

Kcnn4 Is a Regulator of Macrophage Multinucleation in Bone Homeostasis and Inflammatory Disease

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SUMMARY

Macrophages can fuse to form osteoclasts in bone or multinucleate giant cells (MGCs) as part of the immune response. We use a systems genetics approach in rat macrophages to unravel their genetic determinants of multinucleation and investigate their role in both bone homeostasis and inflammatory disease. We identify a *trans*-regulated gene network associated with macrophage multinucleation and *Kcnn4* as being the most significantly *trans*-regulated gene in the network and induced at the onset of fusion. *Kcnn4* is required for osteoclast and MGC formation in rodents and humans. Genetic deletion of *Kcnn4* reduces macrophage multinucleation through modulation of Ca^{2+} signaling, increases bone mass, and improves clinical outcome in arthritis. Pharmacological blockade of *Kcnn4* reduces experimental glomerulonephritis. Our data implicate *Kcnn4* in macrophage multinucleation, identifying it as a potential therapeutic target for inhibition of bone resorption and chronic inflammation.

INTRODUCTION

Macrophages are versatile and plastic cells that have the ability to fuse and give rise to multinucleate cells (Chen and Olson, 2005). Macrophage fusion leads to the formation of osteoclasts in bone and multinucleate giant cells (MGCs) in chronic inflammatory diseases (Brodbeck and Anderson, 2009; Helming and

Gordon, 2009; Vignery, 2005). Thus, both osteoclasts and MGCs are derived from the fusion of macrophages. Multinucleation represents an essential step in osteoclast formation and activation because mononucleate osteoclasts are unable to resorb bone efficiently (Helming and Gordon, 2009; Vignery, 2005, 2008) and the genes that are critical for osteoclast activity are also fundamental to MGC function (Bühling et al., 2001; da Costa et al., 2005; Yagi et al., 2005; Zhu et al., 2007). Despite the well-characterized role of osteoclasts in bone homeostasis, macrophage activation associated with MGC formation in inflammatory diseases remains poorly understood.

Genetic studies in mice and humans suggest that germline sequence variation can regulate macrophage multinucleation and that this can influence disease pathogenesis. In a murine model of chronic tuberculosis infection, genetic loci were associated with production of inflammatory mediators by macrophages (Kramnik et al., 2000; Pichugin et al., 2009), suggesting the presence of a heritable genetic component affecting MGC function, which, in turn, mediates susceptibility to granulomatous diseases. In humans, deletions or loss-of-function mutations in either the *TYROBP* (DAP12) or *TREM2* genes are causally associated with Nasu-Hakola disease (Paloneva et al., 2000, 2002), where defective multinucleation in osteoclasts results in impaired bone resorption (Humphrey et al., 2004). In addition, human genome-wide association studies of MGC-associated diseases such as tuberculosis (Thye et al., 2012) and granulomatosis with polyangiitis (Lyons et al., 2012) identified common sequence variations associated with these disorders. In rheumatoid arthritis (RA), osteoclast differentiation and activation lead to bone erosion associated with prolonged inflammation (McInnes and Schett, 2011). However, to date, genetic studies failed to identify genes and pathways involved in osteoclast activation

resulting from multinucleation. Hence, the key determinants and molecular pathways of multinucleation in macrophages and the resulting pathophysiological effects remain largely unexplored.

RESULTS

Identification of a Multinucleation Gene Network in Macrophages

In order to investigate the genetic components of MGC formation, we took advantage of strain-specific phenotypic differences in spontaneous formation of MGCs in the rat, which have not been described previously. We observed that when bone-marrow-derived macrophages (BMDMs) from Wistar Kyoto (WKY) rats and Lewis (LEW) rats are cultured in vitro, WKY macrophages spontaneously form MGCs in contrast with what is observed in LEW (Figures 1A and S1). These strains have been widely studied for their differential susceptibility to experimental glomerulonephritis through macrophage activation (Aitman et al., 2006; Behmoaras et al., 2008, 2010; Page et al., 2012), and WKY rats also show MGCs in their glomeruli following the induction of nephrotoxic nephritis (NTN) (Figure S1). These marked phenotypic differences suggested genetic determinants of the process underlying MGC formation in the rat, which we set out to investigate by profiling genome-wide expression levels in macrophages of 200 backcross rats derived from WKY and LEW.

We used mRNA expression level as a quantitative trait to carry out genome-wide linkage analysis using a panel of single nucleotide polymorphisms (SNPs) throughout the rat genome. Using multivariate Bayesian regression approaches (Bottolo et al., 2011b), we identified a set of 2,357 transcripts showing significant linkages to discrete genetic loci (posterior probability >80%), which are designated as expression quantitative trait loci (eQTLs, Figure S2). The majority of transcripts were regulated by local genetic variation forming *cis*-eQTLs (67%); however, we also identified many *trans*-regulated transcripts (*trans*-eQTLs) across the rat genome (Figure 1B). As previously reported (Hubner et al., 2005; Langley et al., 2013), *trans*-eQTLs can form clusters that are commonly referred to as *trans*-eQTLs hot spots (Breitling et al., 2008) (Table 1). Notably, we identified a large eQTL hot spot that mapped to three SNPs spanning a genomic region of 1.2 Mbp located on rat chromosome 9q11, which regulated in *trans* the expression of 190 transcripts (Table 1; Figure 1B). These 190 transcripts formed a gene coexpression network, where each gene is regulated in *trans* by the same genetic locus (Figure 1C). Cell-type enrichment analysis using a mouse gene expression atlas showed that the gene network is enriched for osteoclast genes (enrichment p value = 4×10^{-7} , Z test for relative overexpression of the network genes in a tissue/cell type, see Supplemental Experimental Procedures), suggesting a role for the network in macrophage multinucleation (Figure 1C). Closer inspection of the network genes revealed two major determinants of osteoclast activity (*Mmp9* and cathepsin K, reviewed in Helming and Gordon, 2009) as well as several reported regulators of macrophage multinucleation such as *P2rx7* (Lemaire et al., 2006), *Tm7sf4* (also known as DC-STAMP [Yagi et al., 2005]), osteopontin (*Osp*) (Tsai et al., 2005), *Pik3cb* (also known as PI3K [Peng et al., 2010]), tetraspanin *Cd9* (Takeda et al., 2003), and its binding partner *Igsf8*.

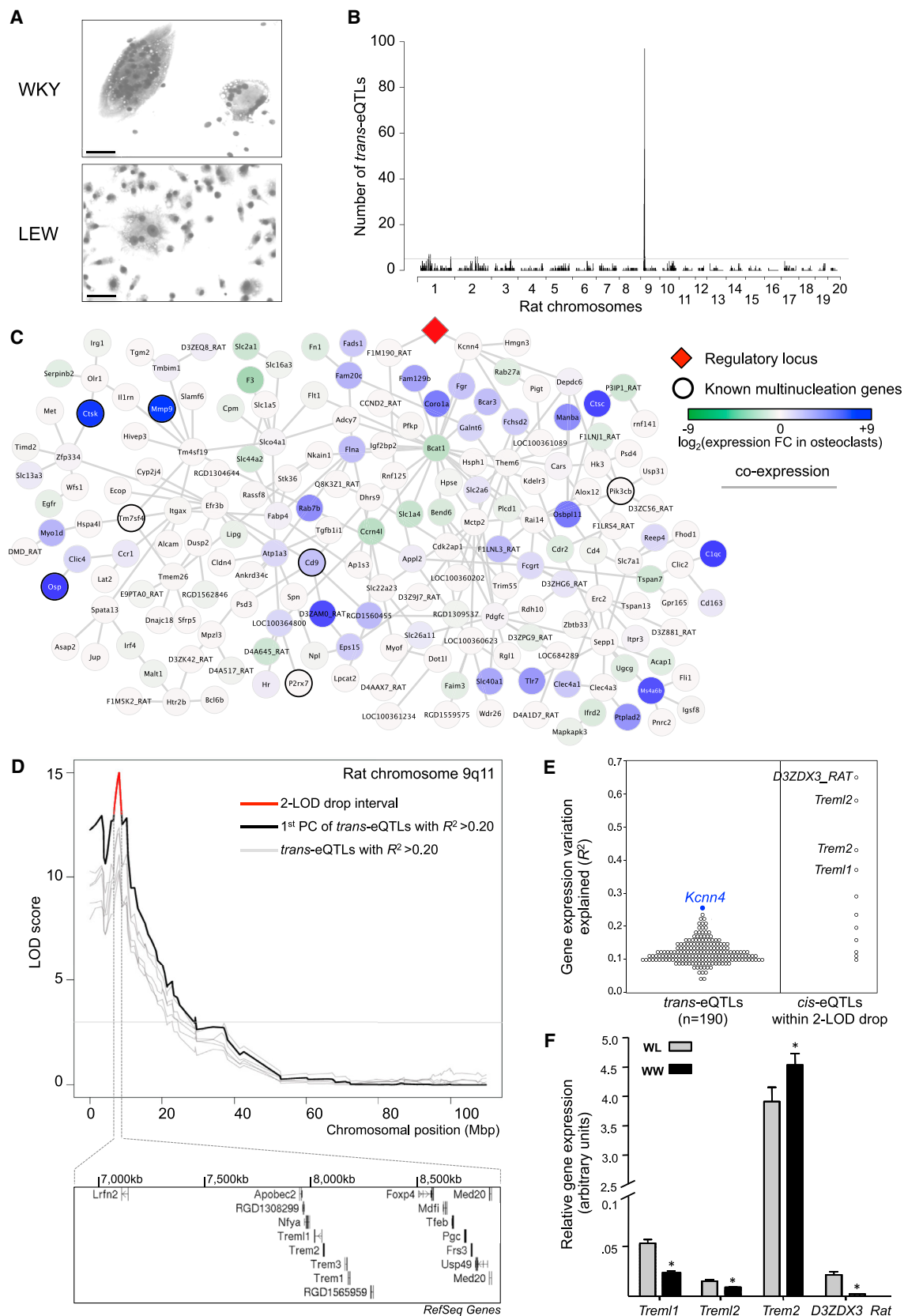
We hypothesize that other genes of the large coexpression network might regulate macrophage multinucleation, therefore defining a “Macrophage Multinucleation Network” or MMnet.

To prioritize master regulator genes at the rat chromosome 9 locus, we carried out fine mapping analysis and, among others, identified the Triggering receptor expressed in myeloid cells (*Trem*) gene family cluster within the 2-LOD drop support interval of the peak of linkage (Figure 1D). At this locus, the *Trem* family genes showed the strongest *cis*-acting genetic regulation in macrophages (variation in gene expression explained by the regulatory locus; $R^2 > 0.25$, Figures 1E), and, among all genes in the MMnet, *Kcnn4* was the most significant *trans*-eQTL (Figure 1E). Quantitative real-time PCR analysis of the expression of the *Trem* family genes in backcross macrophages confirmed their *cis*-regulation and prioritized *Trem2* as the most highly expressed gene in rat macrophages (>90-fold more expression compared to all other genes in the *Trem* cluster, Figure 1F).

Identification of *Trem2* as a Master Genetic Regulator of the MMnet

We observed a positive correlation between the expression of *Trem2* and 125 (66%) MMnet genes and a negative correlation between *Trem1*, *D3ZDX3_Rat*, *Trem12*, and the network genes (Figure 2A). We selected ten genes from the MMnet, including the most strongly *trans*-regulated gene (*Kcnn4*, $R^2 = 0.25$), genes previously reported to play a role in macrophage multinucleation (*P2rx7* [Lemaire et al., 2006], *Tm7sf4* [Yagi et al., 2005], *Pik3cb* [Peng et al., 2010], tetraspanin *Cd9* [Takeda et al., 2003]) and additional *trans*-regulated MMnet genes with varying genetic effects (R^2 ranging from ~ 0.1 to 0.22). These genes recapitulate the correlation pattern observed between the *Trem* cluster genes and the MMnet (Figure 2B). We carried out RNAi experiments to further prioritize candidates within the *Trem* gene cluster as potential master regulators of the MMnet, by using small interfering RNA (siRNA) against *Trem1*, *Trem2*, *D3ZDX3_Rat*, and *Trem12* genes that were differentially expressed between WKY and LEW macrophages (Figure 1F), followed by quantitative real-time PCR assessment of ten *trans*-regulated genes from the network. In keeping with the positive correlation between *Trem2* and the MMnet genes (Figures 2A and 2B), we found significant downregulation of the *trans*-regulated genes after *Trem2* silencing (Figure 2C). On the contrary, silencing of *Trem1*, *D3ZDX3_Rat*, and *Trem12* resulted in a weaker and variable downregulation of the network genes (Figure 2C). This downregulation was not in accordance with the expected transcriptional response based on the negative correlation between *Trem1*, *D3ZDX3_Rat*, *Trem12*, and each *trans*-eQTL gene expression (Figure 2B). These in vitro experiments prioritized *Trem2* as a master regulator gene of the MMnet in the rat. We then explored whether silencing of *TREM2* in monocyte-derived macrophages had a similar effect on the *trans*-eQTL network genes in humans (Figure 2D). We showed that there was a significant correlation between the percentage of knockdown in human MDMs and rat BMDMs following *Trem2* knockdown (Figure 2E). These data suggest that the *Trem2*-regulated MMnet genes identified in rat macrophages are similarly regulated in human macrophages.

Taken together, genome-wide eQTL analysis and in vitro experiments uncovered a large gene network regulating



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Table 1. eQTL Hot Spots Identified in Backcross BMDMs Derived from WKY and LEW

SNP	Chr.	Position (Mb)	Transcripts Mapping to Each SNP	Five Most Strongly Regulated Genes
Rn34_1071564105	1	71564105	7	<i>RGD1562091 / Zfp61 / Vasp / F1LU75_RAT / Slc26a11</i>
Rn34_1071580649	1	71580649	6	<i>Gemin7l1 / TMED5_RAT / LOC691921 / Kcnn4 / Trove2</i>
Rn34_1084462932	1	84462932	7	<i>F1M372_RAT / D3ZBY1_RAT / Flna / Plek / Fn1</i>
Rn34_1253288627	1	253288627	6	<i>Blnk / LOC689756 / Oasl / Apoc1 / Egr3</i>
Rn34_2146742392	2	146742392	6	<i>RGD1359508 / Dtw2 / Hrh1 / RGD1561662 / Lce1l</i>
Rn34_2164452465	2	164452465	6	<i>Sucnr1 / Tuft1 / Mgrn1 / Dlgap4 / Ptch1</i>
Rn34_9003266206	9	3266206	29	<i>Phlda1 / Pak6 / Xylt1 / Slc30a1 / RGD1565705</i>
Rn34_9003796216	9	3796216	9	<i>RGD1566226 / D4AE99_RAT / Slc35e4 / RGD1311946 / Hprt1</i>
Rn34_9004824426	9	4824426	15	<i>Plk3 / Capn5 / Bag3 / F1LTZ3_RAT / Ccl22</i>
Rn34_9005917596	9	5917596	7	<i>Tgfb2 / Leprot / RGD1310371 / Creb3l2 / RTN1_RAT</i>
Rn34_9006863573 ^a	9	6863573	40	<i>Tspan7 / Rdh10 / F1LRS4_RAT / Plcd1 / D3ZC56_RAT</i>
Rn34_9007659609 ^a	9	7659609	53	<i>D3ZC56_RAT / D3ZC56_RAT / Ms4a6b / Myo1d / Bend6</i>
Rn34_9008096016 ^a	9	8096016	97	<i>Kcnn4 / Slco4a1 / Flna / Bcat1 / Atp1a3</i>
Rn34_9010210028	9	10210028	6	<i>D4ACH5_RAT / St6gal1 / Myh9 / Plek / E9PU51_RAT</i>

n = 200. eQTL hot spots were defined as significant overrepresentation of *trans*-eQTLs (i.e., any SNP is associated with more than five transcripts; see [Experimental Procedures](#)).

^aSNPs delineate a genomic region of 1.2 Mb linked to the expression of 190 transcripts, which were located elsewhere in the genome and regulated in *trans* (i.e., *trans*-eQTLs).

macrophage multinucleation, which is under *trans*-acting genetic control by the *Trem2* gene in rats and humans.

Kcnn4 Is Implicated in Macrophage Multinucleation in Rodents and Humans

Because multinucleate osteoclasts and MGCs originate from macrophages (Vignery, 2005), our data suggest that MMnet controlled by *Trem2* may contain novel genes regulating macrophage multinucleation. We therefore hypothesized that the most significant *trans*-eQTL in the network ($R^2 = 0.25$, $p = 3.8 \times 10^{-16}$, [Figure 1E](#)), *Kcnn4*, encoding an intermediate-conductance calcium-activated potassium channel, regulates multinucleation in macrophages. *Kcnn4* expression peaked during macrophage multinucleation ([Figure S3](#)) and a separate time-dependent microarray analysis of fusing rat alveolar macrophages and hu-

man monocytes revealed *Kcnn4* as a differentially expressed gene, which is strongly induced at the onset of fusion ([Figure S3](#)). We therefore investigated whether *Kcnn4* determines multinucleation in osteoclasts and MGCs, two cell types resulting from multinucleation in physiological and pathological conditions, respectively. To this aim, we used two selective distinct pharmacological blockers (ICA-17043 and TRAM-34) of *Kcnn4* in human and rodent osteoclasts and MGCs and confirmed the effect of these blockers with targeted gene deletion and RNAi for *Kcnn4* ([Figure 3](#)). Transfection with siRNA designed against *Kcnn4* ([Figure 3A](#)), selective pharmacological blockade ([Figures 3B and 3D](#)), and targeted gene deletion ([Figure 3C](#)) of *Kcnn4* consistently decreased macrophage multinucleation in rodents. Similar results were obtained in human osteoclasts and MGCs ([Figures 3E and 3F](#)), indicating that *Kcnn4* regulates macrophage

Figure 1. Identification of Kcnn4 within a Genetically Regulated Macrophage Multinucleation Network

(A) Genetic determinants of macrophage multinucleation were explored in WKY and LEW bone-marrow-derived macrophages (BMDMs). WKY macrophages fuse spontaneously to form multinucleate giant cells (MGCs) in vitro and show a marked phenotypic difference when compared to LEW macrophages, which form very few MGCs at day 6 of cell differentiation (original bars, 50 μ m).

(B) eQTL analysis of the backcross (BC) BMDMs identifies a unique master regulatory locus on rat chromosome 9q11. Genome-wide distribution of *trans*-eQTLs shows a single locus regulating the expression of 190 transcripts in *trans*, suggesting master regulation of the *trans*-eQTL cluster. The detailed eQTL hot spots with the corresponding SNP positions are reported in [Table 1](#).

(C) Gene coexpression network of the 190 *trans*-eQTLs is enriched for osteoclast-expressed genes where the master regulatory locus is highlighted in red. Each gene in the network is represented as a circle (node), and the fold change in expression refers to overexpression (blue) or underexpression (green) in osteoclasts as compared to average expression in other cell types ([Supplemental Experimental Procedures](#)). Known multinucleation genes (*Mmp9*, *Ctsk*, *P2rx7*, etc.) are indicated with thick circles. The edges represent coexpression between the two transcripts as identified by ARACNE ([Margolin et al., 2006](#)).

(D) Genetic linkage showing the principal component of all the *trans*-cluster transcripts (LOD > 10) together with *trans* eQTLs with variation in gene expression explained by the SNP (R^2) > 0.2. The 2-LOD drop interval (in red) and the underlying positional candidates within 2 Mb are also shown. The annotated gene names are according to RefSeq.

(E) The *cis*-eQTLs within the 2-LOD drop interval (in red) are shown together with the *trans* cluster. *Trem1*, *Trem2*, *D3ZDX3_Rat*, *Trem2* are positional candidates ($R^2 > 0.25$) and among the *trans*-eQTLs, *Kcnn4* (in blue) is the most significant *trans*-eQTL.

(F) Quantitative real-time PCR analysis on the positional candidates in BC BMDMs according to their genotype (WKY homozygous, WW; WKY/LEW heterozygous, WL). Note that the expression levels of *Trem2* are increased at least 90-fold when compared to other positional candidates.

Error bars indicate SEM, * $p < 0.01$. See also [Figures S1 and S2](#).

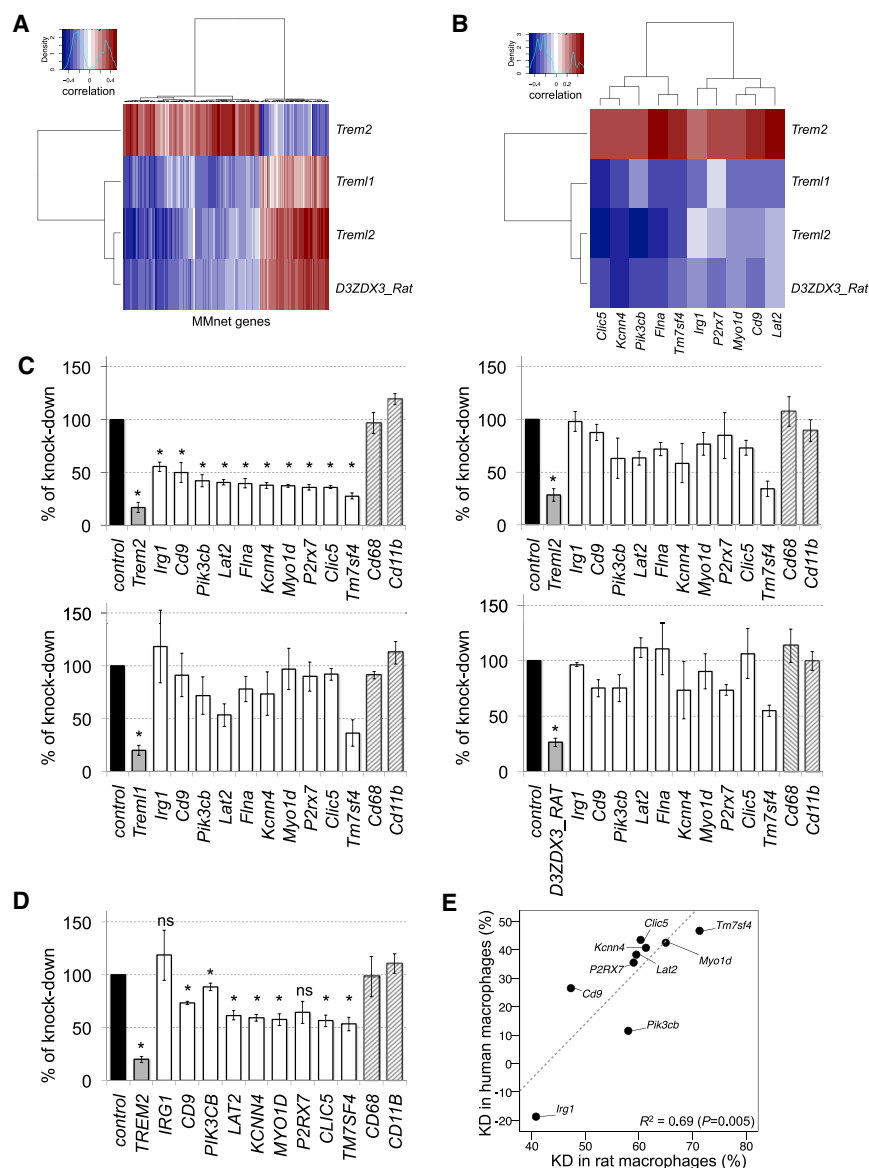


Figure 2. *Trem2* Is a Master Genetic Regulator of Macrophage Multinucleation Network

(A) Heatmap of the correlations between macrophage mRNA levels of positional candidates (rows) and all *trans*-regulated genes of MMnet, showing that *Trem2* was positively correlated with the majority (66%) of MMnet transcripts. Red indicates positive correlation and blue indicates negative correlations.

(B) Heatmap of the correlations between macrophage mRNA levels of positional candidates (rows) and ten *trans*-regulated genes representative of the larger MMnet selected for the siRNA knock-down experiments in rat BMDMs. Red indicates positive correlation and blue indicates negative correlations.

(C) siRNA against *Trem2*, *Trem1*, *Trem2*, *D3ZDX3_Rat* in rat macrophages followed by measurement of 10 transcripts belonging to the chromosome 9q11 *trans* cluster by quantitative real-time PCR. *Cd68* and *Cd11b* expression levels were assessed as control transcripts that do not belong to MMnet. Given the correlation patterns shown in Figure 2B, a one-sample t test was used to test for directionality of the effect in the transcriptional response. Knockdown of *Trem2* led to significant (~50%, $p < 0.001$) downregulation of all genes tested, whereas knockdown of *Trem1*, *D3ZDX3_Rat* did not result in a significant alteration in the expression levels of MMnet genes ($p > 0.1$ for all MMnet genes tested). At least $n = 3$ rats were used in each experiments. Error bars indicate SEM, * $p < 0.001$.

(D) siRNA-mediated knockdown of *TREM2* in human monocyte derived macrophages (MDMs) and the quantitative real-time PCR analysis of ten transcripts from the MMnet. MDMs are from buffy coats from four healthy donors. Error bars indicate SEM, * $p < 0.05$ compared to control (scrambled).

(E) Knockdown of *TREM2* in human MDMs resulted in transcriptional downregulation of MMnet genes, which significantly correlated with downregulation observed in rat BMDMs ($R^2 = 0.69$, $p = 0.005$).

multinucleation in rodents and humans. The inhibitory effect of TRAM-34 on macrophage multinucleation is reversible and TRAM-34 does not affect macrophage differentiation (Figure S3) but changes the macrophage transcriptome assessed by high-throughput sequencing of mRNA (RNA-seq, Figure S3). Furthermore, in order to assess whether the absence of *Kcnn4* affects preosteoclast formation, we have quantified TRAP⁺ mononuclear cells isolated from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice at day 3, 4, and 5 of cell differentiation and showed that early steps of osteoclast differentiation do not seem to be affected by *Kcnn4* deficiency (Figure S3).

***Kcnn4* Modulates Bone Turnover**

Given our data implicating *Kcnn4* in macrophage multinucleation and because defects in osteoclast formation impact bone resorption (Teitelbaum, 2011), we tested whether *Kcnn4* defi-

ciency affects bone mass. Femurs from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} female and male mice were analyzed by X-ray, peripheral quantitative computed tomography (pQCT), and microcomputed tomography (microCT). These analyses consistently revealed increased bone mass and density in both female and male *Kcnn4*^{-/-} mice compared with *Kcnn4*^{+/+} mice (Figure 4). In particular, femurs from *Kcnn4*^{-/-} mice showed increased (1) X-ray absorption (Figure 4A), (2) total bone mineral density (9% and 14% increases in females and males, respectively), (3) trabecular bone density (30% and 29% in females and males), and (4) trabecular bone volume fraction (27% and 26% in females and males) (Figures 4B and 4C). Accordingly, *Kcnn4*^{-/-} mice had reduced numbers of TRAP-positive osteoclasts (Figure S4) and a reduced number of osteoclasts per unit of bone surface (Figures 4D and S4; Table S1), thus supporting a role for *Kcnn4* in osteoclast formation in vivo. Furthermore, *Kcnn4*^{-/-}

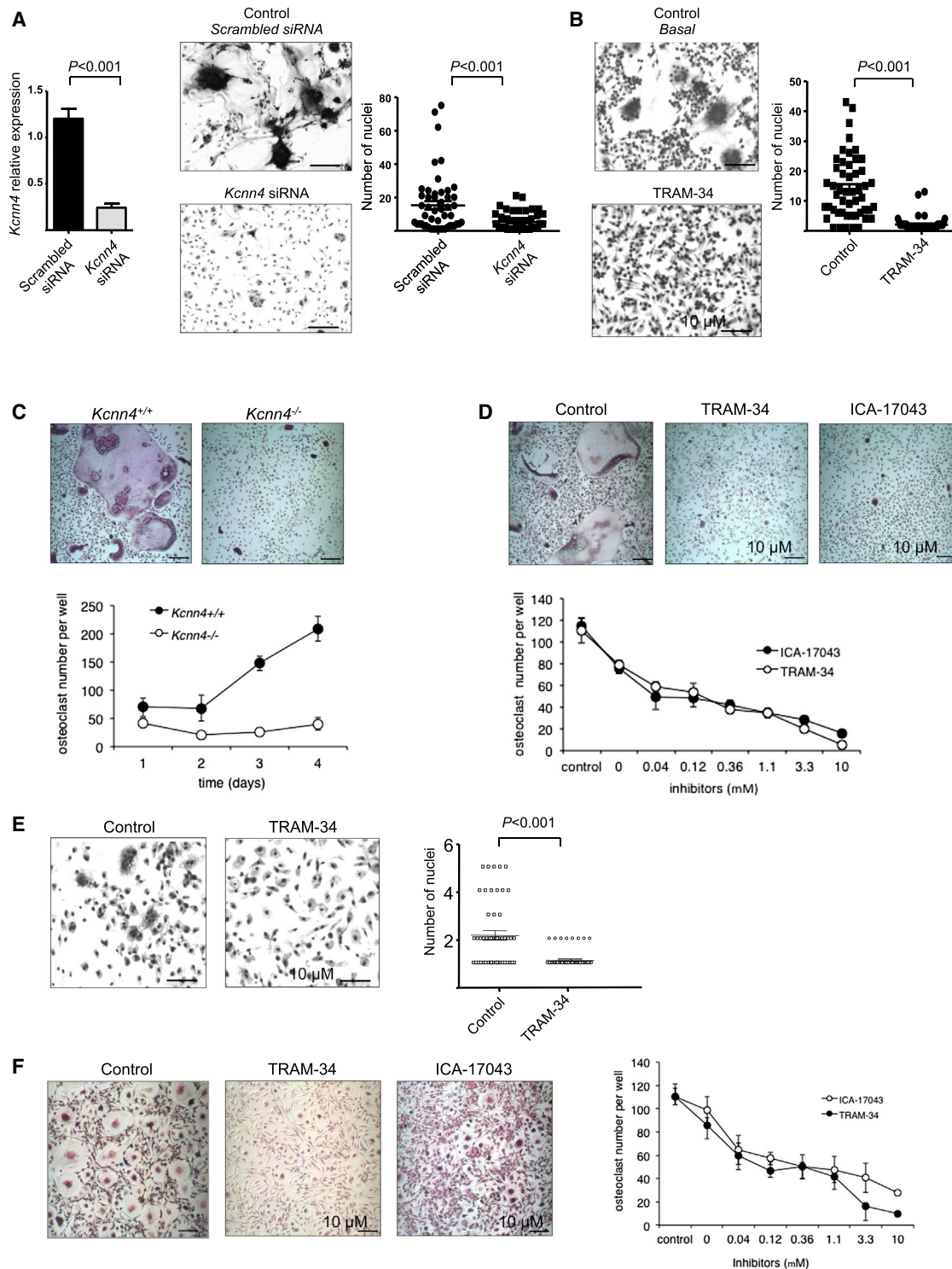


Figure 3. *Kcnn4* Regulates Macrophage Multinucleation in Rodents and Humans

(A) WKY BMDMs were cultured in Lab-Tek chambers and incubated with either scrambled or *Kcnn4* siRNA, and cells were fixed for the assessment of the multinucleation. *Kcnn4* expression levels were measured by quantitative real-time PCR and normalized to *Hprt* expression, showing 80% knockdown in macrophages following incubation with *Kcnn4* siRNA (left panel). Silencing of *Kcnn4* led to a marked reduction in macrophage multinucleation as the number of nuclei in macrophages transfected with *Kcnn4* siRNA was significantly reduced (right panel) when compared to controls (mean $\pm$ SEM; $n = 4$ rats). Original bars, 50 μ m. $p < 0.001$ determined by Kruskal-Wallis test.

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mice also displayed increased bone formation and mineral apposition rates compared with *Kcnn4*^{+/+} mice, indicating the high bone mass phenotype in *Kcnn4*^{-/-} mice results from effects on both bone formation and bone resorption (Table S1). We next investigated whether osteoclast activity was decreased in *Kcnn4*^{-/-} mice in an inflammation-induced calvaria bone resorption provocation model. This study revealed that *Kcnn4*^{-/-} mice lacked the normal osteoclast-mediated bone resorption response to inflammation (Figure 4E). We also showed that deletion of *Kcnn4* results in reduced osteoclastic resorption in vitro (Figure S4). Taken together, these data indicate that *Kcnn4* participates in the control of bone turnover by regulating functional osteoclast formation and suggest that its effects on bone formation may be mediated via a primary role in osteoclasts.

Inactivation of *Kcnn4* Reduces Severity of Glomerulonephritis and Bone Erosion in Arthritis

To study the role of *Kcnn4* in macrophage multinucleation in pathological conditions, we used two inflammatory models in rodents, characterized by MGC and osteoclast activation: glomerulonephritis and arthritis, respectively. In crescentic glomerulonephritis (Crngn), we blocked *Kcnn4* with its selective inhibitor TRAM-34 (Wulff et al., 2000) in the WKY NTN model characterized by glomerular MGCs (Figure S1; Lan et al., 1998). In the WKY NTN model, the severe and reproducible glomerular macrophage infiltration following nephrotoxic serum injection (Tam et al., 1999) is also characterized by approximately 20% of glomeruli presenting MGCs (Figures 5 and S1). It is therefore possible to study the effect of TRAM-34 on glomerular MGC formation and renal injury assessed by proteinuria. TRAM-34 treatment of WKY rats with NTN led to significant reduction of glomerular crescents, glomerular MGC formation, and proteinuria without affecting the number of macrophages infiltrating the glomeruli (Figures 5A and 5B). To evaluate the role of *Kcnn4* in bone erosion in inflammatory arthritis, we assessed the effect of *Kcnn4* deficiency in anticollagen antibody-induced arthritis (CAIA) and showed that *Kcnn4*^{-/-} mice have reduced joint inflammation and tissue damage (Figures 5C, 5D, and S5). Following CAIA serum bone resorption (CTX) and bone formation (P1NP) markers were measured in *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice. *Kcnn4* deficiency resulted in

impaired osteoclastic bone resorption that was not affected by CAIA but did not affect osteoblastic bone formation (Figure S5). This is consistent with the results obtained in the lipopolysaccharide (LPS)-induced calvaria model (Figure 4E) showing reduced osteoclast activity in vivo. Taken together, the results obtained from the LPS and CAIA experiments suggest that reduced bone loss in *Kcnn4*^{-/-} mice can be attributed to decreased osteoclast activity resulting from impaired multinucleation.

Kcnn4 Regulates Ca²⁺ Signaling in Macrophage Multinucleation

In order to investigate the mechanism by which *Kcnn4* regulates multinucleation, we focused on intracellular Ca²⁺ levels because of the established link between K⁺ channels and Ca²⁺ influx during lymphocyte activation (Feske et al., 2012). We assessed whether pharmacological blockade or gene deletion of *Kcnn4* results in disturbed Ca²⁺ signaling and oscillations and revealed that there are decreased Ca²⁺ oscillations in *Kcnn4*^{-/-} osteoclasts compared to *Kcnn4*^{+/+} (Figure 6A). We also detected differences between *Kcnn4*^{+/+} and *Kcnn4*^{-/-} macrophages in patch clamp experiments, including a shift in observed reversal potential following gene deletion or inhibition of *Kcnn4* (Figure 6B). One of the major downstream effects of receptor activator of nuclear factor kappa-B ligand (RANKL)-induced activation of Ca²⁺ signaling is nuclear translocation of dephosphorylated nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) (Takayanagi, 2007), and, consistent with this, there was reduced expression of NFATc1 in *Kcnn4*^{-/-} osteoclasts (Figure 6C). The reduced NFATc1 expression was associated with decreased nuclear translocation in *Kcnn4*^{-/-} bone-marrow-derived macrophages in response to RANKL (Figure 6D). Similarly, WKY BMDMs treated with TRAM-34 exhibit reduced *Nfatc1* expression associated with decreased multinucleation (data not shown). To elucidate the mechanisms by which *Kcnn4* affects osteoclast differentiation, we stimulated macrophages with RANKL or LPS for increasing time periods and found that RANKL-induced p38 and ERK activation were not modified by *Kcnn4* deficiency (data not shown). In contrast, RANKL-induced Akt activation was reduced (Figure S6). This result was confirmed in human macrophages treated with TRAM-34 (Figure S6). Because Akt has been shown to be a

(B) When macrophages were cultured in the presence of TRAM-34 (10 μ M), the number of nuclei per 100 macrophages was significantly reduced when compared to cells incubated with the media only (control, basal cells). The results are representative of five independent experiments. Original bars, 50 μ m. $p < 0.001$ determined by Kruskal-Wallis test.

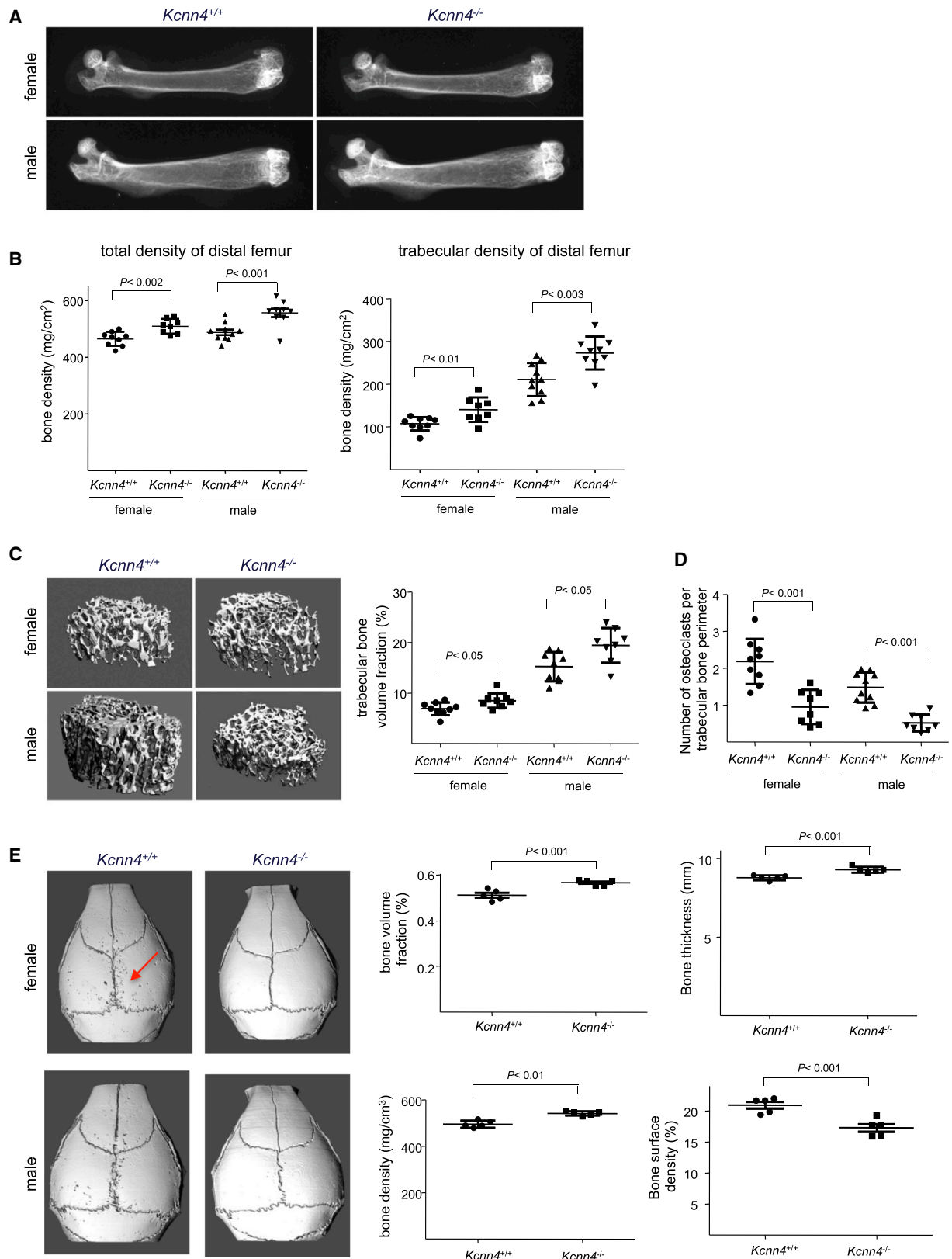
(C) BMDMs from 6-week-old *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice were cultured in the presence of macrophage colony stimulating factor (M-CSF) (25 ng/ml) and RANKL (20 ng/ml) for 4 days to induce the differentiation of osteoclasts (upper panel). The formation of osteoclasts measured as the number of TRAP⁺ multinucleated osteoclasts per well increased with time in *Kcnn4*^{+/+} cells but not in *Kcnn4*^{-/-} cells. Mean $\pm$ SD; $n = 5$; the osteoclast number/well was significantly different ($p < 0.001$) at all time points; the results are representative of three independent experiments. Original bars, 100 μ m.

(D) Mouse BMDMs were cultured in the presence of M-CSF (25 ng/ml) and RANKL (40 ng/ml) and treated with TRAM-34 or ICA-17043 (both 10 μ M, upper panel). TRAM-34 and ICA-17043 treatment on mouse osteoclasts reduced the number of osteoclasts per well in a dose-dependent manner (lower panel, mean $\pm$ SD; $n = 5$). Original bars, 100 μ m.

(E) Human-monocyte-derived macrophages were cultured in Lab-Tek chambers, and spontaneous MGC formation was observed in some buffy coats, and this was significantly reduced when cells were incubated with TRAM-34 (10 μ M). At least three separate buffy coats were used to differentiate human macrophages. Original bars, 50 μ m. $p < 0.001$ determined by Kruskal-Wallis test.

(F) Human peripheral blood monocytes were cultured in the presence of M-CSF (20 ng/ml) and RANKL (10 ng/ml) for 5 days to induce the differentiation of osteoclasts. Cells were treated with TRAM-34 or ICA-17043 (both 10 μ M, left panel). Quantification of osteoclast number per well shows that TRAM-34 and ICA-17043 inhibited the formation of monocyte-derived human osteoclasts in a dose-dependent manner (mean $\pm$ SD; $n = 4$). Original bars, 100 μ m.

See also Figure S3.



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critical mediator of cell proliferation and survival in a variety of cell types (Scheid and Woodgett, 2001), our results showing impaired Akt phosphorylation in association with *Kcnn4* deficiency suggested that *Kcnn4* may regulate apoptosis. Supporting this hypothesis, deletion of *Kcnn4* was associated with increased apoptosis in osteoclasts (Figure S6).

DISCUSSION

Using systems genetics approaches, our study demonstrates how genomic sequence variation affects a unique regulatory network that is associated with macrophage multinucleation in the rat. By conducting an eQTL study in the primary macrophages of a backcross rat population derived from two inbred rat strains with contrasting phenotypes (“markedly fusogenic WKY macrophages” versus “lowly fusogenic LEW macrophages”), we have identified a unique gene regulatory network (MMnet) associated with macrophage multinucleation in the rat. We show that *Trem2* regulate MMnet, which was enriched for genes regulating osteoclast formation in the rat, a result that was confirmed in human macrophages. Consistent with the previously established role of *Trem2* in macrophage fusion (Helming et al., 2008), activation (Turnbull et al., 2006), and osteoclast formation (Cella et al., 2003; Humphrey et al., 2006; Otero et al., 2012), here, we demonstrate that the strongest *trans*-eQTL in the *Trem2*-controlled gene network, *Kcnn4*, is a key determinant of macrophage multinucleation in rodent and human osteoclasts and MGCs. We independently confirmed the role of *Kcnn4* as a potential modulator of multinucleation by microarray analysis of fusing rat macrophages and human monocytes that form osteoclasts. We show that *Kcnn4* inhibitors prevent the formation of monocyte-derived human osteoclasts and MGCs, in addition to mouse bone-marrow-derived osteoclasts and rat MGCs.

The intermediate-conductance Ca^{2+} -activated K^{+} channel *Kcnn4* (also known as *Kca3.1* or *IK1*) is activated in response to increases in intracellular Ca^{2+} through calmodulin bound to its intracellular C terminus (Lam and Wulff, 2011). The blockade of this channel by a selective small molecule inhibitor (TRAM-34) was first shown to have immunosuppressive effects on T lymphocytes (Wulff et al., 2000) and further tested on T cell-mediated diseases such as inflammatory bowel disease, experimental autoimmune encephalomyelitis, rheumatoid arthritis, and asthma (reviewed in Lam and Wulff [2011]). Although

Kcnn4 is expressed in macrophages, its role in macrophage multinucleation has not previously been reported. Treatment with TRAM-34 improved atherosclerosis in *ApoE*^{−/−} mice aortas and carotid arteries by decreasing macrophage infiltration and by possibly inhibiting their migration (Toyama et al., 2008). Similarly, TRAM-34 treatment reduced ED1⁺ macrophages and microglia in the rat brain following ischemia/reperfusion stroke (Chen et al., 2011b), and, more recently, it has been shown that blockade of *Kcnn4* reduced F4/80⁺ cells in a streptozotocin-induced diabetic mouse model (Huang et al., 2013).

Although it had been reported that Ca^{2+} -activated K^{+} currents are involved in the regulation of osteoclast movement and spreading on bone substrates, and that increase in intracellular Ca^{2+} blocks osteoclast bone resorption in vitro (Valverde et al., 2005), the specific contribution of *Kcnn4* in inflammation-induced osteoclast development in vivo was not previously reported. The effect of *Kcnn4* on bone mass is modest compared to its critical regulatory role in osteoclast and macrophage multinucleation. This is consistent with previous findings demonstrating a relatively mild effect of macrophage fusion marker (DC-STAMP) on bone mass (Yagi et al., 2005). At this stage, the bone formation abnormality observed in *Kcnn4*^{−/−} mice remains incompletely understood and additional studies to investigate further will require detailed analysis of *Kcnn4*^{+/+} and *Kcnn4*^{−/−} mice at various ages during development and adulthood, as well as cell-specific gene targeting approaches because it was previously used for studying the effect of cathepsin-k deletion on bone formation (Lotinun et al., 2013).

We also show that *Kcnn4* affects macrophage multinucleation without having an effect on preosteoclast formation, a result previously documented for *Atp6v0d2*, a regulator of osteoclast fusion (Lee et al., 2006). Here, we show that *Kcnn4*-deficient mice fail to mount an osteoclastic response to local injection of LPS into calvaria. Local osteoclast formation and bone loss are a major component of the pathogenesis of metabolic and inflammatory bone diseases (Boyle et al., 2003). Thus, *Kcnn4*, which was previously reported as a potential target for immunosuppression (Cahalan and Chandy, 1997; Jensen et al., 2001), autoimmune diseases (Wulff et al., 2003), and periodontal disease (Valverde et al., 2005), also represents a potential new target for the prevention of inflammation-related bone loss.

The WKY NTN model of Crgn is characterized by glomerular MGC formation (Kaneko et al., 2003) and the renal injury is entirely dependent on macrophage infiltration and activation

Figure 4. *Kcnn4* Modulates Bone Homeostasis via Osteoclasts

(A) Genetic deletion of *Kcnn4* increases bone mass as shown by X-ray analysis performed on femurs from 8-week-old *Kcnn4*^{+/+} and *Kcnn4*^{−/−} female and male mice. Note the stronger radio-opacity in femurs from *Kcnn4*^{−/−} mice when compared with *Kcnn4*^{+/+} mice.

(B) Peripheral quantitative computed tomography (pQCT) analysis of femoral bones showing total bone density and trabecular bone density of distal femurs from 8-week-old *Kcnn4*^{−/−} mice compared with *Kcnn4*^{+/+} mice (mean ± SD; n = 7–9).

(C) Representative images of microcomputed tomography (microCT) analysis of distal femurs (left panel) from 8-week-old female and male *Kcnn4*^{+/+} and *Kcnn4*^{−/−} mice. Trabecular bone volume fraction from *Kcnn4*^{+/+} and *Kcnn4*^{−/−} female and male mice is shown in the right panel (mean ± SD; n = 7–9).

(D) Histomorphometry analysis of tibiae from 8-week-old *Kcnn4*^{+/+} and *Kcnn4*^{−/−} female and male mice showing the number of osteoclasts per trabecular bone perimeter (mean ± SD; n = 10).

(E) Microcomputed tomography analysis of calvaria from 8-week-old *Kcnn4*^{−/−} and *Kcnn4*^{+/+} male and female mice that had received a 2 μl of injection of 25 μg LPS on the right calvaria. Mice were sacrificed 5 days later. Note the low abundance of resorption lacunae in *Kcnn4*^{−/−} mice when compared with wild-type. Calvarial bone volume fraction, bone thickness and bone density were higher, whereas bone surface density was lower in *Kcnn4*^{−/−} mice when compared with wild-type (right panel; mean ± SD; n = 5).

See also Figure S4 and Table S1.

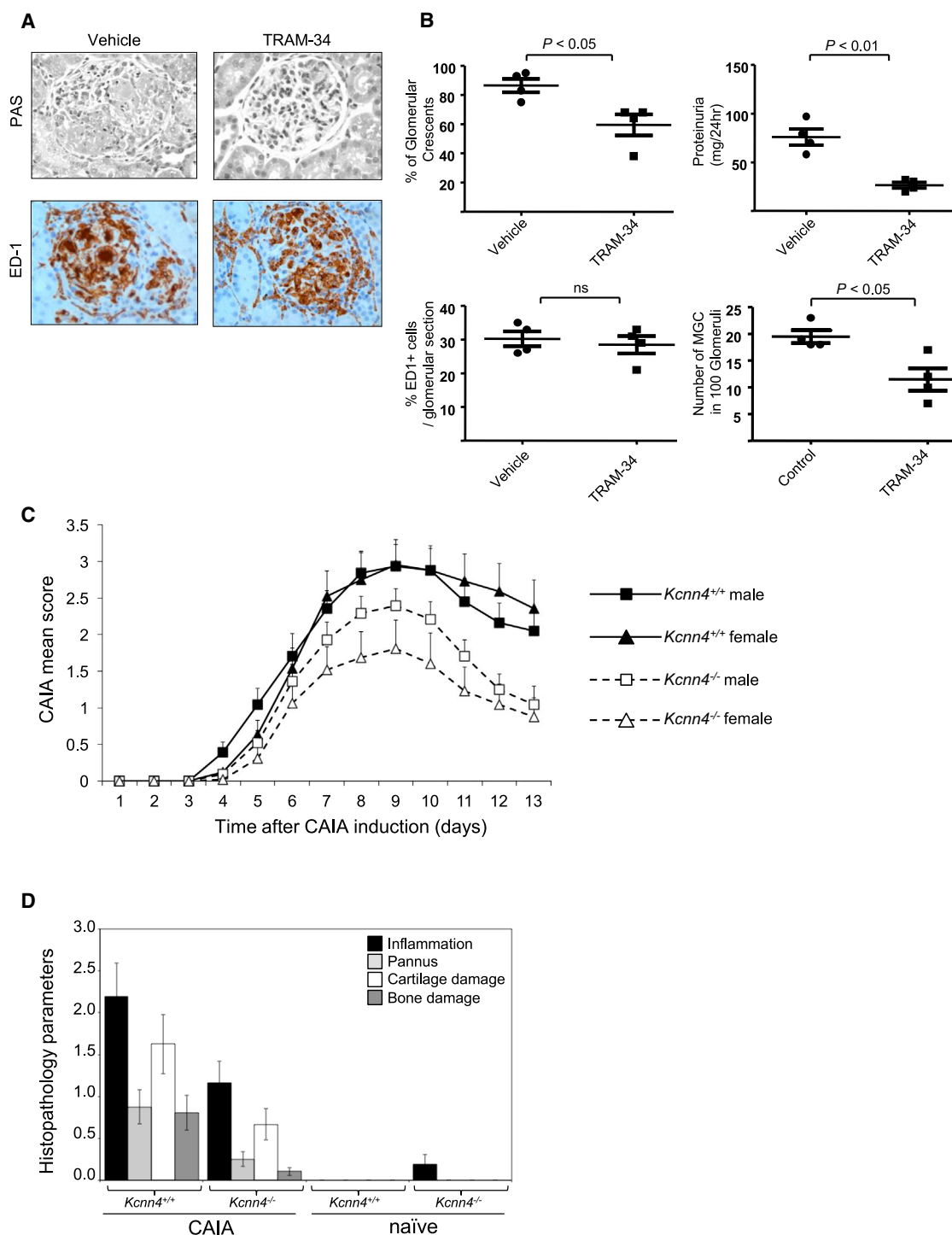


Figure 5. Reduced Severity of Glomerulonephritis and Arthritis by *Kcnn4* Inactivation

(A) Glomerular immunohistochemistry showing the effect of specific *Kcnn4* blocker TRAM-34 in renal injury in the WKY nephrotoxic nephritis model. Following nephrotoxic serum injection, glomerular crescents (PAS) and percentage of ED1+ macrophages were assessed in control (vehicle) and TRAM-34-treated rats ($n = 4$ rats were used per group of treatment). Original bars, 20 μ m.

(B) Quantification of glomerular crescents, proteinuria, percentage of ED1+ cells and number of glomerular MGCs.

(C) Collagen antibody-induced arthritis (CAIA) was induced in 2-month-old male and female *Kcnn4*^{-/-} and *Kcnn4*^{+/+} mice (mean $\pm$ SD; $n = 12-15$). Arthritic severity was monitored daily using a visual scoring system as detailed in [Supplemental Experimental Procedures](#). Based on inflammation scoring, *Kcnn4* deletion

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(Aitman et al., 2006; Behmoaras et al., 2008, 2010; D'Souza et al., 2013; Deplano et al., 2013; Hull et al., 2013; Page et al., 2012). We show that blocking *Kcnn4* in vivo by administration of TRAM-34 significantly reduces glomerular injury in the WKY NTN model. Although we did not observe different numbers of glomerular macrophages between TRAM-34 and vehicle-treated animals, there was a reduction in glomerular MGCs, which suggests that *Kcnn4* blockade reduced multinucleation in vivo by preventing disease. In keeping with this, we show that *Kcnn4* deletion leads to a reduced severity in chronic inflammatory arthritis characterized by osteoclast activation. Collagen-induced arthritis is characterized by focal collections of osteoclasts at sites of bone destruction, and previous reports showed that osteoclast depletion ameliorated bone erosion in this model (Romas et al., 2002; Sims et al., 2004). Our results showing reduced bone loss in anticollagen antibody-induced arthritis in *Kcnn4*^{-/-} mice support these findings. However, the effect of *Kcnn4* deletion in the CAIA model could be driven by either inflammation or osteoclast-mediated bone erosion/resorption. Further studies using osteoclast-specific *Kcnn4* knockout mice will be crucial in dissecting the relative contribution of inflammation and osteoclast-driven responses in this model.

The measurements of intracellular Ca²⁺ reveal that *Kcnn4* deficiency reduces the amplitude of Ca²⁺ oscillations, but not their frequency, suggesting that K⁺ currents through the *Kcnn4* channel contribute to the sustained Ca²⁺ oscillations mainly via the regulation of membrane potential, rather than Ca²⁺ mobilization from intracellular Ca²⁺ stores. Absence of *Kcnn4* appears to affect several downstream signaling pathways, including NFATc1 via calcium signaling, and Akt activation, which together lead to decrease survival of multinucleate macrophages.

In summary, using a systems genetics approach, we identified a *trans*-regulated multinucleation network in macrophages and provided compelling evidence that at least one gene within the network (*Kcnn4*) regulates macrophage multinucleation in bone homeostasis. We also show that *Kcnn4* can be effectively targeted to decrease severity of inflammatory conditions characterized by MGC or osteoclast activation.

EXPERIMENTAL PROCEDURES

Animals, Genetic Crosses, and Genotyping

Wistar-Kyoto (WKY/NCrl) rats and Lewis (LEW/Crl) were purchased from Charles River UK. Two hundred Backcross (BC) rats were produced by breeding WKY with LEW rats (congenic WKY rats were used where the previously identified crescentic glomerulonephritis QTLs on chromosome 13 and 16 were introgressed from the LEW rats [Behmoaras et al., 2010]). The (WKY x LEW) F1 animals were backcrossed to the congenic WKY rats. For genotyping, DNA was isolated from the BC rat spleens using a standard phenol-chloroform extraction. DNA (250 ng) was used for Nspl and Styl fragmentation according to Affymetrix SNP6.0 GeneChip instructions. Custom designed Whole Genome Rat Genotyping arrays (RATDIV arrays, MDC) that

contain 803,484 SNPs covering the rat genome (based on Rn3.4 version) were used. SNP calling was performed using the BRLMM algorithm and led to the identification of 785,247 SNPs with >95% call rate (see [Supplemental Experimental Procedures](#) for additional details). *Kcnn4*^{-/-} mice were generated on a mixed 129J/C57BL6 background, bred, and genotyped as previously described (Begenisich et al., 2004). The animals were housed in standard caging on a 12-hr-light cycle and were offered free access to rodent chow (Harlan Teklad #2018 Rodent Diet) and water.

Bone X-Ray Radiography, pQCT, microCT, and Histomorphometry in Mice

Excised femurs were subjected to X-ray using a MX-20 (Faxitron X-ray) at 30 kV for 3 s. X-ray films were scanned using an Epson Perfection 4870. Bone density was determined as described previously (Ballica et al., 1999) by Peripheral Quantitative Computed Tomography (pQCT, XCT Research M; Norland Medical Systems) of a virtual 1 mm cross-section of the distal femur 0.25 mm proximal to the growth plate. In addition, distal femurs were scanned with a Microcomputed Tomography (microCT) scanner (MicroCT 40; Scanco) with a 2,048 x 2,048 matrix and isotropic resolution of 12 μm³ voxel size. 3D trabecular measurements in the secondary spongiosa were made directly, as previously described (Li et al., 2005). Femurs and tibiae from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice were dehydrated in a graded ethanol series and embedded without decalcification in methylmethacrylate, as we described previously (Baron et al., 1984). Four-micrometer-thick cross-sections of the distal femur were stained with Villanueva Mineralized Bone Stain for static histomorphometric analysis (see [Supplemental Experimental Procedures](#)), whereas 8-μm-thick sections were left unstained for dynamic bone histomorphometric parameters.

All procedures were approved by the Yale University Institutional Animal Care and Use Committee. The care and treatment of experimental mice complied with all applicable federal guidelines and was approved by the Institutional Animal Care and Use Committee at Yale University.

Microarray Expression Profiling, eQTL Mapping, and Network Analysis

Total RNA was extracted from BMDMs from 200 backcross rats using TRIzol (Invitrogen), and gene expression was measured using Affymetrix 1.0 ST Rat Gene array. WKY-LEW next-generation sequencing data (Illumina HiSeq 2000) was used to remove SNP-containing probes prior to normalization. Arrays were normalized using RMA normalization from the Affymetrix package (Irizarry et al., 2003). Normalization step included Norm-exp background correction and quantile normalization. Probes signal were summarized at a transcript level using median polish. The resulting data were log transformed (Figure S2). Remaining batch effects were removed using ComBat (Chen et al., 2011a; Johnson et al., 2007) with nonparametric prior. All other parameters were left to default. The first two principal components of the normalized data before and after correcting for batch effects are shown in Figure S2. The eQTL mapping was performed using ESS++ (Bottolo et al., 2011a, 2011b). Fixed effects on each individual were added as covariates in the variable selection process to account for potential outliers or genotyping errors. eQTL analysis details, network inference, and cell-type enrichment analysis are described in [Supplemental Experimental Procedures](#).

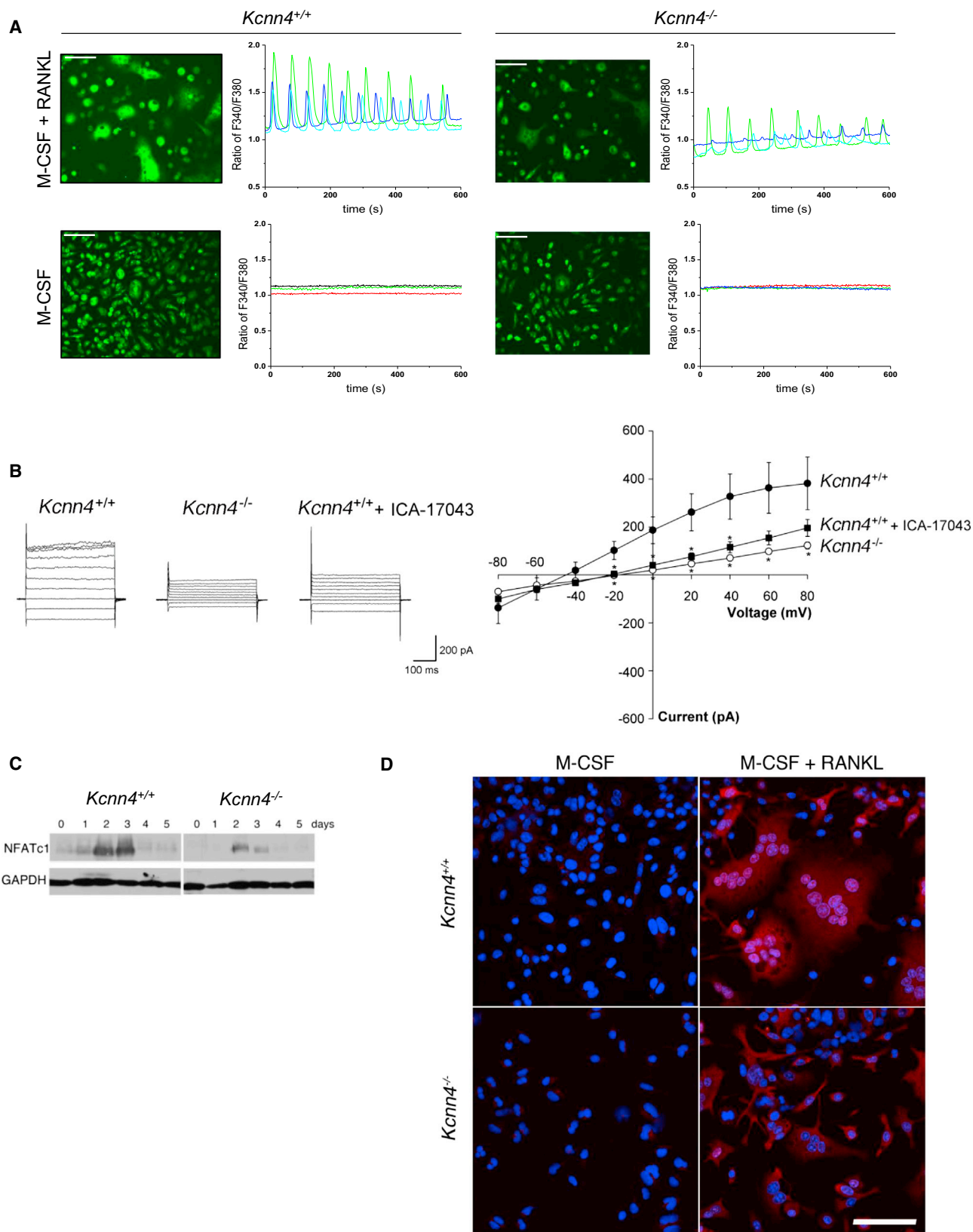
Nephrotoxic Nephritis in Rats and Collagen Antibody-Induced Arthritis in Mice

The effect of TRAM-34 on nephrotoxic nephritis (NTN) in the WKY rat was assessed as follows: eight male WKY rats were injected with the nephrotoxic serum (NTS) as previously described (D'Souza et al., 2013). A group of four rats received intraperitoneal injections of TRAM-34 (40 mg/kg) in Miglyol 812

reduced inflammation in males and females, though the prevention was more efficient in female mice. From day 8 onward, the CAIA mean score is significantly different between *Kcnn4*^{+/+} and *Kcnn4*^{-/-} animals for both genders ($p < 0.001$).

(D) Histopathomorphometry analysis of the paws and the ankles of *Kcnn4*^{+/+} and *Kcnn4*^{-/-} naive and CAIA-induced female mice. All the histomorphometric parameters (inflammation, pannus, cartilage damage, and bone damage) were compared to CAIA *Kcnn4*^{+/+} mice and are significant (mean ± SEM; $n = 10$ –12 mice in each group; $p < 0.05$). Similar results were obtained in male mice (data not shown).

See also [Figure S5](#).



neutral oil (Kemcare) at 1 μ l/g, twice a day with 10 hr intervals for 7 days. The control group ($n = 4$) had IP injections of Miglyol (vehicle). Rats were killed 7 days following injection of NTS, and glomerular crescents and proteinuria were measured as previously described (D'Souza et al., 2013). Chronic inflammatory arthritis was induced in 8-week-old male and female *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice by intraperitoneal (i.p.) injection of 7 mg (700 μ l) of Arthrogen monoclonal antibodies (ArthoMAB) blend (Millipore) on day 0. This cocktail of monoclonal antibodies is directed against epitopes recognized in the region CB11 of collagen type II. On day 3, 50 μ g (<100 μ l volume) of LPS was administered i.p. Beginning on day 4, the animals were monitored daily for the onset and development of collagen-antibody-induced arthritis (CAIA), and the injection site on each animal was evaluated for signs of infection, such as heat, redness, and/or exudation. Mice that felt as "cold," which likely underwent cachexia in response to LPS, received subcutaneous injection of 500 μ l of warm Ringer solution. Arthritic severity was monitored daily using a visual scoring system (Supplemental Experimental Procedures). The histopathological evaluation of CAIA is described in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) accession number for the rat-bone-marrow-derived macrophage microarray data reported in this paper is E-MTAB-2719.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.07.032>.

AUTHOR CONTRIBUTIONS

A.V., J.L., G.N., E.P., and J.B. designed the study. H.K. completed the cell biology experiments on human and mouse osteoclasts. A.K. performed the sample preparation for microarrays, RNAi experiments, RNA-seq, and cell biology experiments on rat and human macrophages with contributions from J.B. and J.H.K. M.R. has performed the eQTL mapping and network analysis with contributions from E.P. X.X. analyzed the role of *Kcnn4* in the formation of human and mouse osteoclasts and contributed to histomorphometry analysis and the calvaria assay. Q.Z. completed the phenotyping of the mouse bones and contributed to the histomorphometry analysis of the mouse femurs and to the CAIA study. Z.D. performed the in vivo studies in rats and analyzed the results with J.B. and H.T.C. M.K. completed the pilot experiments on human and mouse osteoclasts. J.C.S. contributed to the in vivo experiments in

mice. J.H.K., J.H.D.B., and G.R.W. performed ELISA for bone turnover markers. P.K.S. analyzed RNA-seq studies. J.R.G. completed the Ca²⁺ flux studies. L.G. performed the microarrays on rat macrophages. J.E.M. provided the *Kcnn4*^{-/-} mice. A.H. performed microarray experiments of rat alveolar macrophages and human osteoclasts. J.D. performed compound inhibition studies in human multinucleate giant cells. J.Z. performed microCT analysis. D.S. performed the CAIA study. A.K.B. performed expression analysis of mouse osteoclasts. J.B., E.P., and A.V. coordinated the project and wrote the manuscript with contributions from W.C., H.K., T.J.A., H.T.C., J.E.M., J.H.D.B., G.R.W., and J.L.

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Figure 6. *Kcnn4* Regulates Ca²⁺-NFATc1 Signaling in Multinucleate Macrophages

(A) Ca²⁺ oscillations were recorded by intracellular Ca²⁺ imaging using fura-2 in multinucleate osteoclasts from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice. Fluorescence images of cells is shown on the left panel, whereas traces of change in fura-2 fluorescence ratio in single cells treated with M-CSF only or M-CSF and RANKL for 72 hr are shown on the right panel. Note the decrease in Ca²⁺ oscillations associated with *Kcnn4* deficiency, and the absence of Ca²⁺ oscillations in the presence of M-CSF alone, independent of *Kcnn4*. These experiments were repeated multiple times with similar results, and one representative result is shown. Original bars, 50 μ m.

(B) Representative current responses to increasing voltage steps (–100 mV to +80 mV; 400 ms) observed in mouse BMDMs from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice treated with M-CSF (25 ng/ml) and RANKL (40 ng/ml) for 5 days (left panel). I–V plot demonstrates the average current/voltage relationship observed (right panel). Data points for *Kcnn4*^{-/-} and *Kcnn4*^{+/+} macrophages treated with the *Kcnn4* inhibitor ICA-17043 (1 μ M) were statistically different from *Kcnn4*^{+/+} in the range of –20 mV to +80 mV (mean $\pm$ SD, * $p < 0.05$; $n = 10$), with the exception of *Kcnn4*^{+/+} + ICA-17043 at +60 mV (mean $\pm$ SD, $p = 0.06$; $n = 10$) and +80 (mean $\pm$ SD, $p = 0.11$; $n = 10$). There was no significant difference between *Kcnn4*^{-/-} macrophages and *Kcnn4*^{+/+} treated with ICA-17043.

(C) NFATc1 protein levels in BMDMs from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice. BMDMs isolated from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice were cultured in the presence of M-CSF (25 ng/ml) and RANKL (40 ng/ml) and subjected to western blot analysis at the indicated times using antibodies directed against NFATc1. GAPDH served as an internal control for equal loading of proteins on a SDS-PAGE protein gel. Note the lower abundance of NFATc1 in *Kcnn4*^{-/-} macrophages compared with *Kcnn4*^{+/+} cells. This figure is representative of several experiments performed with similar results.

(D) BMDMs isolated from *Kcnn4*^{-/-} and *Kcnn4*^{+/+} mice were treated with M-CSF (25 ng/ml) alone or supplemented with RANKL (40 ng/ml) for 5 days and subjected to immunocytochemistry using anti-NFATc1 antibody. Note the decrease in immunoreactive NFATc1 in the nuclei of *Kcnn4*^{-/-} macrophages treated with RANKL compared with *Kcnn4*^{+/+} cells.

Original bars, 100 μ m. See also Figure S6.

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SUPPLEMENTARY INFORMATION

***Kcnn4* is a regulator of macrophage multinucleation in bone homeostasis and inflammatory disease**

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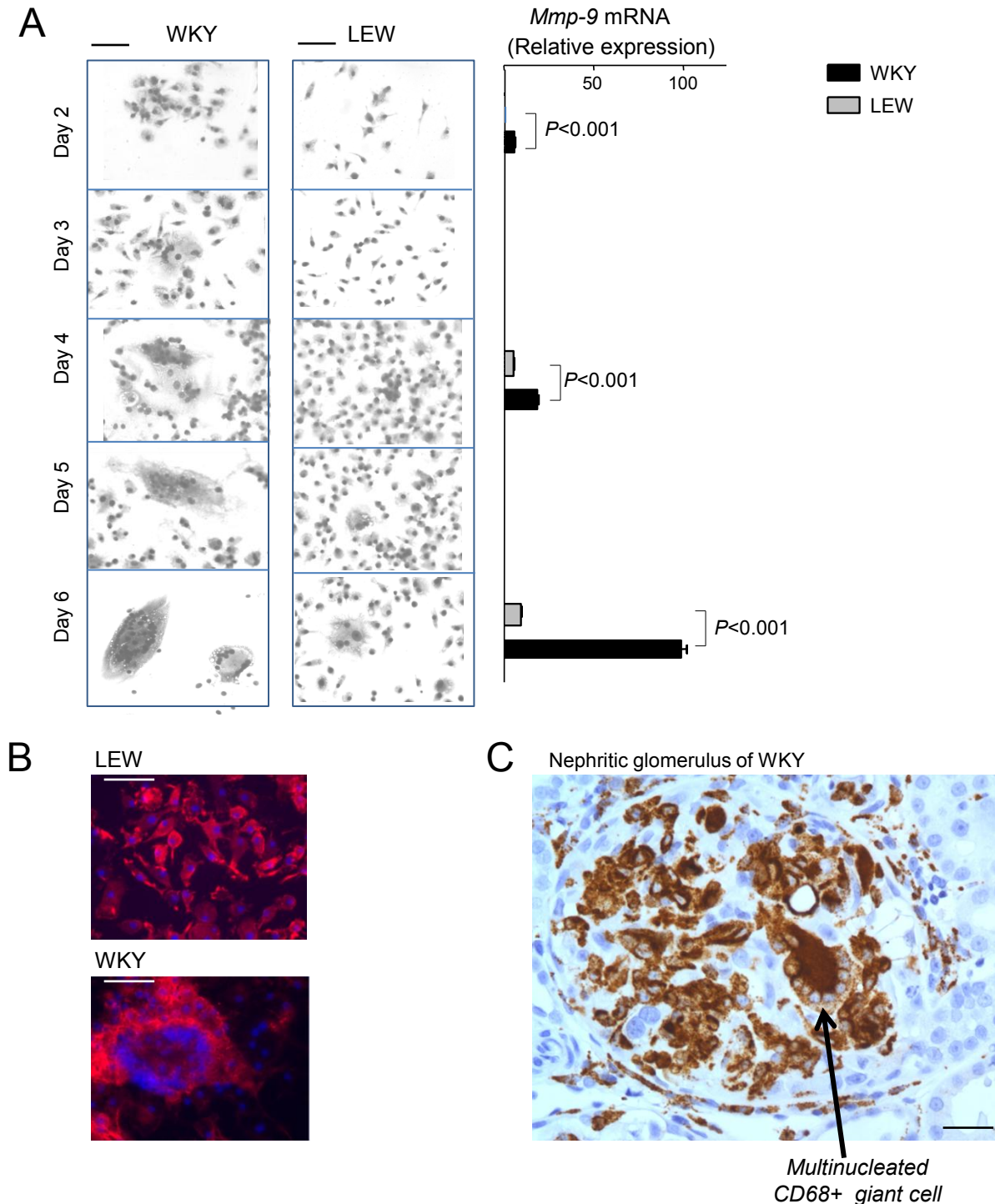
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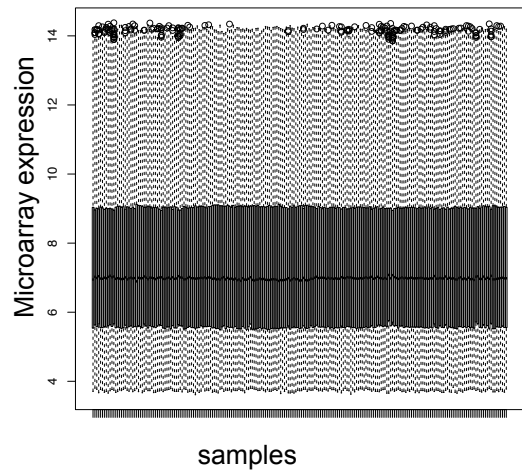
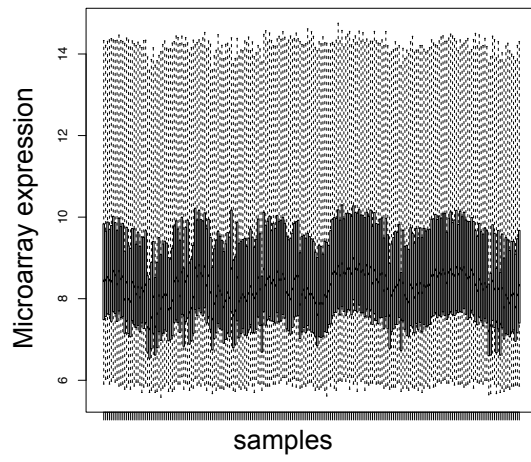
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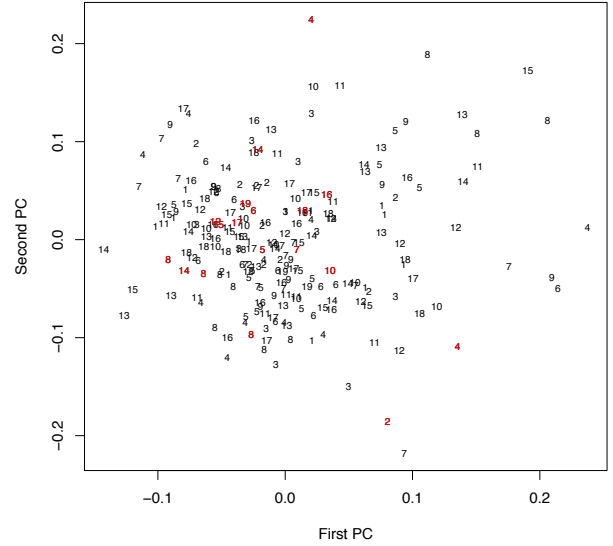
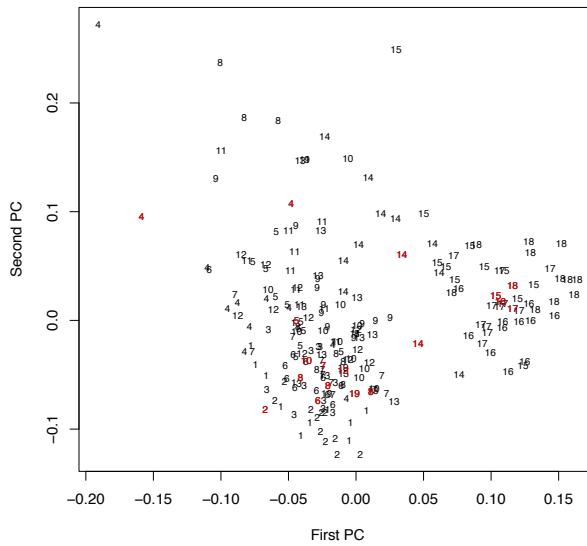
Figure S1. Related to Figure 1. WKY macrophages fuse spontaneously to form multinucleated giant cells *in vitro* and during crescentic glomerulonephritis. **(A)** BMDMs from NTN-susceptible WKY and NTN-resistant LEW rats were cultured and cells were fixed at different time points (Day 2 to Day 6). Total RNA was extracted at Day 2, Day 4 and Day 6 to assess *Mmp-9* expression as readout of macrophage giant cell formation by qRT-PCR. Original bars = 25 μ m **(B)** Actin cytoskeleton (phalloidin) in spontaneously formed multinucleated giant cells in WKY BMDMs (nuclei staining: DAPI, original bars = 50 μ m). **(C)** CD68 (ED-1) staining of a WKY glomerulus showing multinucleated ED1+ giant cells following the induction of NTN (day 10); original bars = 25 μ m. Error bars indicate SEM.



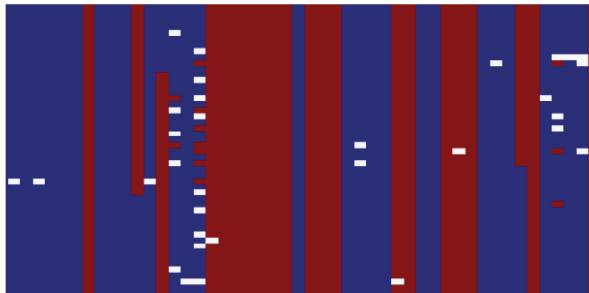
A



B



C



Blue : WKY/WKY
Red : LEW/WKY
White: missing value

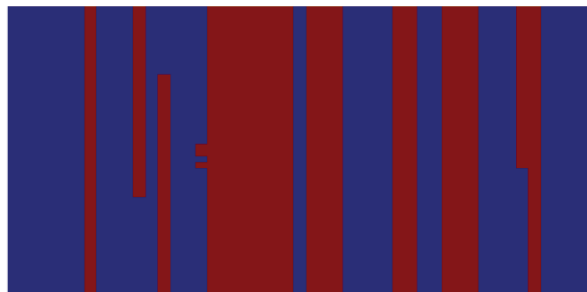
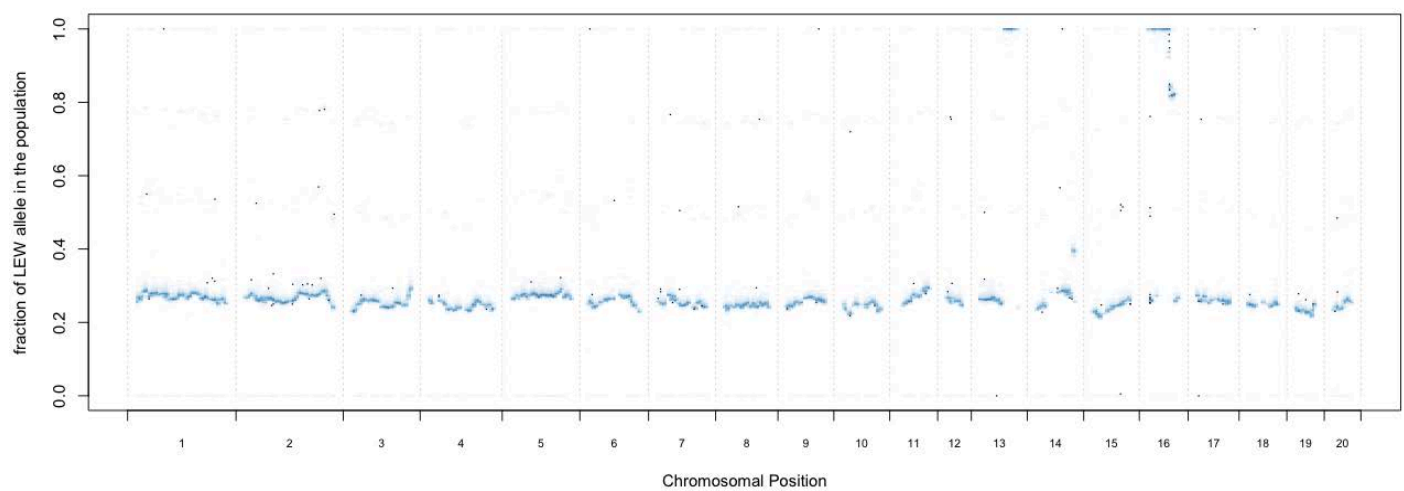


Figure S2

D



E

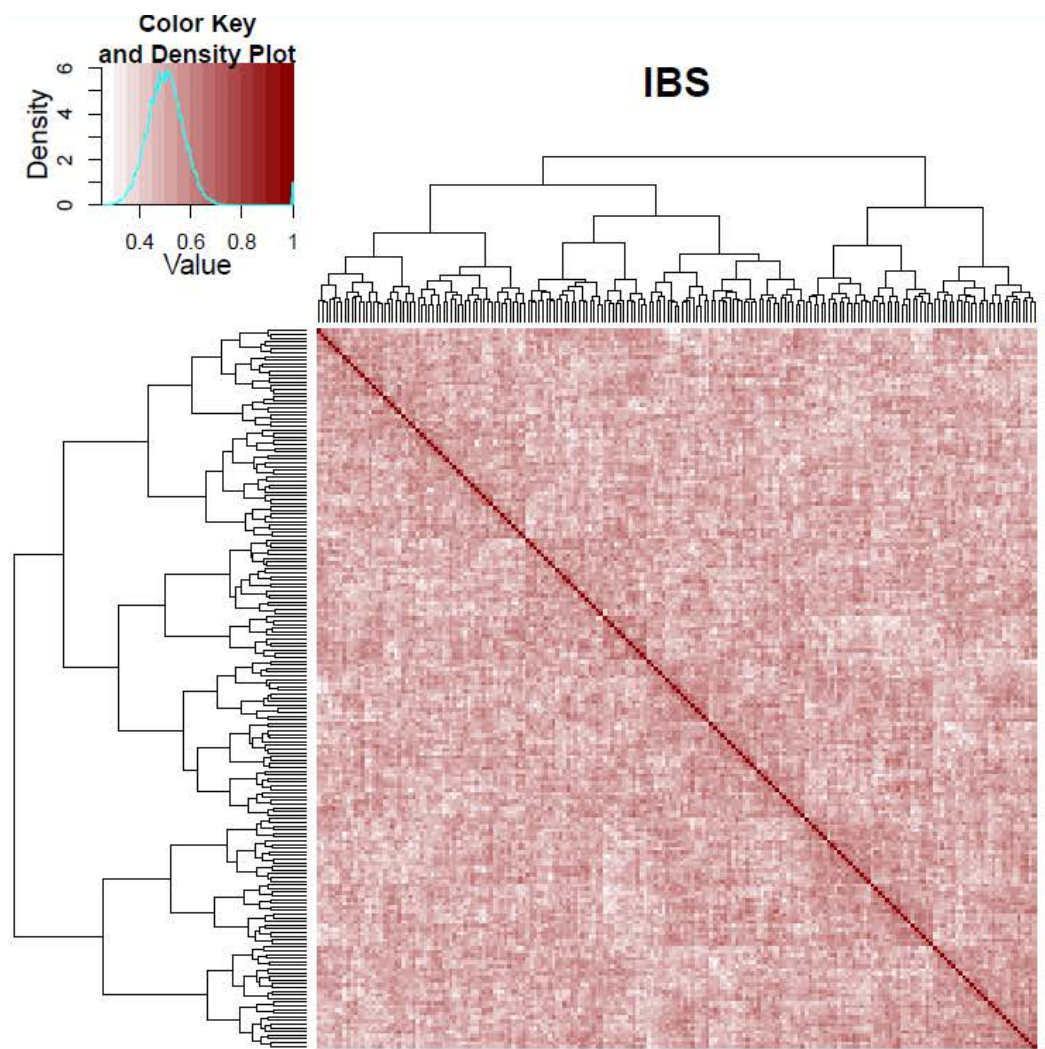
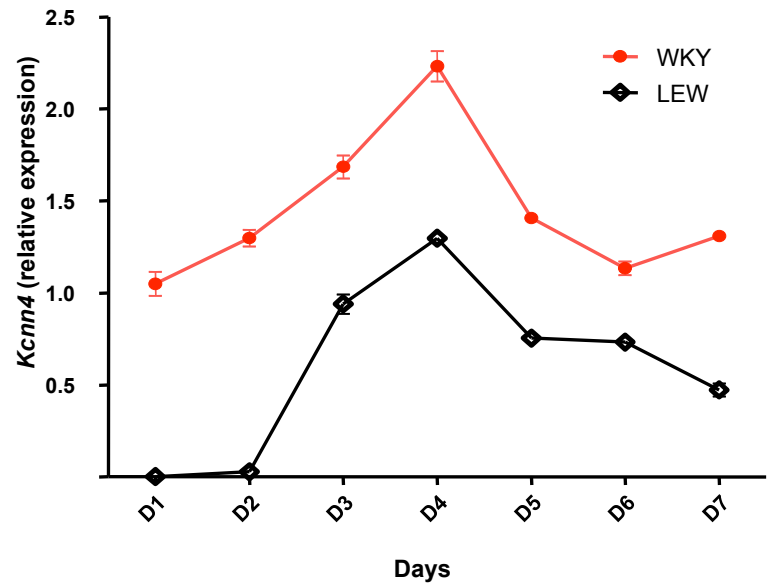


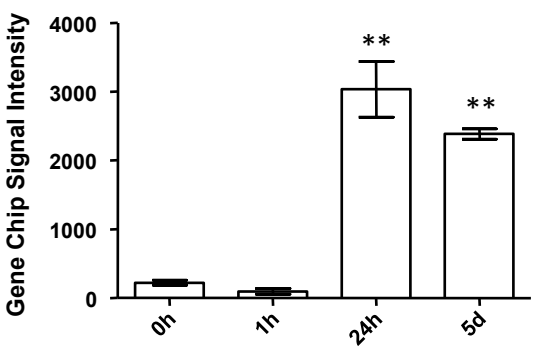
Figure S2. Related to Figure 1. eQTL analysis in the BMDMs derived from 200 back-cross rats. **(A)** Boxplots of expression data before and after normalization. Boxplots of log₂-transformed expression data before (left panel) and after (right panel) RMA normalization, respectively. Each boxplot corresponds to one sample. Samples are shown in processing order. **(B)** Position of individual BC rats on first two principal components (PC) of the genome-wide expression data, before (left panel) and after (right panel) batch-effect corrections. Each rat is designated by a number corresponding to the batch it was processed. Parental congenic rats were culled and processed together with the rest of the BC rats controls (one for each batch) and they are shown in red. **(C)** Imputation of the missing genotypes in the BC population (n=200). All three panels represent a fraction of chromosome 20 genotype data in BC rats, where each row represents a SNP and each column represents an individual BC rat. Blue and red cells represent homozygous WKY-WKY and WKY-LEW heterozygous genotypes respectively, whereas white cells indicate missing genotypes. Genotyping data before (left panel) and after imputation (right panel) are shown. Genotyping errors were corrected in the lower panel. Imputation fills each missing value with the most likely genotype according to surrounding haplotype whereas genotype correction replaces isolated genotype calls according to their haplotypic context. **(D)** Average fraction of Lewis DNA in the population across the genome. 2D density plot of LEW allele frequency in the BC population according to genomic position, based on the 278,124 SNPs where the WKY genome differs from LEW. For the largest majority of SNPs, the frequency of LEW allele is close to 25%, consistent with the chosen back-cross strategy. SNPs where the frequency differs significantly from 0.25 reflect either congenics regions (see Methods), or failure of the genotype calling algorithm (isolated dots). All these SNPs were not taken into account for the eQTL analysis. **(E)** Heatmap of Identity by Sharing (IBS) in the WKY x LEW back-cross population. The normal distribution of IBS around 0.5 is consistent with the distribution expected under the absence of population structure.

A



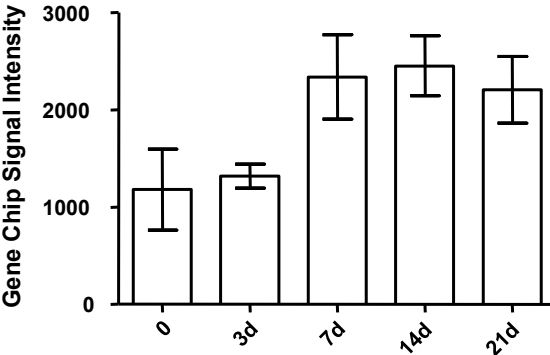
B

Rat alveolar macrophages (Microarray)

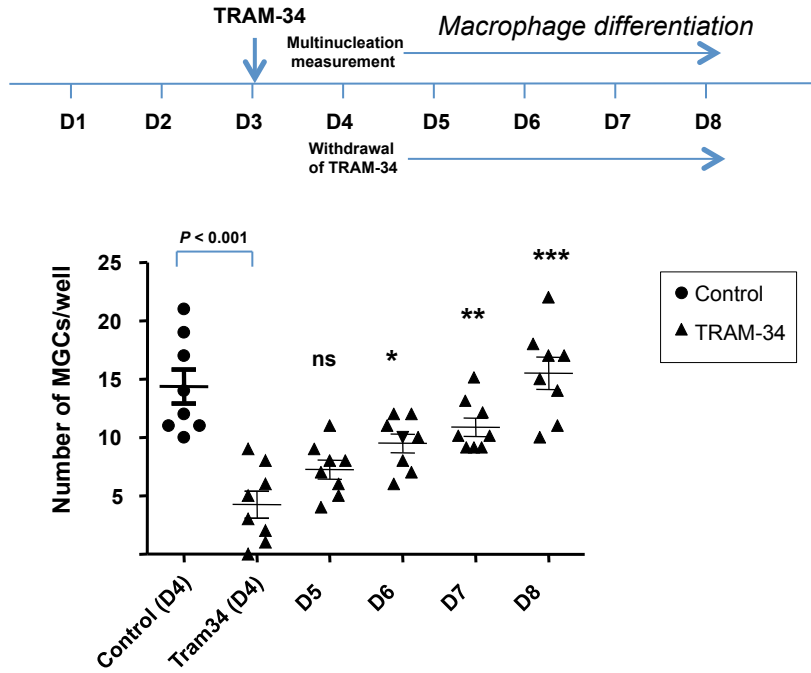


C

Human osteoclasts (Microarray)



D



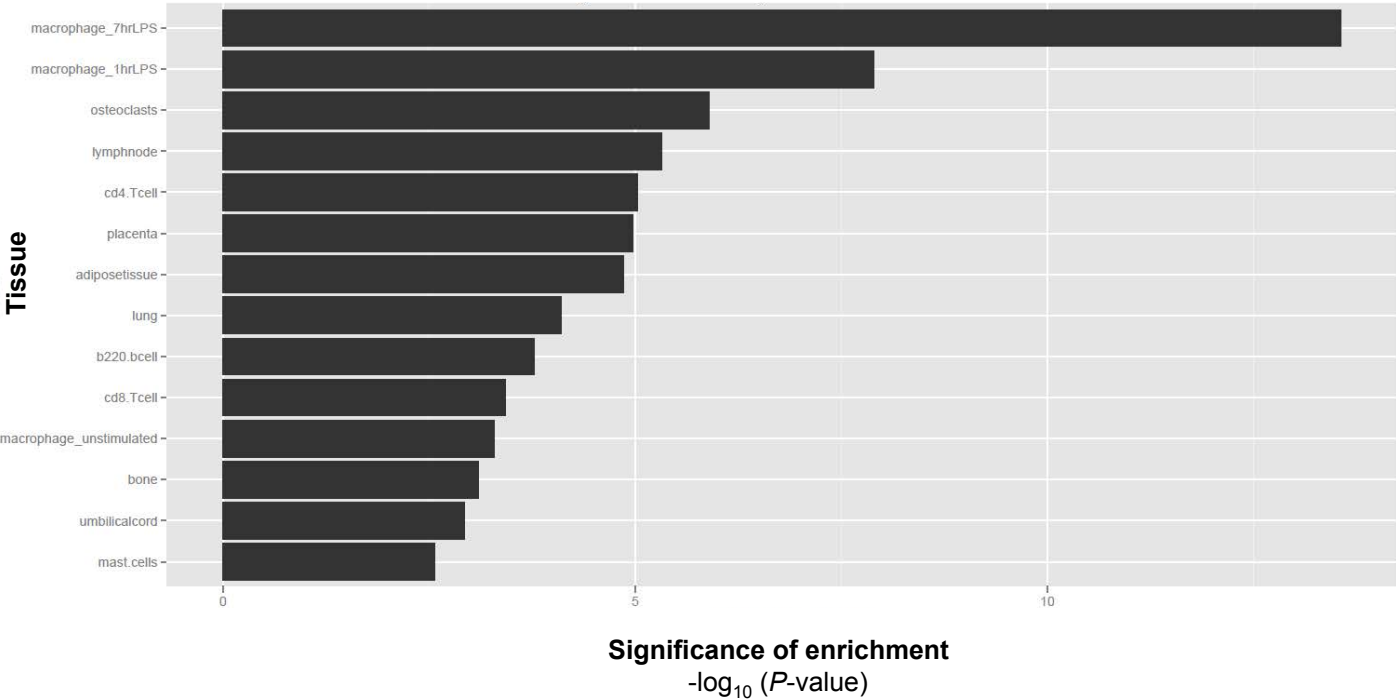
E

Differentiation (days)	D2	D3	D4	D5	D6	D7	D8
% of CD68+ cells (Control)	98	98	99	100	98	97	100
% of CD68+ cells (TRAM34)	99	100	97	100	100	98	99

F

Log(FC)	P-value	FDR	Gene symbol
-1.23	1.45×10^{-71}	2.17×10^{-67}	<i>Abcg1</i>
-1.71	2.82×10^{-71}	2.17×10^{-67}	<i>Serpinb2</i>
-1.34	2.17×10^{-57}	1.12×10^{-53}	<i>Scd</i>
-1.00	1.60×10^{-54}	6.19×10^{-51}	<i>Abca1</i>
-1.38	1.25×10^{-51}	3.86×10^{-48}	<i>Alpk2</i>
-0.92	5.57×10^{-45}	1.43×10^{-41}	<i>Irg1</i>
-0.80	1.27×10^{-43}	2.79×10^{-40}	<i>Slamf6</i>
-1.28	4.31×10^{-42}	8.32×10^{-39}	<i>P97600_RAT</i>
-1.10	5.36×10^{-42}	9.18×10^{-39}	<i>Marcks1</i>
-1.17	6.24×10^{-42}	9.63×10^{-39}	<i>Olr1</i>
-1.31	2.91×10^{-39}	4.08×10^{-36}	<i>E9PSM5_RAT</i>
-1.22	8.49×10^{-39}	1.09×10^{-35}	<i>Mfng</i>
-0.91	1.09×10^{-38}	1.29×10^{-35}	<i>Cd8a</i>
-2.47	4.61×10^{-38}	5.08×10^{-35}	<i>LOC689963</i>
-1.33	2.35×10^{-33}	2.42×10^{-30}	<i>Kit</i>
-1.02	4.74×10^{-32}	4.57×10^{-29}	<i>Nrxn2</i>
-2.46	1.94×10^{-31}	1.76×10^{-28}	<i>Ccl6</i>
-1.58	2.67×10^{-30}	2.29×10^{-27}	<i>Myliip</i>
-0.62	1.86×10^{-29}	1.51×10^{-26}	<i>Malt1</i>
-0.88	3.38×10^{-28}	2.61×10^{-25}	<i>Atp2b4</i>

G



H

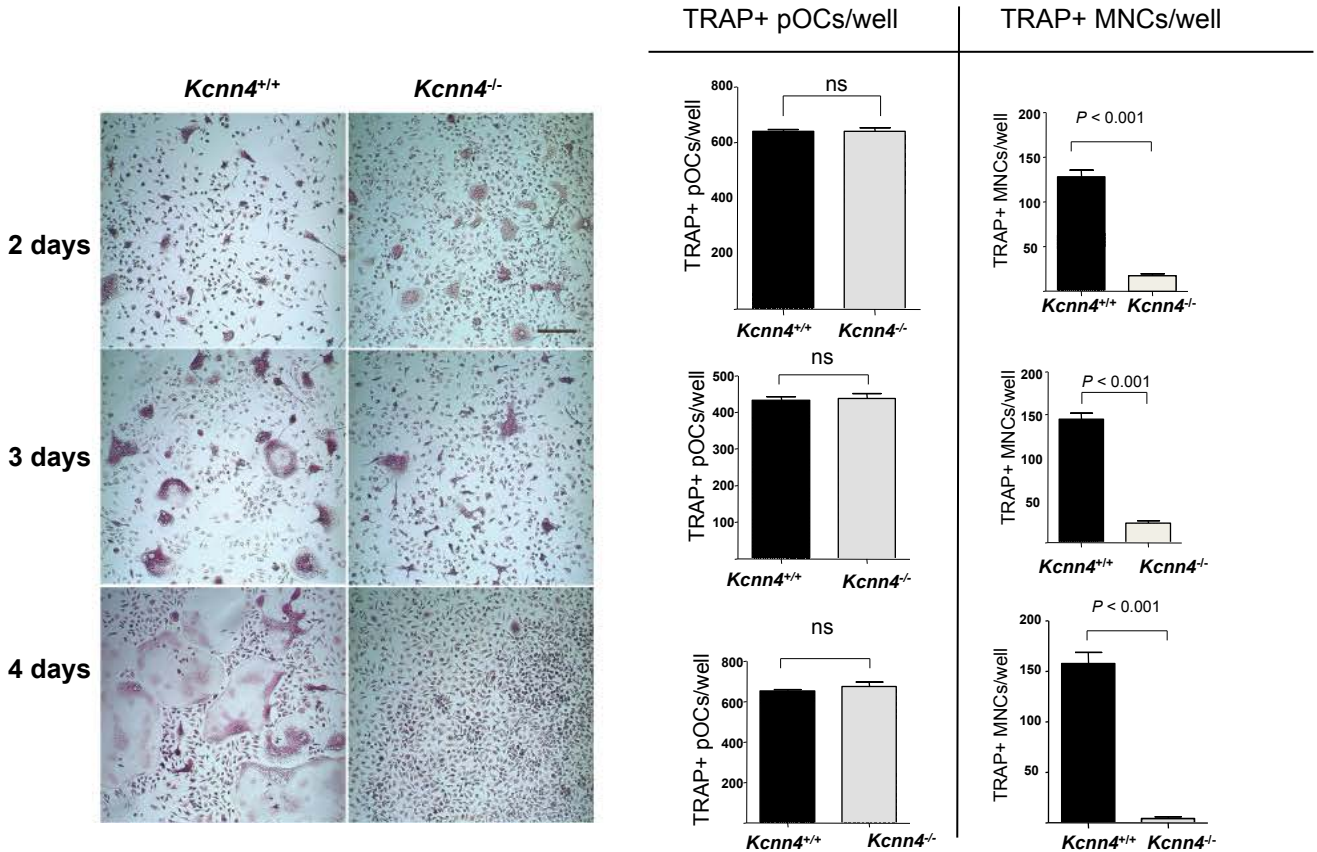
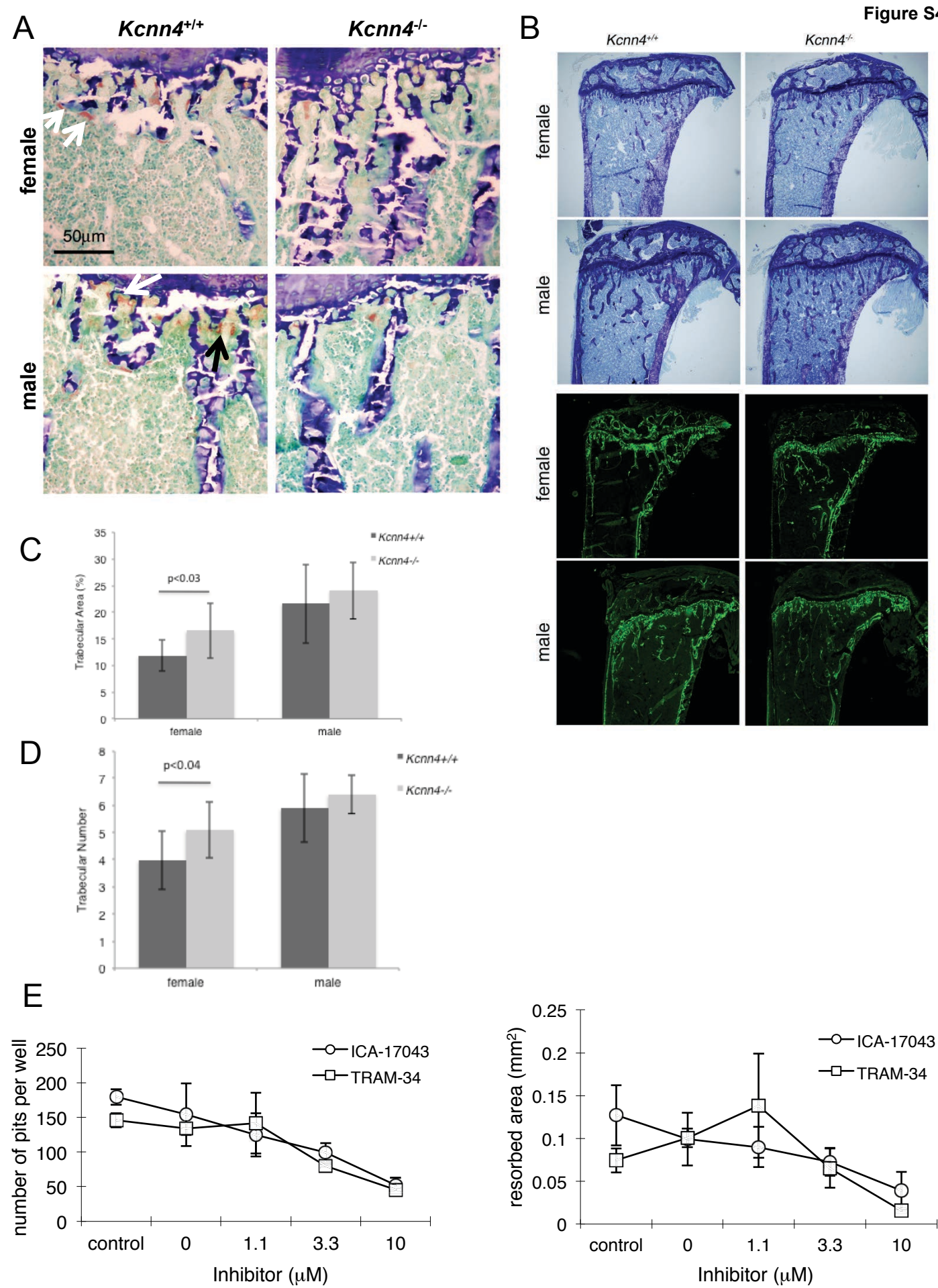


Figure S3. Related to Figure 3. *Kcnn4* expression is induced at the onset of macrophage fusion; *Kcnn4* blockade is reversible and its genetic deletion does not affect pre-osteoclast formation. **(A)** Total RNA was extracted at Day 1 until Day 7 to assess *Kcnn4* expression by qRT-PCR in progressively multinucleating WKY BMDMs. Note the peak of *Kcnn4* expression at day 4 in both strains and the relative increase in the fusion-competent WKY BMDMs. Relative expression values were normalised to *Hprt* expression. Error bars indicate SEM. **(B)** Total RNA was isolated from fusing rat alveolar macrophages (n=4) and subjected to Affymetrix Genechip analysis at different time points (0h, 1h, 24h, 5 days). Mean signal probe-set intensities are shown for rat *Kcnn4*. **(C)** Human monocyte-derived macrophages were cultured in the presence of M-CSF (20 ng/ml) and RANKL (10 ng/ml) for the indicated times to induce osteoclast differentiation and subjected to Affymetrix Genechip analysis. Mean signal probe-set intensities are shown for human *Kcnn4*. **, $P < 0.001$ when compared to 0h; error bars indicate SEM.

Figure S3. Related to Figure 3. (D) Bone marrow-derived macrophages from WKY rats were cultured at indicated times and incubated with TRAM-34 (10 μ M) at day 3 (D3) where multinucleation occurs. TRAM-34 was then withdrawn from the culture media and the number of MGCs/well were determined as the number of cells containing > 10 nuclei at day 4 onwards. The lower panel shows the quantification of MGCs from D4. ns, non significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ when compared with TRAM-34 (D4). **(E)** % of CD68⁺ cells quantified by counting ED-1⁺ (rat CD68) cells during macrophage differentiation in control and media supplemented with TRAM-34. **(F)** RNA-seq analysis following selective pharmacological blockade of *Kcnn4*. The table shows the top 20 differentially expressed genes between WKY BMDMs treated with TRAM-34 (10 μ M) when compared with untreated ones. FDR, False Discovery Rate; FC, fold change. **(G)** Tissue enrichment analysis of all the differentially expressed genes between control and TRAM-34 treated cells. **(H)** Bone marrow-derived macrophages from 6-week old *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice were cultured in the presence of M-CSF (25 ng/ml) and RANKL (20 ng/ml) for 2, 3 and 4 days to induce the differentiation of osteoclasts (Bar =100 μ m, left panel). TRAP⁺ mononuclear cells (pre-osteoclasts) were quantified at each day during differentiation and the number of TRAP⁺ pre-osteoclasts/well reported (TRAP + pOCs/well). When the fusion outcome was measured as the number of large TRAP⁺ multinucleated cells (TRAP⁺ MNCs/well), there was a marked reduction in these cells in *Kcnn4*^{-/-} mice at all considered time points. ns, non-significant. Error bars indicate SEM.



F



G

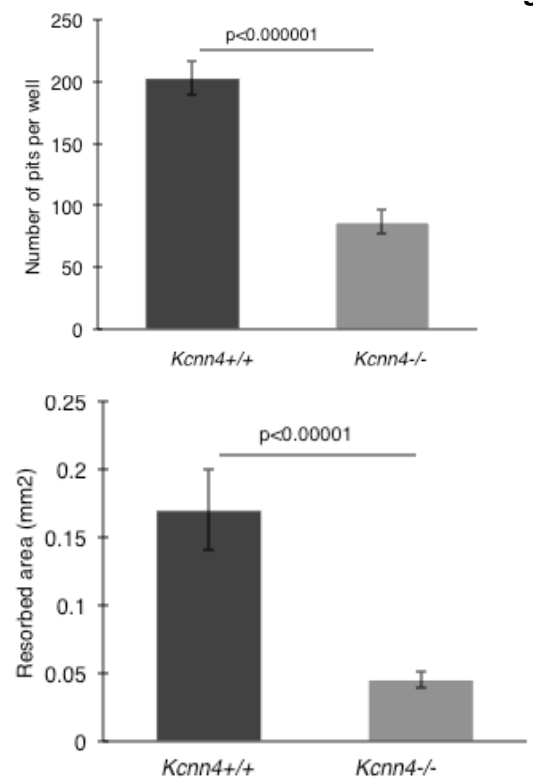


Figure S4. Related to Figure 4. *Kcnn4* affects osteoclast formation and activity. (A) Representative TRAP staining images of proximal tibiae from 8-week old *Kcnn4*^{+/+} and *Kcnn4*^{-/-} female and male mice. Note the reduced TRAP⁺ osteoclasts (in red) in male and female *Kcnn4*^{-/-} mice tibiae. Arrows show the TRAP⁺ red staining in *Kcnn4*^{+/+} mice. Original bar = 50μm. (B) Representative images of tibial bone sections for histomorphometry (static, upper two panels; dynamic, lower two panels; 8-week-old *Kcnn4*^{+/+} and *Kcnn4*^{-/-} female and male mice). (C-D) Histomorphometry analysis of Trabecular Area (C) and Trabecular Number (D). Data are reported as mean±SD (n=10 mice). (E) Mouse bone-marrow-derived macrophages were cultured in the presence of M-CSF (20 ng/ml) and RANKL (40 ng/ml) on calcium phosphate substrate coated chambers and treated with *Kcnn4* inhibitors, TRAM-34 or ICA-17043. Graphs show quantitation data for the number of resorption pits per well (left panel) and the resorbed area per well (right panel). Error bars indicate SD. (F, G) Bone-marrow-derived macrophages from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice were cultured in the presence of M-CSF (20 ng/ml) and RANKL (40 ng/ml) on calcium phosphate substrate coated chambers. (F) Representative images displaying resorption pits by osteoclasts. (G) Quantitation data for the number of resorption pits (upper) and the resorbed area per well (lower). Error bars indicate SD.

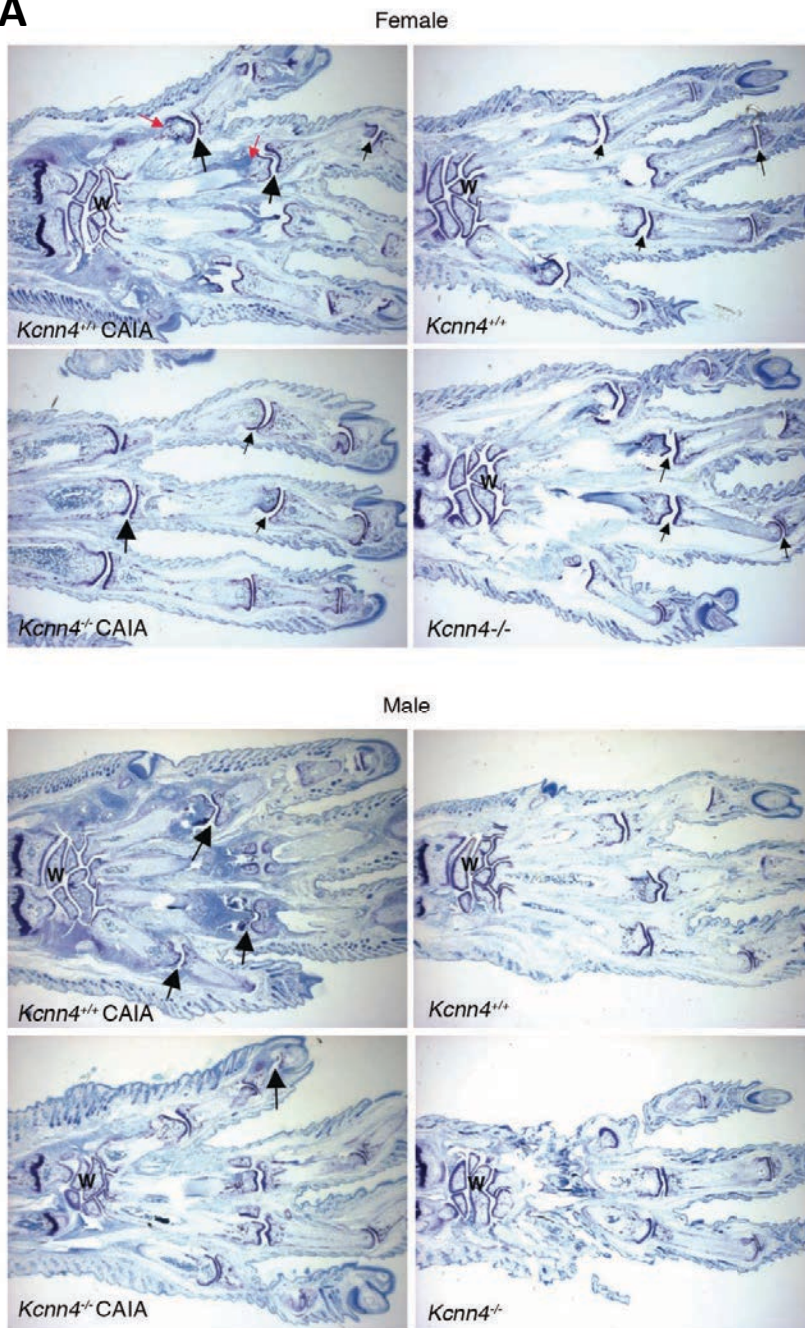
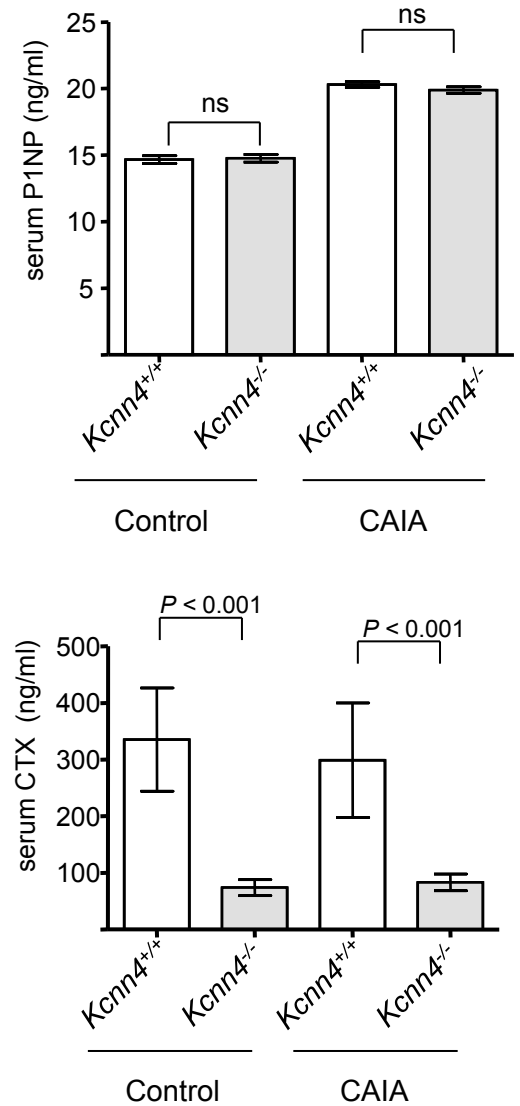
A**B**

Figure S5. Related to Figure S5. Deletion of *Kcnn4* reduces anti-collagen antibody-induced bone erosion in chronic inflammatory arthritis. **(A)** Representative micrographs of the fore paws from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} female (upper panel) and male (lower panel) naïve and CAIA-induced mice. Small arrows point out representative normal joints. Red arrows point out areas of subchondral bone resorption. W = wrist. Note the severe inflammation and cartilage damage with moderate pannus and bone resorption in joints of CAIA-induced *Kcnn4*^{+/+} mice. This is in contrast with naïve and CAIA-induced *Kcnn4*^{-/-} mice, which show no and minimal inflammation and cartilage damage, respectively. **(B)** Measurement of serum markers of bone formation (P1NP) and bone resorption (CTX) in control (naïve) and following CAIA induction in *Kcnn4*^{+/+} and *Kcnn4*^{-/-} animals. At least n=8 mice were used in each group. Error bars indicate SEM.

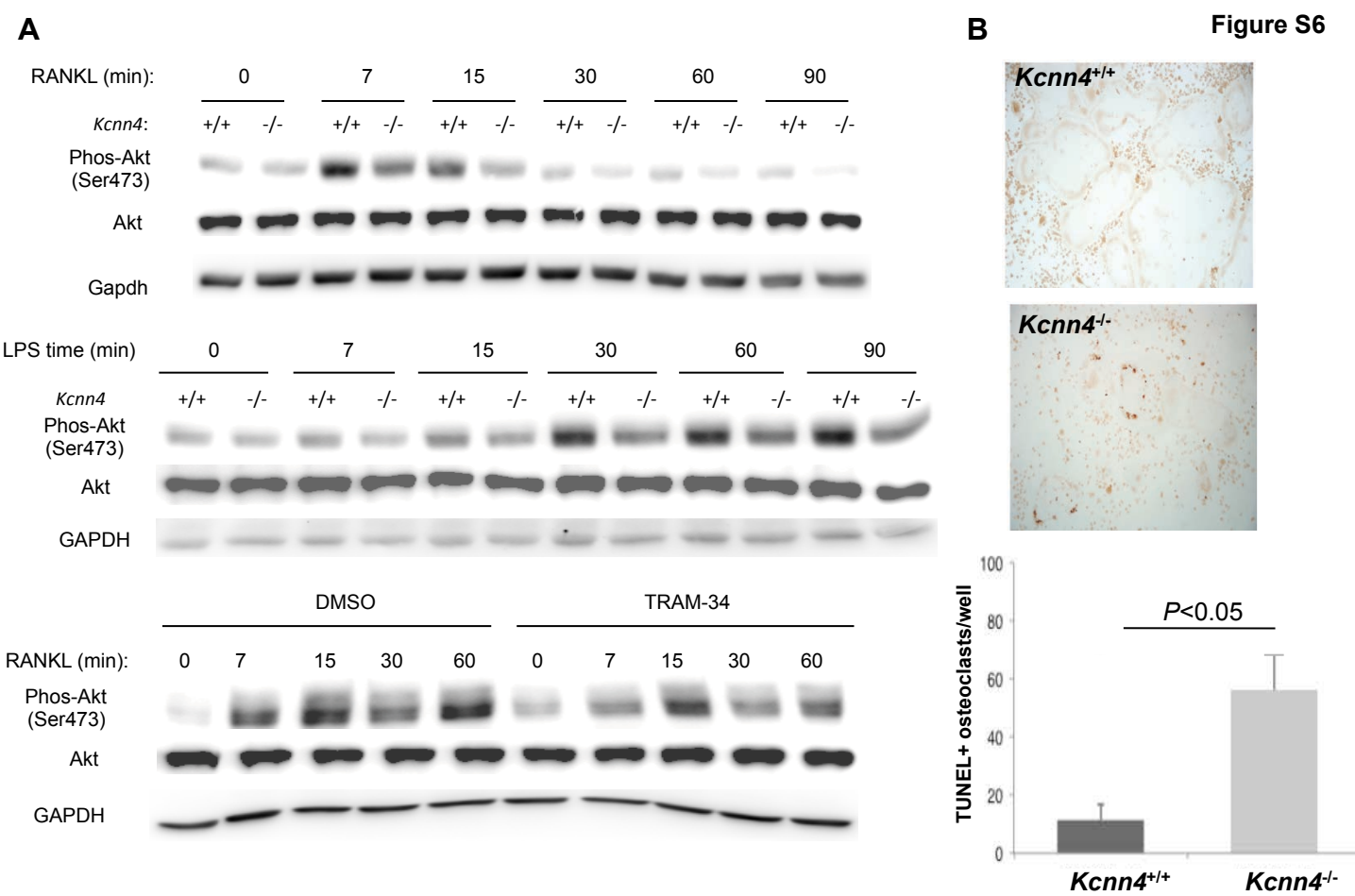


Figure S6. Related to Figure 6. AKT activation in the absence of *Kcnn4* in macrophages and the effect of *Kcnn4* deficiency on apoptosis in osteoclasts. **(A)** Western Blot analysis of BMDMs isolated from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice were cultured in the presence of M-CSF (25 ng/ml) and RANKL (40 ng/ml) for 4 days, then cultured in the presence of M-CSF alone, with or without RANKL (40 ng/ml) or LPS (100 ng/ml) for the indicated times. Note the decrease in Akt (Ser473) phosphorylation in *Kcnn4*^{-/-} macrophages treated with RANKL when compared with *Kcnn4*^{+/+}. Human monocyte-derived macrophages were pre-treated with TRAM-34 (10 mM) for 40 minutes and then stimulated with RANKL (100 ng/ml) for the indicated times. Similarly TRAM-34 prevented activation of Akt in human osteoclasts. These experiments were repeated 3 times with similar results and representative blots are shown. **(B)** Bone marrow derived macrophages from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice were cultured in presence of M-CSF (25ng/ml) and RANKL (10ng/ml) for 4 days to induce the differentiation of osteoclasts. Cell were then incubated in serum and ligand (M-CSF/RANKL)-free media for 12 hours to induce apoptosis. TUNEL positive (dark brown colour) is observed in the nuclei of multinucleated osteoclasts that are undergoing apoptosis (upper panel). Following quantification, osteoclasts from *Kcnn4*^{-/-} mice contained significantly more TUNEL-positive nuclei when compared to osteoclasts from *Kcnn4*^{+/+} mice (lower panel). Error bars indicate SD.

Table S1. Related to Figure 4. Histomorphometric analysis of proximal tibiae from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice. See extended protocols for the detailed description of all parameters.

[illegible]

Extended experimental procedures

Reagents

Rabbit polyclonal antibodies directed against p-38, phosphorylated-p38 (Thr180/Tyr182), p42/44MAPK, phosphorylated -p42/44MAPK (Thr202/Tyr204), Akt, phosphorylated -Akt (Ser473), CREB, phosphorylated -CREB (Ser133) were obtained from Cell Signaling Technology (Beverly, MA). A rabbit polyclonal antibody directed against the intracellular domain of MFR was published previously (Han et al., 2000). A rabbit polyclonal antibody directed against c-Fos, and a mouse monoclonal antibody directed against NFATc1 were purchased from Santa Cruz (Santa Cruz, CA). Rat monoclonal antibodies directed against mouse CD200 and CD44 were purchased from AbD Serotec (Kidlington, UK) and BD Biosciences (San Diego, CA), respectively. Mouse anti-GAPDH was purchased from Ambion (Austin, Texas). Horseradish peroxidase-conjugated F(ab')₂ from goats directed against rabbit, rat and mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA). Phalloidin-Alexa fluor 568 and Tropo-3 was purchased from Invitrogen (Carlsbad, CA) and Osteologic slides from BD (Franklin Lakes, NJ). All supplies and reagents for tissue culture were endotoxin-free. Unless otherwise stated, all chemicals were from Sigma Chemical Co. (St Louis, MO). The Kcnn4 inhibitors, TRAM-34 and ICA-17043, were dissolved in 100% DMSO, and the final concentration of DMSO in culture was 0.1%, independent of the concentration of the inhibitors.

Genotype cleaning and imputation

The distribution of haplotype length shows a great overrepresentation of single SNP haplotypes. This overrepresentation can be attributed to genotyping errors that occur randomly in an independent manner. Thus isolated single SNP haplotype are likely to be caused by genotyping errors rather than true recombination events. Therefore, we reversed the genotype calling for all single SNP haplotype surrounded by 2 haplotypes of length

greater than 2 or for all cluster of several single SNP haplotype separated by one SNP each and surrounded by 2 haplotypes of length greater than 2. An example of the genotype Imputation and cleaning process can be seen of **Figure S2** (panel C).

The WKY and LEW whole genome sequence data (Illumina HiSeq 2000; at least 10X coverage for both strains as described in (Atanur et al., 2013)) was then used to define the WKY and LEW allele at each individual SNP. SNPs where the WKY and LEW alleles could not be determined from the sequencing data were discarded resulting in 278,124 SNPs that can discriminate both alleles. Frequency of the WKY and LEW alleles across the whole genome can be seen in **Figure S2** (panel D). SNPs where the BRLMM clustering was not consistent with the breeding strategy (Heterozygous frequency < 0.35 or > 0.65 , presence of Lewis homozygous genotypes) were discarded leading to a final number of 242,252 SNPs. Missing genotypes were then imputed using fastPhase with the 2 founder haplotypes (**Figure S2**). The total number of SNPs for eQTL analysis were 1,974 following removal of those in complete linkage disequilibrium ($r^2=1$). The distribution of Identity by Sharing (IBS) was verified in all BC animals and confirmed the absence of any population structure (shown in **Figure S2**, panel E).

Primary macrophage, osteoclast cultures and assessment of cell multinucleation

Rat bone marrow derived macrophages (BMDMs) were cultured as previously described (Behmoaras et al., 2010). BMDMs were flushed from femur and tibia bones from rats and cultured in presence of L929-conditioned media for 4 days. To assess spontaneous MGC formation, macrophages were first dissociated using non enzymatic cell dissociation buffer (Sigma) and re-plated in Lab-Tek chambers (Fisher Scientific, UK). MGCs were then fixed at day 5 using Reastain Quick Diff and MGC quantification was performed by counting the number of nuclei in 100 macrophages using light microscopy. In some experiments TRAM-34 (10 μ M, Tocris Bioscience, UK) was added to the cell culture during macrophage

differentiation to assess the effect of *Kcnn4* blockade on macrophage multinucleation and MGC formation. Bone marrow-derived osteoclasts were generated from four- to twelve-week old *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice as previously described (Cui et al., 2007). Bone marrow cells were isolated, mechanically dissociated and cultured at a density of 1.33×10^6 cells per cm² in a-MEM containing 10% heat inactivated FBS, 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA), 1% MEM vitamins, 1 mM glutamine, and 5 to 30 ng/ml M-CSF (R&D, Minneapolis, MN). RANKL (R&D) was added at a concentration of 7 or 40 ng/ml. Culture medium was refreshed every three days. At the end of the culture, cells were fixed and reacted for tartrate-resistant acid phosphatase according to the supplier's directions. TRAP-positive cells with 3 nuclei or more were counted as osteoclasts as previously described (Li et al., 2005). Human monocyte derived macrophages were differentiated from buffy coats from healthy donors using gradient separation (Histopaque 1077, Sigma) and adhesion purification. Following Histopaque separation, peripheral blood mononuclear cells were re-suspended in RPMI (Life Technologies) and monocytes were purified by adherence for 1h at 37°C, 5% CO₂. The monolayer was washed 6 times with HBSS to remove non-adherent cells and monocytes were matured for 5 days in RPMI containing 100 ng/ml M-CSF (PeproTech, UK). Macrophage purity was confirmed by immunohistochemical assessment of CD68 and > 99% cells were CD68+. In some experiments human peripheral blood monocytes were obtained from AllCells LLC (Emeryville, CA). Human osteoclasts were generated from normal peripheral blood monocytes or positively selected normal peripheral blood CD14+ cells (AllCells, Emeryville, CA). Peripheral blood monocytes were isolated using Ficoll-Paque and cultured at a concentration of 3×10^6 cells/ml in low glucose DMEM supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, NC), recombinant human M-CSF (20 ng/ml) and recombinant human RANKL (10 ng/ml). The medium was refreshed twice a week. At the end of the culture, the cells were fixed and reacted for tartrate-

resistant acid phosphatase according to the supplier's directions. TRAP-positive cells with 3 nuclei or more were counted as osteoclasts.

eQTL analysis, network inference and cell type enrichment analysis

A transcript was designated as an eQTL when it mapped with a probability of 0.8 to a genomic region of less than 10 Mb. When multiple SNPs are located within the 10 Mb window, we referred to the SNP with the best marginal probability in that region as the associated SNP. We defined eQTL hotspots as SNPs linked to a significant over-representation of *trans* eQTLs by assuming that each of the 1071 *trans* eQTLs is associated at random to one of the 1974 haplotype tagging SNPs. The number of transcripts associated to a SNP follows a binomial distribution with parameters (1071, 1/1974). The Bonferroni-corrected 95% upper limit of the expected number of associated transcripts at each position, is then obtained by taking the α^{th} upper quantile of the distribution with $\alpha = 0.05/1974$. A *trans* eQTL hotspot is defined by any SNP associated to more than 5 transcripts. eQTL hotspots located in the same LD block ($r^2 > 0.8$) were grouped and the *trans* cluster is defined as the whole set of transcripts mapping in *trans* to the LD block.

The network shown in Figure 1C was built by first extracting gene expression from the 184 genes of the network (190 transcripts) and adjusting the gene expression for the effect of *cis*-regulatory SNPs. R package *minet* was used to compute the mutual information between all pairs of gene as well as between all pair of genes and the chromosome 9 locus SNP, and ARACNE (Basso et al., 2005) was used to remove indirect edges from the network. Cell type enrichment analysis was performed using the Mouse GNF1M Gene Atlas data (GSE1133), containing expression from 78 tissues and cell types. Log transformed normalized expression was used and for each gene g and cell type c , a Z-score was computed to measure the relative overexpression of gene g in cell type c compared to other cell types. Each Z-score was then assigned a P -value for significance of the overexpression assuming normal distribution of the

Z-scores. Finally, for each cell type, enrichment of the network was computed by counting the number of genes with a significant *P*-values at a marginal 0.05 level, inside and outside of the network and using an hypergeometric test for enrichment.

LPS-induced calvarial bone resorption assay

To assess osteoclast formation and activity *in vivo*, we administered a single local subperiosteal calvarial injection of LPS (25 µg in 2 µl; Escherichia coli O55:B5, Sigma Chemical, St. Louis, MO) to 8-week-old male and female wild type and *Kcnn4*^{-/-} mice that were sacrificed 5 days later. Because the angle of the needle is kept fixed during the injection, the osteoclast pits tend to remain in the vicinity of the injected site. Animals were checked daily for the five-day duration of the experiment, including weekends and holidays, when applicable. Those animals showing evidence of pain, were administered analgesics based on consultation with a veterinarian. Calvariae were subjected to microCT analysis, and resorbed areas were quantified using ImageJ software (NIH, Bethesda, MD). All procedures were approved by the Yale University Institutional Animal Care and Use Committee.

Measurement of bone turnover markers

Serum samples were obtained from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice at sacrifice in control and following CAIA and were stored at -80 °C. N-terminal propeptide of type 1 procollagen (P1NP) and C-terminal cross-linked telopeptide of type I collagen (CTX) levels were determined by enzyme immunoassay using IDS kits AC-33F1 and AC-06F1, respectively (Immunodiagnostic Systems).

RNA-seq library preparation and data analysis in primary macrophages

Total RNA was extracted from WKY macrophages (+/- TRAM-34, 10µM) using Trizol (Invitrogen) according to manufacturer's instructions with an additional purification step by on-column DNase treatment using the RNase-free DNase Kit (Qiagen) to ensure elimination of any genomic DNA. The integrity and quantity of total RNA was determined using a

NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies). 1 μ g of total RNA was used to generate RNA-seq libraries using TruSeq RNA sample preparation kit (Illumina) according to the manufacturer's instructions. Briefly, RNA was purified and fragmented using poly-T oligo-attached magnetic beads using two rounds of purification followed by the first and second cDNA strand synthesis. Next, cDNA 3' ends were adenylated and adapters ligated followed by 10 cycles of library amplification. Finally, the libraries were size selected using AMPure XP Beads (Beckman Coulter) purified and their quality was checked using Agilent 2100 Bioanalyzer. Samples were randomized to avoid batch effects and libraries were run on a single lane per sample of the HiSeq 2000 platform (Illumina) to generate 75bp paired-end reads. RNA-seq reads were then aligned to the rn4 reference genome using tophat 2 and non-uniquely aligned reads were discarded leading to coverage of ~40M reads and 35M uniquely mapped reads. Gene level read counts were computed using HT-Seq and genes with less than 10 aligned reads across all samples were discarded prior to analysis leading to 15,155 genes. Differential expression analyses between the two groups were carried out using edgeR and using a 5% FDR to call significant differentially expressed genes. Cell type enrichment analysis was performed using the Mouse GNF1M Gene Atlas data (GSE1133), containing expression from 78 tissues and cell types. Log transformed normalized expression was used and for each gene g and cell type c , a Z-score was computed to measure the relative overexpression of gene g in cell type c compared to other cell types. Each Z-score was then assigned a P -value for significance of the over-expression assuming normal distribution of the Z-scores. For each cell type, enrichment of the set of differentially expressed genes was computed by counting the number of genes with significant P -values at a marginal 0.05 level, inside and outside of the set of differentially expressed genes and using an hypergeometric test for enrichment.

siRNA and qRT-PCR

On day 5 of culture, WKY BMDMs were replated in six-well plates (1×10^6 cells per well) in DMEM (Invitrogen) overnight and transfected for 48 hours with siGENOME SMARTpool for either human or rat *Trem2*, and rat *Trem11*, *Trem1*, *D3ZDX3_Rat* (100 nM, Dharmacon) or siGENOME non-targeting siRNA pool as the scrambled control siRNA using Dharmafect 1 (1:50, Dharmacon) as a transfection reagent in OPTIMEM medium (Invitrogen). For human monocytes-derived macrophages (MDMs), cells were incubated on day 5 of culture with either human *TREM2* siRNA or siGENOME non-targeting siRNA pool. The siRNA sequences used in the siGENOME SMARTpool for all transcripts are available upon request. All qRT-PCRs were performed with an ABI 7900 Sequence Detection System (Applied Biosystems, Warrington, UK). A two-step protocol was used beginning with cDNA synthesis with iScript select (Bio-Rad) followed by PCR using SYBR Green Jumpstart Taq Ready Mix (Sigma). A total of 10ng of cDNA per sample was used. All samples were amplified using a set of 4 biological replicates with three technical replicates used per sample in the PCR analysis. Sequence detection software (SDS) version (Applied Biosystems) was used to obtain the Ct values. Results were analysed using the comparative Ct method and each sample was normalised to the reference gene *Hprt*, to account for any cDNA loading differences. The primer sequences are available upon request.

Histomorphometric analysis of the bone

Histomorphometry was performed 0.25 mm proximal to the growth plate using Osteomeasure software (Osteometrics, Atlanta, GA). The following parameters were measured: the relative tissue surface occupied by bone (B.Ar/T.Ar; %); the number of trabeculae per mm (Tb.N/mm); the relative surface of bone occupied by trabeculae (Tb.Ar; %); the distance/separation between trabeculae (μm); the number of osteoclasts per total bone surface (Oc/T.Ar); the perimeter of osteoclasts per total bone perimeter (Oc.Pm./B.Pm); the perimeter of osteoblasts

per total bone perimeter (Ob.Pm/B.Pm), mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BV).

Scoring and histopathological evaluation of collagen antibody-induced arthritis (CAIA)

The scoring system for CAIA was 0 = normal; 1 = erythema and edema in 1-2 digits; 2 = erythema and edema in >2 digits or mild erythema and edema, usually in the ankle joint; 3 = moderate erythema and edema encompassing the tarsal joint; 4 = severe erythema and edema encompassing the tarsal and metatarsal joint. This analysis was completed by Bolder BioPath (Boulder, Co). Formalin fixed joints (right and left fore and hind paws, both ankles and both knees) were decalcified in 5% formic acid for 2-3 days; tissues were trimmed, processed for paraffin embedding, sectioned at 8 μ m and stained with toluidine blue. Both hind paws, both fore paws, and both knees were embedded and sectioned in the frontal plane while ankles were sectioned in the sagittal plane or ankles may be sectioned with hind paws in the frontal plane. All sections were scored without knowledge of the treatment groups. Groups were later identified as follows. When scoring paws or ankles from mice with arthritic lesions, severity of changes as well as number of individual joints affected were recorded. When only 1-3 joints of the paws or ankles out of a possibility of numerous metacarpal/metatarsal/digit or tarsal/tibio-tarsal joints were affected, an arbitrary assignment of a maximum score of 1, 2 or 3 for parameters below was given depending on severity of changes. If more than 3 joints were involved, the criteria below were applied to the most severely affected/majority of joints. Inflammation: 0 = Normal; 1 = Minimal infiltration of inflammatory cells in synovium and periarticular tissue of affected joints; 2 = Mild infiltration of inflammatory cells. If referring to paws, generally restricted to affected joints (1-3 affected); 3 = Moderate infiltration with moderate edema. If referring to paws, restricted to affected joints, generally 3-4 joints + wrist or ankle; 4 = Marked infiltration affecting most areas with marked edema, 1 or 2 unaffected joints may be present; 5 = Severe diffuse infiltration with severe edema

affecting all joints and periarticular tissues. Pannus: 0 = Normal; 1 = Minimal infiltration of pannus in cartilage and subchondral bone, marginal zones; 2 = Mild infiltration with marginal zone destruction of hard tissue in affected joints; 3 = Moderate infiltration with moderate hard tissue destruction in affected joints; 4 = Marked infiltration with marked destruction of joint architecture, affecting most joints; 5 = Severe infiltration associated with total or near total destruction of joint architecture, affects all joints. Cartilage Damage: 0 = Normal; 1 = Minimal = generally minimal to mild loss of toluidine blue staining with no obvious chondrocyte loss or collagen disruption in affected joints; 2 = Mild = generally mild loss of toluidine blue staining with focal areas of chondrocyte loss and/or collagen disruption in some affected joints; 3 = Moderate = generally moderate loss of toluidine blue staining with multifocal chondrocyte loss and/or collagen disruption in affected joints, some matrix remains on any affected surface with areas of severe matrix loss; 4 = Marked = marked loss of toluidine blue staining with multifocal marked (depth to deep zone) chondrocyte loss and/or collagen disruption in most joints, if knee-one surface with total to near total cartilage loss; 5 = Severe = severe diffuse loss of toluidine blue staining with multifocal severe (depth to tide mark) chondrocyte loss and/or collagen disruption in all joints, if knee-2 or more surfaces with total to near total cartilage loss. Bone Resorption: 0 = Normal; 1 = Minimal = small areas of resorption, not readily apparent on low magnification, rare osteoclasts in affected joints, restricted to marginal zones; 2 = Mild = more numerous areas of resorption, not readily apparent on low magnification, osteoclasts more numerous in affected joints, restricted to marginal zones; 3 = Moderate = obvious resorption of medullary trabecular and cortical bone without full thickness defects in cortex, loss of some medullary trabeculae, lesion apparent on low magnification, osteoclasts more numerous in affected joints; 4 = Marked = full thickness defects in cortical bone, often with distortion of profile of remaining cortical surface, marked loss of medullary bone, numerous osteoclasts, affects most joints; 5

= Severe = full thickness defects in cortical bone and destruction of joint architecture of all joints. For each animal, the inflammation, pannus, cartilage damage and bone damage scores were determined for each of the 8 joints submitted. A sum total (all 8 joints) animal score and an eight joint mean animal score was determined as well as sums and means for each of the individual parameters. Parameters for the various groups were then compared with Group A (vehicle) using a Student's t-test or other appropriate analysis methods with significance set at $p \leq 0.05$.

Calcium oscillations and electrophysiology

Bone marrow-derived macrophages isolated from *Kcnn4*^{+/+} and *Kcnn4*^{-/-} mice were plated on 12-mm diameter coverglass that were placed in 24-well dishes. Cells were cultured in the presence of M-CSF (25 ng/ml) supplemented or not with RANKL (10 ng/ml). Oscillations were recorded 3 days later. Cells were incubated for 30 minutes at 30°C in loading solution (115 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM HEPES, and 10 mM glucose, pH 7.42) supplemented with 5 μM Fura-2/AM (Invitrogen). Fura-2 fluorescent images were analyzed using an inverted microscope (ECLIPSE TE300, Nikon) and a video image analysis system (Argus-50/CA, Hamamatsu Photonics) with excitation filters at 340 ± 10 and 380 ± 10 nm, a dichroic beam splitter at 400 nm, and a bandpass emission filter at 510–550 nm. The recording solution contained 115 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM HEPES, and 10 mM glucose, pH 7.42. Data are presented as mean ± standard error of the mean (SEM) and statistical significance was determined using the Student's t-test.

Electrophysiology

For electrophysiology, macrophages plated on 12-mm diameter coverslips were transferred to a recording chamber and superfused (~1 ml/min) with external solution: 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 2.5 mM Glucose, pH 7.4. Pipettes

had a resistance of 3-9 M Ω when filled with internal solution: 120 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 20 mM glucose, 10 mM EGTA, pH 7.3. To achieve a free internal Ca²⁺ concentration of 10⁻⁶ M in the presence of 10 mM EGTA, 8.98 mM CaCl₂ was added to the internal solution (23). In some experiments, 1 μ M ICA-1703 was included in the external solution. Experiments were performed using an upright microscope (Olympus BX51WI, Olympus, Center Valley, PA) under phase-contrast optics (60x objective, NA 0.9) and at room temperature. An Axopatch 200B patch clamp amplifier, Digidata 1322A digitizer, and PClamp9 software (Axon Instruments, CA) were used for recordings (2 kHz low-pass filtered; 5 kHz sampling rate). Cells were maintained at a holding potential of -60 mV. Voltage steps from -100 mV to +80 mV (400 ms) and voltage ramps (-140 mV to +120 mV over 200 ms) were applied to generate current / voltage plots in Clampfit 9.0 (Axon Instruments, CA).

Immunohistochemistry and Immunofluorescence

For ED-1 (AbD Serotec, Oxford, UK) immunostaining, formalin-fixed, paraffin-embedded rat tissues were microwaved in sodium citrate buffer for antigen retrieval. Following consecutive blocking steps (peroxide block for 15 min, 10% goat serum block for 15 min, followed by a 10% milk block for 15 min), sections were incubated with the primary antibody ED-1 (1:500) and the slides left for 1 h at room temperature. The slides were then washed and incubated in the polymer-HRP (Dako mouse EnVision kit, Dako, Denmark) for 30 min at room temperature. Staining was visualized using DAB (Dako EnVision kit) and sections counterstained with hematoxylin, dehydrated, and mounted with cover slips. For visualization of the actin cytoskeleton and nuclei, cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature and stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) and 4',6-diamidino-2-phenylindole (DAPI; Sigma) as described (MacLauchlan et al., 2009).

Western Blotting

After four to seven days of culture, osteoclasts were serum-starved for three to four hours and stimulated with the indicated reagents. The cells were then lysed in Laemmli sample buffer supplemented with protease inhibitors (cOmplete™ protease inhibitor tablets; Roche Molecular Biochemicals), sodium fluoride, and a phosphatase inhibitor cocktail II (Roche). The lysates were sonicated and resolved by SDS-PAGE, transferred to PVDF membranes, and subjected to immunoblotting with appropriate primary and secondary antibodies. The probed proteins were detected using SuperSignal West Femto Cheluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL).

Statistical Analysis

Unless otherwise stated, data represent the mean $\pm$ one standard deviation (SD). Treatment groups were compared using the analysis of variance. Unless otherwise stated, pairwise comparison *p*-values between the treatment groups were adjusted using Tukey multiple comparison procedure. Statistical significance was declared if the two-sided *p*-value is < 0.05 .

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