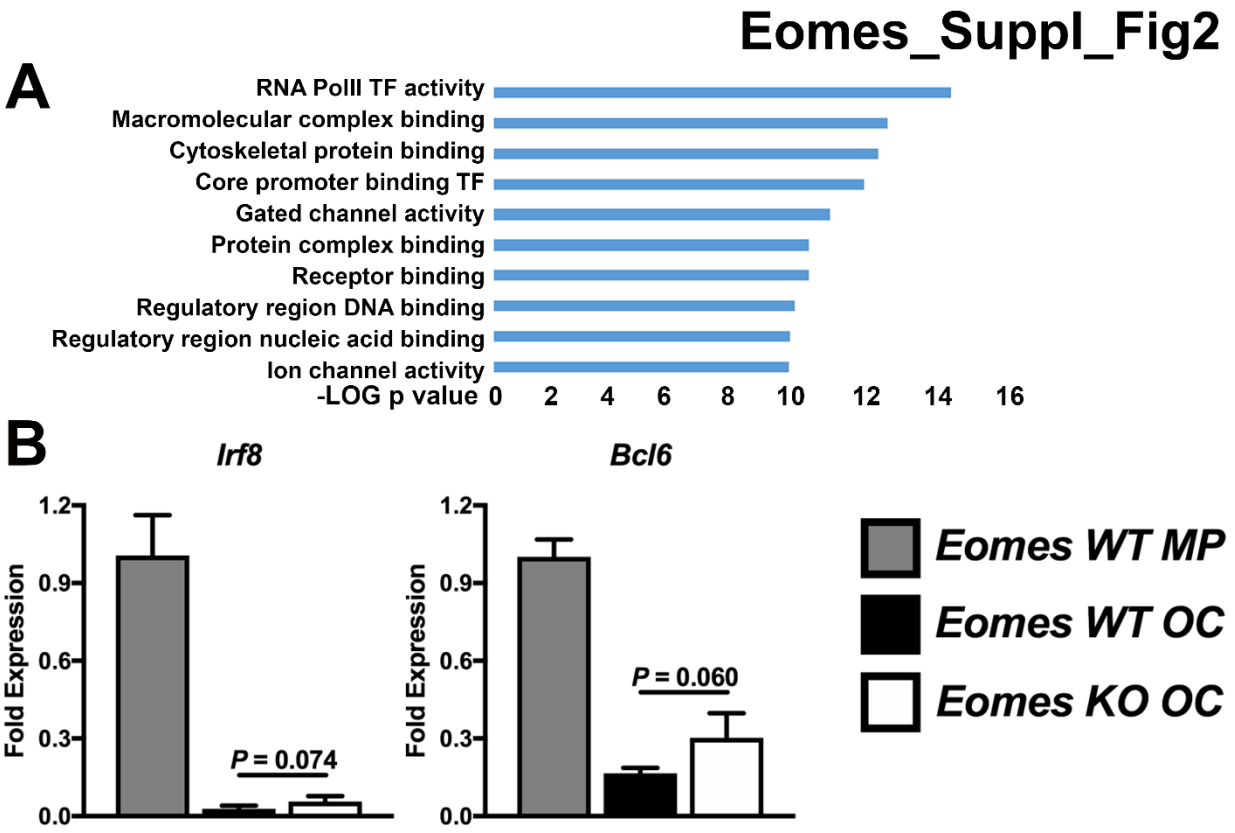
Eomes_Suppl_Fig1



Supplemental Figure 1, Related to Figure 1: A) Tag densities of PU.1 occupancy from both MPs and OCs around MECC EOMES ChIP-Seq peaks. B) Tag densities of MITF occupancy from both MPs and OCs around MECC EOMES ChIP-Seq peaks. For both A) and B), 0 on the X axis indicates the EOMES peak center.



Supplemental Figure 2, Related to Figure 2: A) ToppGene enrichment of EOMES-associated genes to evaluate the molecular functions regulated by EOMES. B) Evaluation of key anti-osteoclastogenic TFs by quantitative real-time RT-PCR (n=2-4).

Transparent Methods

Cell culture and transfection

Cells were grown in complete medium – DMEM (high glucose) with 10% FBS, 2 mM L-glutamine, and 50 U/ml penicillin/streptomycin (Invitrogen). For transient transfections, $2x10^5$ HEK-293 cells were transfected with 2 µg of plasmid using Lipofectamine® 3000 (Invitrogen). The following plasmids were used – FLAG-MITF, HA-PU.1, V5-EOMES, and control V5-His.

Genomic analysis

An external dataset for EOMES ChIP-seq (Sessa et al., 2017) was aligned to the mouse genome *mm9* using Bowtie2 software (Langmead et al., 2009). Peak calling and motif analysis were performed with HOMER software (Heinz et al., 2010). Centered K means clustering was done using Cluster 3.0 and visualized using the Java-Treeview program (de Hoon et al., 2004).

Animals

All use was approved by the Ohio State University and Medical University of South Carolina IACUCs (Protocols: 2007A0120-R2, 2016A00000035, and IACUC-2017-00064). Mice were on a C57BL/6J background (F10 or further). C57BL/6J wild type and *Eomes*$^{fl/fl}$ mice were purchased from Jackson Laboratories. Mice possessing either tamoxifen-inducible or constitutively active myeloid lineage-specific *Csf1r* promoter driven *Cre* ((*Csf1rTAMCre* and *Csf1rCre*, respectively) were from Dr. Jeffrey Pollard (Albert Einstein College of Medicine) (Deng et al., 2010; Qian et al., 2011). Tamoxifen (Sigma-Aldrich) was dissolved in corn oil with 5% ethanol. For inducible myeloid-specific *Eomes* deletion (*Eomes*$^{\Delta MP/\Delta MP}$), *Csf1rTAMCre+;Eomes*$^{fl/fl}$ and *Eomes*$^{fl/fl}$ control littermate mice were injected I.P. with 50 µg tamoxifen/mouse/day on days 1, 2, and 3 after birth (Figure 2A). Since only neonatal mice were used in this study, both male and female mice were used concurrently.

Immunoprecipitation and Western blot

For IP experiments, different combinations of FLAG-MITF, HA-PU.1, and V5-EOMES were transfected into HEK-293 cells. Preparation of cell lysates, IP, and Western blot analysis were as previously described (Carey et al., 2016). Briefly, 200 µg of protein was incubated with **1)** anti-FLAG (Sigma-Aldrich Cat #1804); **2)** anti-HA (Cell Signaling Cat #3724); or **3)** anti-V5 (Sigma-Aldrich Cat #V8012) antibody overnight at 4°C while shaken. Immunoprecipitated proteins were recovered, washed in lysis buffer, and immunocomplexes released with 2X SDS sample buffer.

In vitro OC differentiation and TRAP staining

Mice with MP-specific deletion of *Eomes* (*Eomes*$^{\Delta MP/\Delta MP}$) using constitutive *Csf1rCre* and *Eomes*$^{fl/fl}$ control littermate mice were used. For confirmation of EOMES knockdown in MPs from *Eomes*$^{\Delta MP/\Delta MP}$ mice, genotyping PCR and confirmatory Western blot (described above) using rabbit anti-mouse EOMES primary antibody (Cell Signaling Technology Cat #4540) was performed after combined protein and DNA extraction using TRIzol (Invitrogen). Digested spleens or bone marrow flushes were enriched for MPs and differentiated into OCs for three days as previously described for gene expression analysis (Carey et al., 2018). To evaluate *in vitro* OC differentiation by the formation of TRAP-positive multinucleated OCs, mice with MP-specific deletion of *Eomes* (*Eomes*$^{\Delta MP/\Delta MP}$) using tamoxifen-inducible *Csf1rTAMCre* were used. *Csf1rTAMCre+;Eomes*$^{fl/fl}$ and *Eomes*$^{fl/fl}$ control littermate mice were injected I.P. with 50 µg tamoxifen/mouse/day on days 1, 2, and 3 after birth. Mice were harvested at eight days of age and bone marrow flushes enriched for MPs and differentiated into OCs for five days with tamoxifen in the medium. Cells were stained for TRAP using a Leukocyte Acid Phosphatase kit (Sigma-Aldrich). OCs were defined as TRAP-positive cells having three or more visible nuclei.

RT-qPCR

Total RNA isolation, cDNA synthesis, qPCR using Taqman Master Mix and Universal Probe Library probes and primers (Roche), and data analysis was performed as previously described (Carey et al., 2018).

ChIP and Re-ChIP

Rabbit anti-mouse PU.1 and MITF antibodies used for ChIP have been described previously (Sharma et al., 2007). The same rabbit anti-mouse EOMES antibody used for Western blot was also used. ChIP and Re-ChIP were performed as previously described (Sharma et al., 2007).

μCT and bone morphometry

For myeloid-specific *Eomes* deletion (*Eomes*$^{\Delta MP/\Delta MP}$), inducible *Csf1rTAMCre+;Eomes*$^{fl/fl}$ and *Eomes*$^{fl/fl}$ control littermate mice were used. Femurs were dissected at 8 days of age, formalin-fixed for 24 hours, and maintained in 70% ethanol. μCT analysis was performed on an Inveon Preclinical CT scanner (Siemens AG) with a 9.7 μm resolution. The diaphyseal volume of interest (VOI) was defined as the central 5% and the distal metaphyseal VOI was 2.5% of the femoral length.Bones were analyzed using 3D bone morphometry analysis software from the μCT scanner manufacturer. Segmentation thresholds were constant and VOIs analyzed in a blinded manner.

In vivo TRAP staining and histomorphometry

Femurs from *Eomes*$^{\Delta MP/\Delta MP}$ and *Eomes*$^{fl/fl}$ control mice used for μCT were decalcified in 14% EDTA for 2 weeks, embedded in paraffin, and cut to 4 μm thick sagittal sections. Sections were stained for TRAP (Sigma-Aldrich) and counterstained with hematoxylin. OCs were defined as TRAP positive cells having three or more visible nuclei. Slides were scanned (Aperio

ScanScope XT) and OC number and surface and trabecular surface measured to calculate Oc.S/BS and Oc.N/BS (Aperio ImageScope software).

Statistical analysis

Data are expressed as mean ± 1 standard deviation. Non-normally distributed data and/or data with unequal variance underwent inverse transformation. Comparisons were then performed between **1)** two groups with an unpaired t-test and **2)** three or more groups with a one-way ANOVA and Holm-Sidak post-hoc analysis (normal distribution and equal variance). Quantified EOMES Western blot protein levels were compared between controls and $Eomes^{\Delta MP/\Delta MP}$ mice with a one-tailed t-test. ReChIP comparisons were done using a one-way ANOVA and Holm-Sidak post-hoc analysis with IgG as the control group. Gene expression and ChIP comparisons were performed with ΔCt values. Statistical analyses were conducted with Prism 7 (GraphPad Software) with a statistical significance of $P < 0.05$.

Data and Software Availability

Raw images of immunoprecipitation and western blot data used in this has been deposited to Mendeley depository and can be accessed at URL: http://dx.doi.org/10.17632/wdv72kxgjg.1

Supplemntary References

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