Supplemental Information

Hypoxia-sensitive COMMD1 Integrates Signaling and Cellular Metabolism in Human Macrophages and Suppresses Osteoclastogenesis

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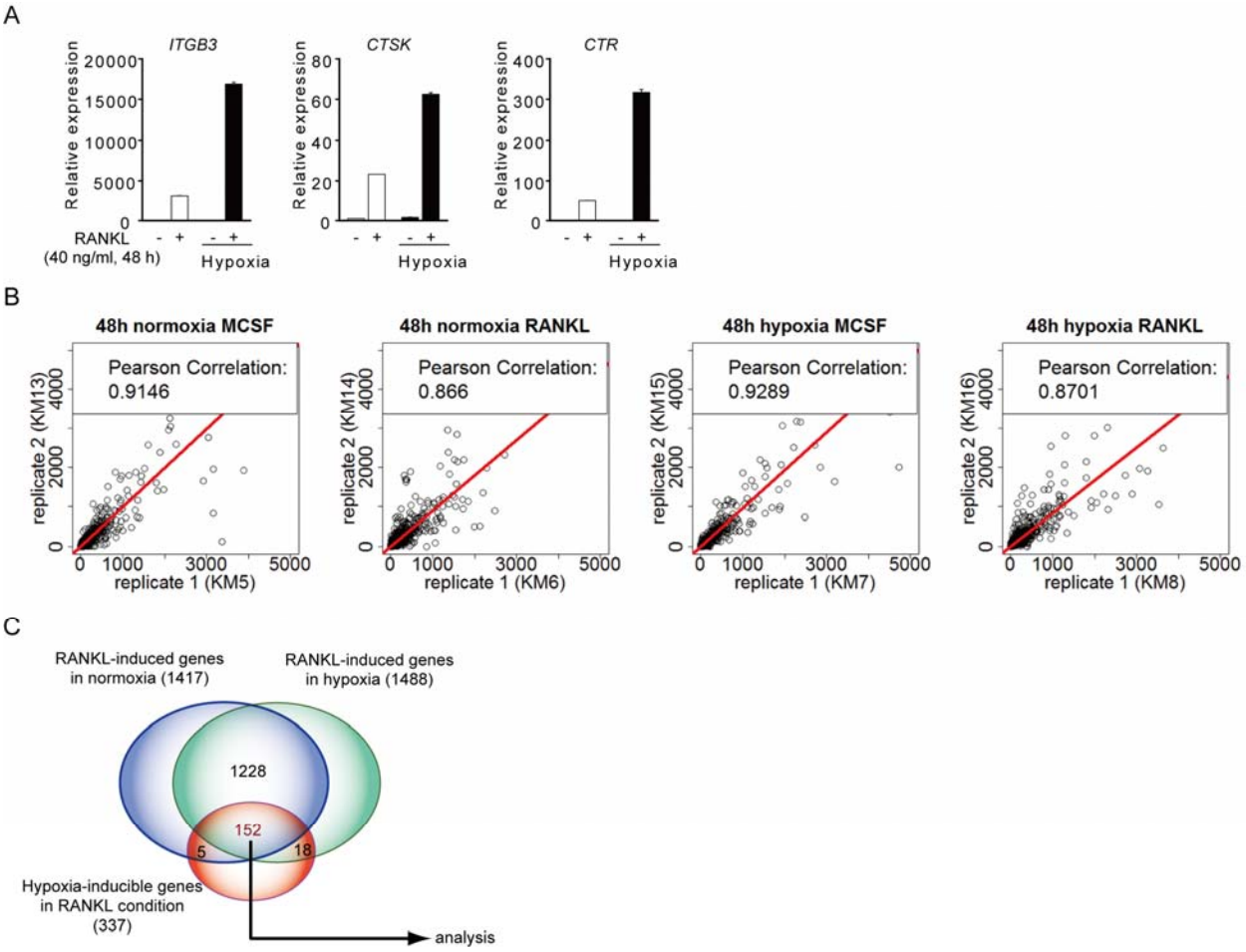
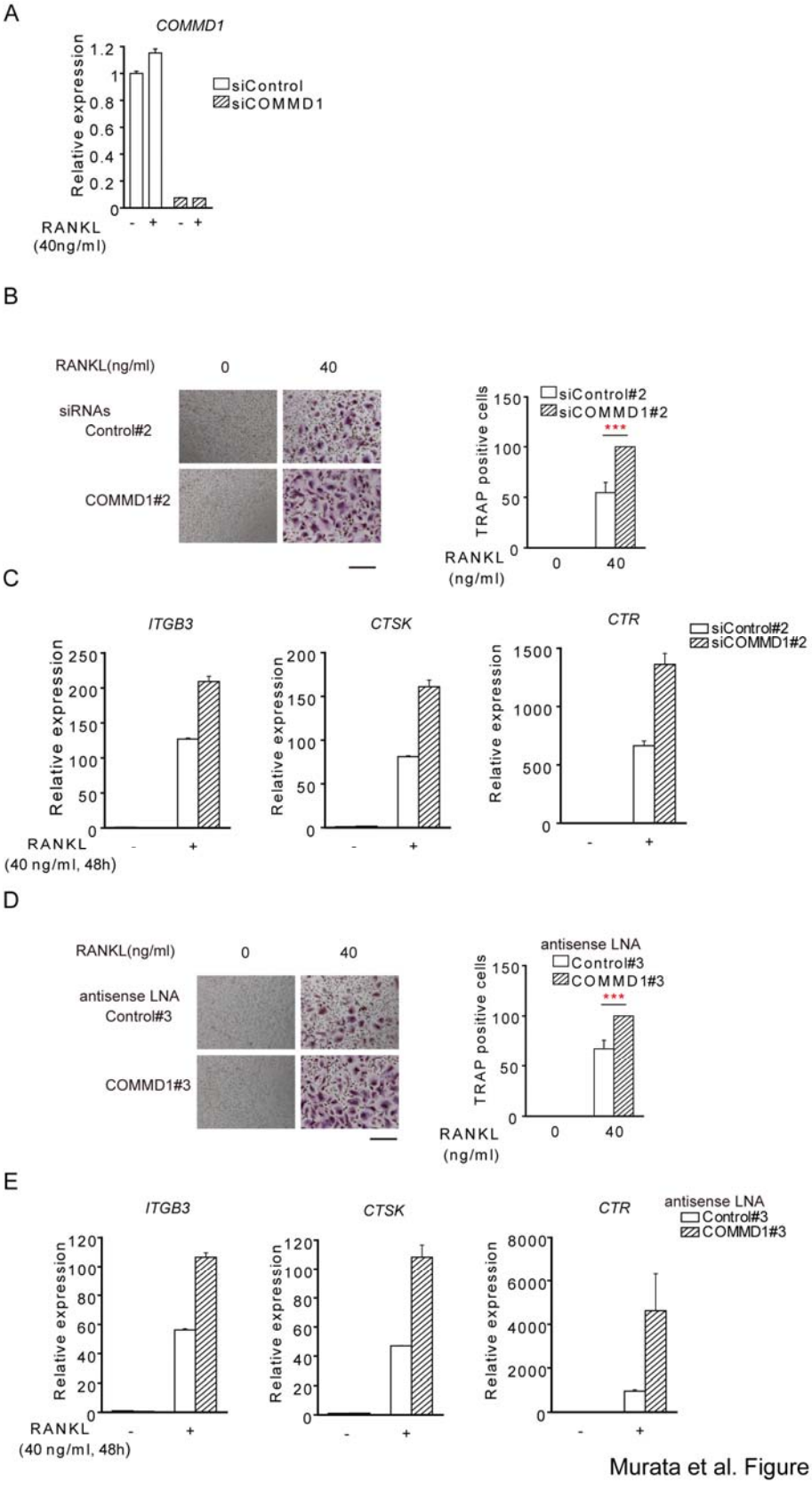


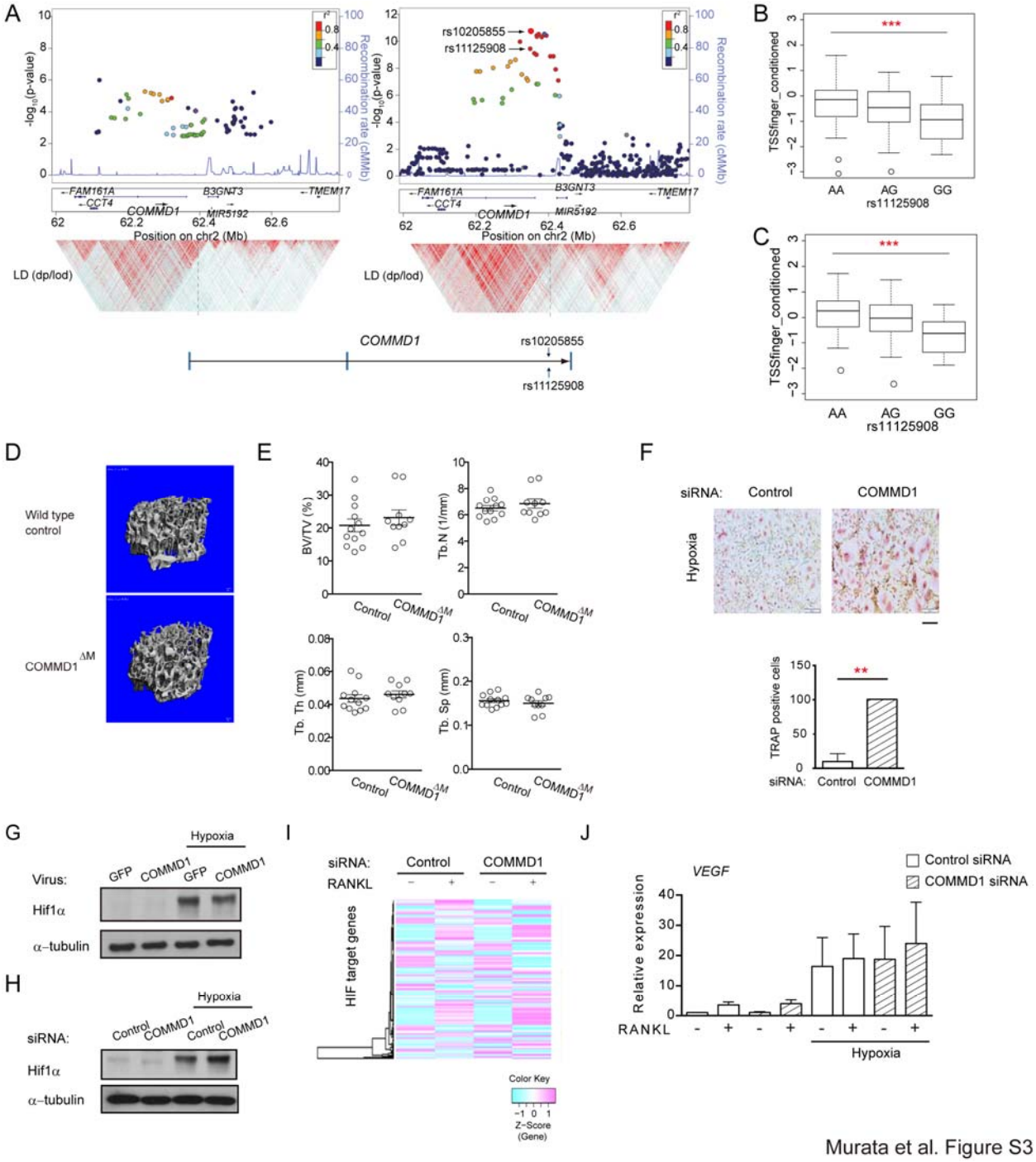
Murata et al. Figure S1

Supplementary Figure 1. Hypoxia enhances osteoclastogenesis, Related to Figure 1
(A) RT-qPCR analysis of *ITGB3*, *CTSK* and *CTR* mRNA normalized relative to *TBP* mRNA.
(B) Pearson correlation analysis of RNAseq (48 h of RANKL treatment) data obtained from two independent donors.
(C) Venn diagram of RANKL-induced and hypoxia-regulated genes, made from two pooled RNA-seq experiments.
Bar graphs show means and error bars represent SEM. The result (A) is representative of at least 3 experiments



Murata et al. Figure S2

Supplementary Figure 2. **COMMD1 is a negative regulator of osteoclastogenesis,** Related to Figure 2.
(A) RT-qPCR analysis of *COMMD1* mRNA normalized relative to *TBP* mRNA.
(B) TRAP staining of human osteoclasts nucleofected with a different set of control or COMMD1-specific siRNAs. Right panel shows pooled data from 7 experiments with different donors normalized relative to the number of osteoclasts obtained using COMMD1 siRNA#2 with RANKL (40 ng/ml) (set at 100%).
(C) RT-qPCR analysis of *ITGB3*, *CTSK* and *CTR* mRNA normalized relative to *TBP* mRNA.
(D) TRAP staining of human osteoclasts nucleofected with COMMD1-specific antisense locked nucleic acids (LNA) or control LNA. Right panel shows pooled data from 4 experiments with different donors normalized relative to the number of osteoclasts obtained using COMMD1 LNA#3 with RANKL (40 ng/ml) (set at 100%).
(E) RT-qPCR analysis of *ITGB3*, *CTSK* and *CTR* mRNA normalized relative to *TBP* mRNA.
Bar graphs show means and error bars represent SEM. *** $p<0.001$. Bar, 200 μm. The results are representative of at least 3 (A, C, E), 7 (B), and 4 (D) experiments.

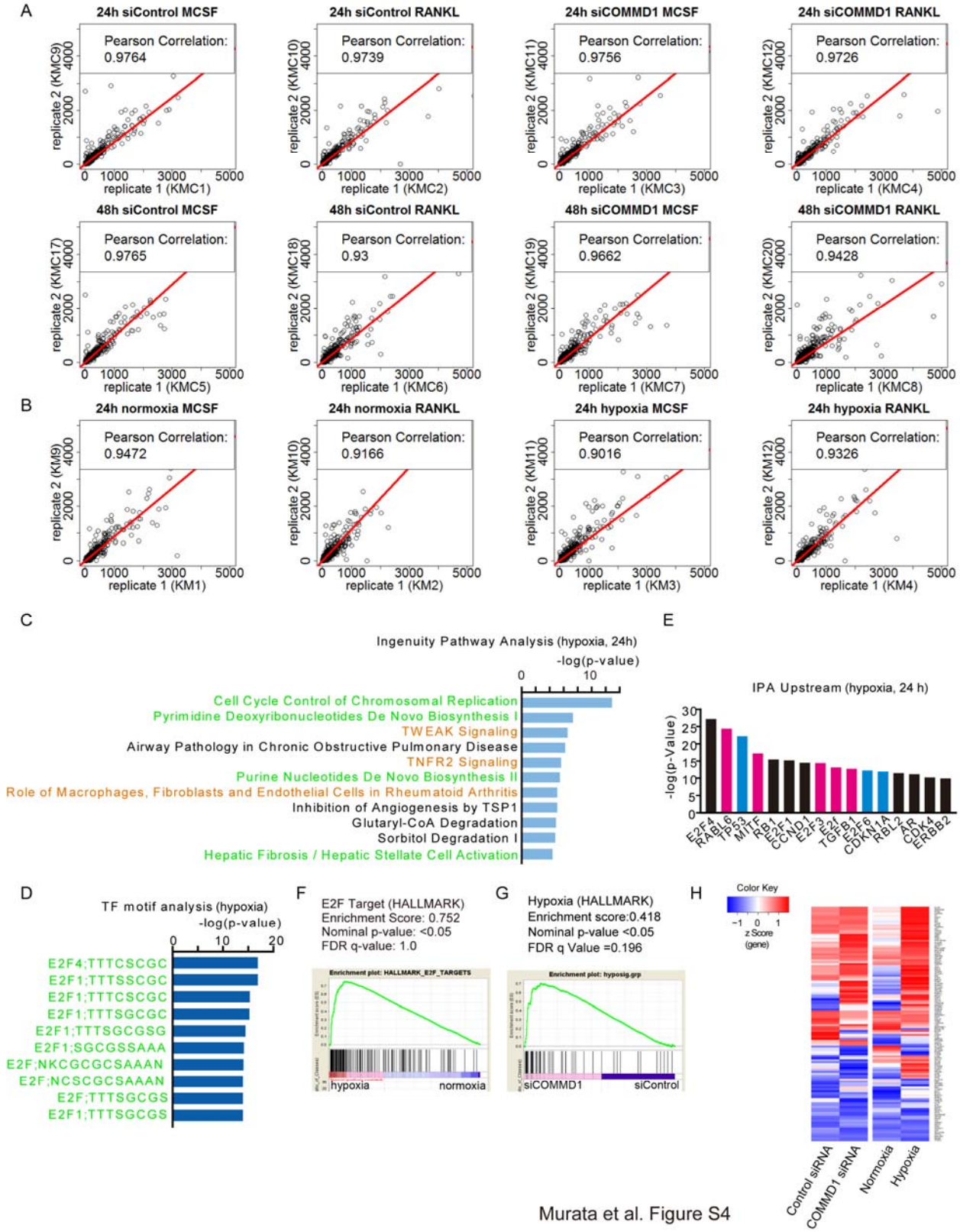


Murata et al. Figure S3

Supplementary Figure 3. **COMMD1 and bone resorption in RA patients and mouse models**, Related to Figure 3

(A) Expression quantitative trait loci (eQTL) associations and similar linkage disequilibrium (LD) structure in European and Japanese populations. Variants' locations and eQTL p-values are shown in European and Japanese populations for upper left and right panels, respectively. The purple circles in the two panels indicate rs1466124 showing significant associations in the two populations. LD structures are

drawn based on the 1000 Genomes data or the previous Japanese study for European and Japanese populations, respectively. The locations of rs10205855 and rs11125908 in COMMD1 region are indicated in the lower panel.
(B) Total Sharp score (TSS) in finger joints adjusted by RF positivity and rs11125908
(C) TSS in finger joints adjusted by RF level and rs11125908
The boxes represent the 25th to 75th percentiles (upper and lower quartiles (UQ and LQ), respectively) and the lines within the boxes represent the median. The lines outside the boxes represent the smallest value bigger than 1.5*inner quartile range (IQR, UQ-LQ) below LQ and the biggest value smaller than 1.5*IQR above UQ. Symbols indicate outliers.
(D-E) Myeloid COMMD1 deficiency does not affect basal bone phenotype in mice. Microcomputed tomography and bone parameters of epiphyseal region of the femurs of 12-week-old wild type control and COMMD1$^{\Delta M}$ mice. n = 10–12 from 3 experiments. Bar graphs show means and error bars represent SEM.
(F) Human monocytes under hypoxic conditions were nucleofected with control or COMMD1 siRNAs and were cultured with M-CSF for two days. Cells were stimulated with RANKL for additional four days and osteoclastogenesis assays were performed under hypoxia. Upper panel showed the representative images from three independent experiments. Lower panel shows pooled data from 3 experiments with different donors, normalized relative to the number of COMMD1-deficient osteoclasts treated with RANKL under hypoxia (set at 100%).
(G) Immunoblot with HIF1α antibody of lysates from human macrophages transduced with adenoviral particles encoding GFP or COMMD1-HA and cultured either under normoxic or hypoxic conditions for three hours. α-tubulin was used as a control. Representative images from three independent experiments.
(H-J) Human monocytes were nucleofected with control or COMMD1 siRNAs and cultured with M-CSF for two days. (H) Immunoblot with HIF1α antibody. Cells were cultured either under normoxic or hypoxic conditions for three hours. α-tubulin was used as a control. Representative images from three independent experiments. (I) Heat map of RNA-seq FPKM values for HIF1α target genes (Benita et al., 2009) in control and COMMD1-deficient macrophages following RANKL stimulation for one day. RNA-seq data from two biological replicates were used. (J) *VEGF* mRNA expression was measured by qPCR. Cumulative results from three independent experiments. Not significant for all lane comparison by one-way ANOVA.



Murata et al. Figure S4

Supplementary Figure 4. **RNA-seq revealed COMMD1 and hypoxia reciprocally regulate cell cycle, metabolic and NF-κB target genes**, Related to Figure 5

(A) Pearson correlation analysis of COMMD1 silenced RNAseq (24 or 48 h of RANKL treatment) data obtained from two independent donors.

(B) Pearson correlation analysis of hypoxia RNAseq (24 h of RANKL treatment) data obtained from two independent donors.

(C, E) Ingenuity pathway analysis (Westra et al.) analysis of RANKL-inducible genes (24 h RANKL treatment) whose expression was superinduced by hypoxia.

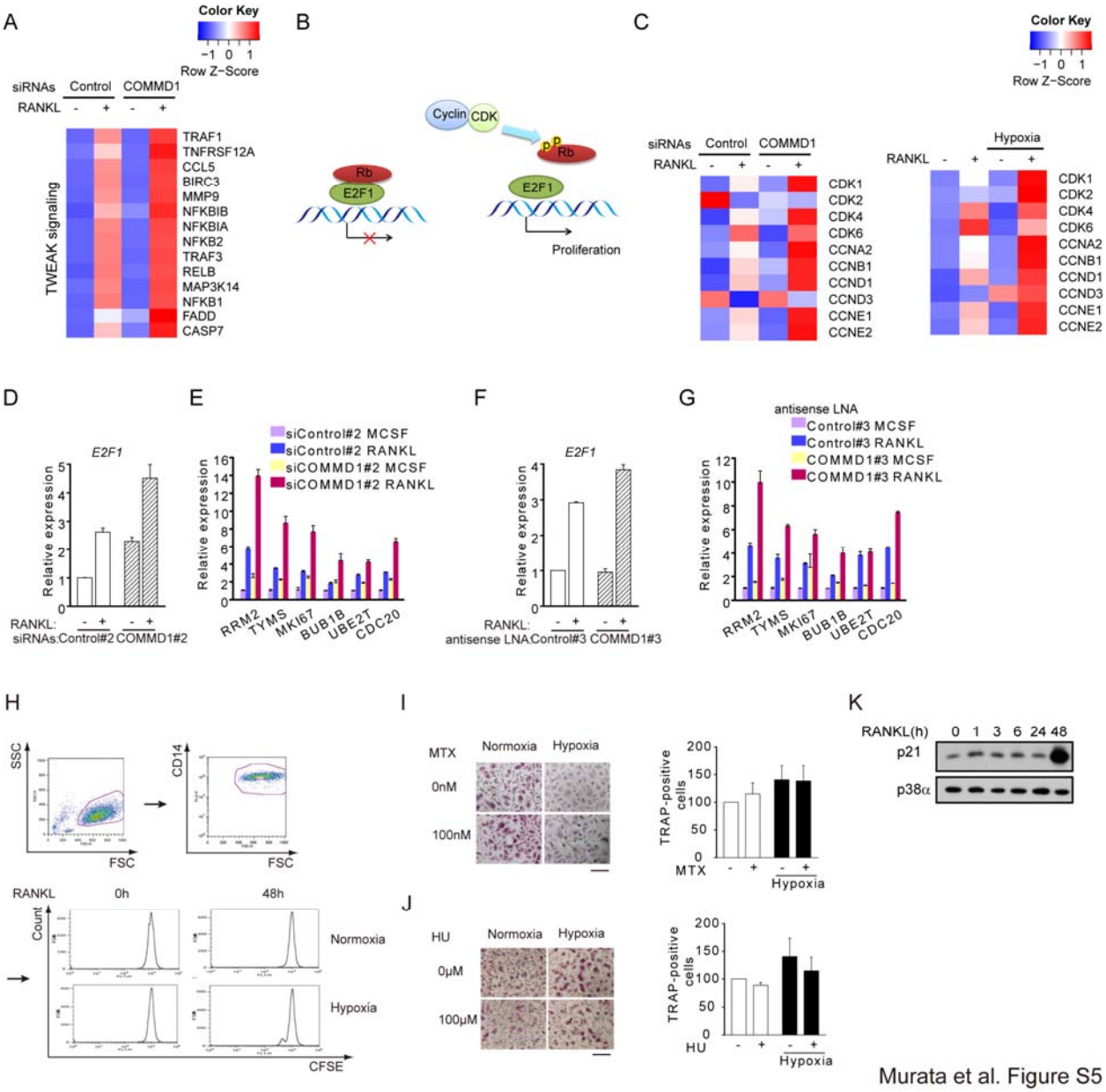
(D) Enriched transcription factor binding motifs in RANKL-inducible hypoxia-dependent gene promoters, using gene set enrichment analysis (GSEA).

(F) GSEA showing significant enrichment of E2F target genes in the hypoxia-regulated gene set (24 h RANKL treatment) using two independent biological replicates.

(G) GSEA showing significant enrichment hypoxia target genes in the COMMD1-regulated gene set (48 h RANKL treatment) using two independent biological replicates.

(H) Heat map showing relative expression of HALLMARK_hypoxia gene sets in Macrophages treated with COMMD1 siRNA (columns 1 and 2) or with hypoxia (columns 3 and 4).

The results are derived from analysis of two pooled biological replicates (C, D, E, H).



Supplementary Figure 5. **COMMD1 and hypoxia reciprocally regulate RANKL activation of NF-κB target genes and pRB-E2F1 pathway**, Related to Figure 5

(A) Heat map showing relative expression of TWEAK pathway genes in macrophages treated with COMMD1 siRNA. Results from pooled data from two biological replicates are shown.

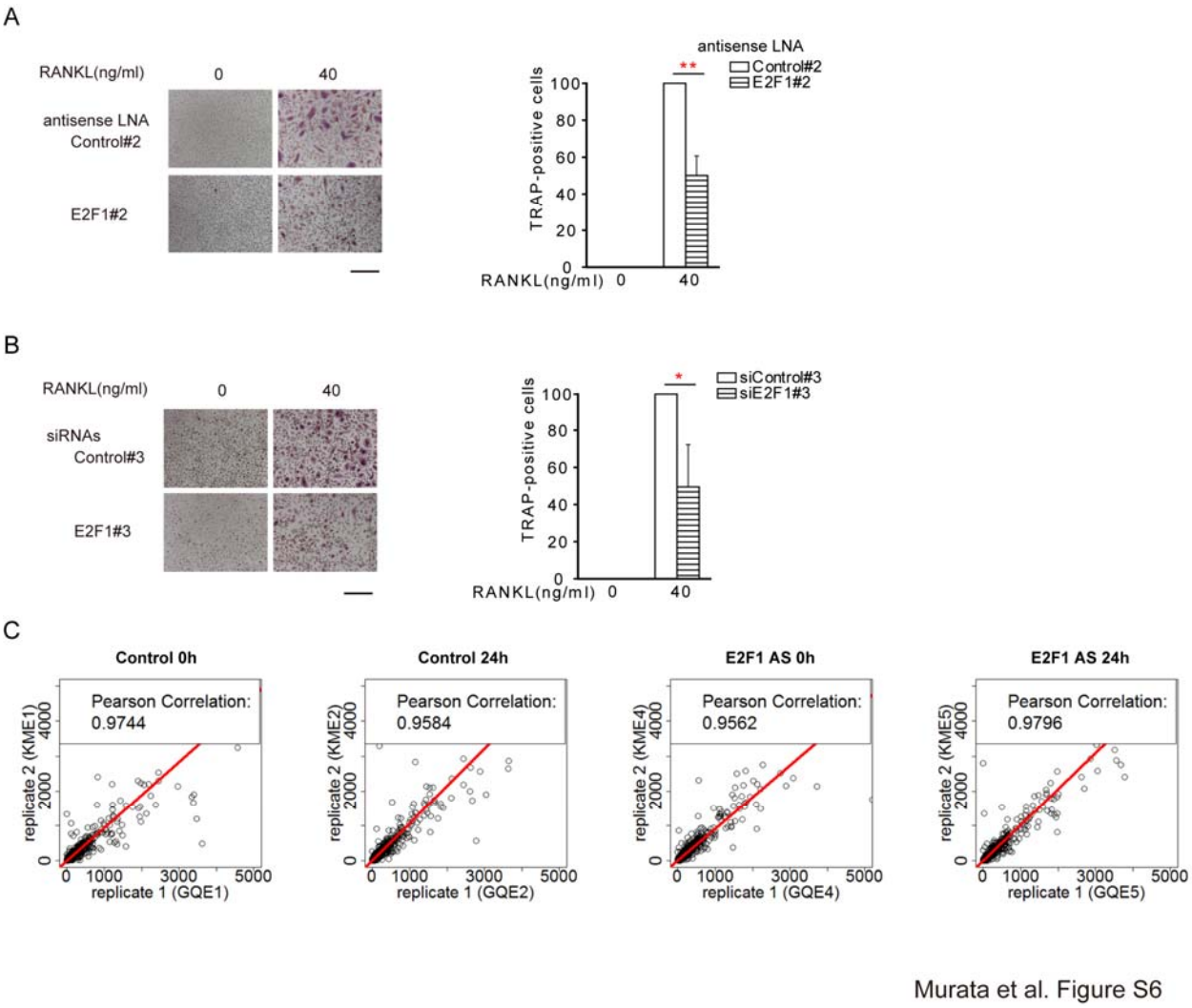
(B) Schematic showing the mechanism of E2F1 activation. Binding of Rb protein with E2F1 inhibits E2F1 transcriptional activity. Phosphorylation of Rb protein by cyclin-CDK complex releases Rb from E2F1, releasing E2F1 from repression and enabling E2F1-mediated gene transcription.

(C) Heat map showing relative expression of cyclins and CDKs (24 h RANKL treatment). Results from pooled data from two biological replicates are shown.

(D-E) RT-qPCR analysis of mRNAs of *E2F1* or E2F1-specific target genes normalized relative to *TBP* mRNA using a second set of control or COMMD1-specific siRNAs.

(F-G) RT-qPCR analysis of mRNAs of *E2F1* or E2F1-specific target genes normalized relative to *TBP* mRNA using COMMD1-specific antisense or control LNA.

(H) Flow cytometric analysis of carboxyfluorescein succinimidyl ester (CFSE)-stained cells.
(I-J) TRAP staining of human osteoclasts treated with methotrexate (MTX) or hydroxyurea (HU). Right panel shows pooled data from 3 experiments with different donors, normalized relative to the number of osteoclasts treated with RANKL alone under normoxia (set at 100%).
(K) Immunoblot with p21 antibody of lysates from human macrophages treated with RANKL for the indicated times. p38α was used as control. Representative images from 4 independent experiments. Bar graphs show means and error bars represent SEM. ** $p<0.01$. Bar, 200 μm. The results are representative of at least 3 (D, E, F, G) and 3 (H, I, J) experiments.



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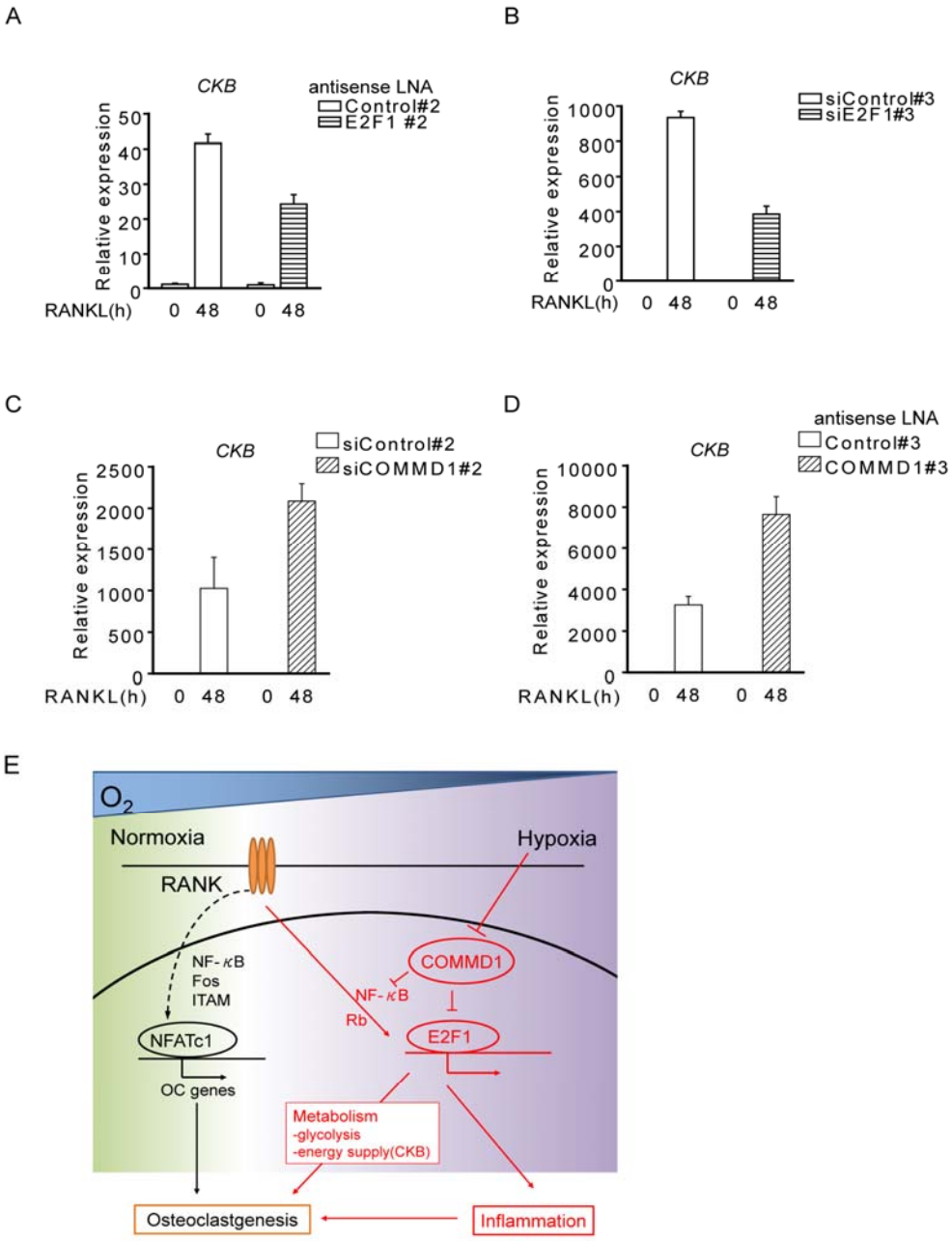
Supplementary Figure 6. Osteoclastogenesis was positively regulated by E2F1 confirmed using additional sets of LNAs and siRNA, Related to Figure 6

(A) TRAP staining of human osteoclasts nucleofected with a second set of control or E2F1-specific antisense LNAs. Right panel shows pooled data from 3 experiments with different donors normalized relative to the number of osteoclasts obtained using Control LNA#2 with RANKL (40 ng/ml) (set at 100%).

(B) TRAP staining of human osteoclasts nucleofected with a set of control or E2F1-specific siRNAs. Right panel shows pooled data from 3 experiments with different donors normalized relative to the number of osteoclasts obtained using Control siRNA#3 with RANKL (40 ng/ml) (set at 100%).

(C) Pearson correlation analysis of E2F1 silenced RNAseq (24 h of RANKL treatment) data obtained from two independent donors.

Bar graphs show means and error bars represent SEM. * $p<0.05$, ** $p<0.01$. Bar, 200 μm. The results are representative of 3 (A, B) experiments.



Murata et al. Figure S7

Supplementary Figure 7. **Regulation of *CKB* expression by COMMD1 and E2F1 confirmed using additional sets of siRNA or antisense LNA**, Related to Figure 7.
(A-D) RT-qPCR analysis of *CKB* mRNA normalized relative to *TBP* mRNA using additional sets of siRNAs or antisense LNAs. (E) Schematic showing the role of the COMMD1-E2F-metabolic pathway in osteoclastogenesis. Hypoxia suppresses COMMD1 to release the E2F-metabolic pathway from repression and thereby promote osteoclastogenesis.
Bar graphs show means and error bars represent SEM. The results are representative of 2 (A) and 3 (B, C, D) experiments.

Supplementary Table 1. Sequence of primers, siRNAs and antisense LNAs used in this study, Related to STAR methods

Real-time PCR primers	Sequences
NFATc1 Fwd	AAAGACGCAGAAACGACG
NFATc1 Rev	TCTCACTAACGGGACATCAC
Itgb3 Fwd	GGAAGAACGCGCCAGAGCAAAATG
Itgb3 Rev	CCCCAAATCCCTCCCCACAAATAC
Ctsk Fwd	CTCTTCCATTTCTTCCACGAT
Ctsk Rev	ACACCAACTCCCTTCCAAAG
Ctr Fwd	CTGAAGCTTGAGCGCCTGAGTC
Ctr Rev	TGGGGTTGGGTGATTTAGAAGAAG
COMMD1 Fwd	CTGTTGCCATTATAGAGCTGGAA
COMMD1 Rev	GCGTCTTCAGAATTTGGTTGACT
E2F1 Fwd	TGCAGAGCAGATGGTTATGG
E2F1 Rev	CTGATCCCACCTACGGTCTC
RRM2 Fwd	AGCCCACGGAGCCGAAAACT
RRM2 Rev	CGGCGGTCCAAAAGGAAGCC
TYMS Fwd	AGTACCTGGGGCAGATCCAACAC
TYMS Rev	CGTTTGGTTGTCAGCAGAGGGA
MKI67 Fwd	CCGGATCGTCCCAGTGGAAG
MIK67 Rev	AAAGTGGGGACCGTCGACCC
BUB1B Fwd	TAGCTCCGAGGGCAGGTTGC
BUB1B Rev	ATGGCTTCACTCAGAGCACCCC
UBE2T Fwd	TGCCACCAAAAGGTGCTTGGA
UBE2T Rev	AGCGGGTCATCAGGGTTGGG
CDC20 Fwd	CCCATGGCACAGTTCGCGTT

CDC20 Rev	GATTTGCCAGGAGTTCGGCCC
CKB Fwd	ACCGGCCTCACCCAGATTGA
CKB Rev	AGCCGCTTAAGCACCTCCGA
siRNAs	
COMMD1#1	AGUCUAUUGCGUCUGCAGAtt
	UCUGCAGACGCAAUAGACUta
COMMD1#2	CUUGACUGCUCAAACCAAAtt
	UUUGGUUUGAGCAGUCAAGaa
E2F1#3	GUCACGCUAUGAGACCUCAtt
	UGAGGUCUCAUAGCGUGACtt
Control #1	Ambion proprietary information Product number: 4390843
Control #2	Ambion proprietary information Product number: 4390846
antisense LNA	
COMMD1#3	TGCAGACGCAATAGA
E2F1#1	GGTGGTCAGATTCAGT
E2F1#2	GATTCAGTGAGGTCT
Negative control A	AACACGTCTATACGC
Negative control B	GCTCCCTTCAATCCAA