Identification and characterization of a transporter complex responsible for the cytosolic entry of nitrogen-containing-bisphosphonates

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Abstract: Nitrogen-containing-bisphosphonates (N-BPs) are a class of drugs widely prescribed to treat osteoporosis and other bone-related diseases. Although previous studies have established that N-BPs function by inhibiting the mevalonate pathway in osteoclasts, the mechanism by which N-BPs enter the cytosol from the extracellular space to reach their molecular target is not understood. Here we implemented a CRISPRi-mediated genome-wide screen and identified *SLC37A3* (solute carrier family 37 member A3) as a gene required for the action of N-BPs. We observed that *SLC37A3* forms a complex with ATRAID (all-trans retinoic acid-induced differentiation factor), a previously identified genetic target of N-BPs. *SLC37A3* and ATRAID localize to lysosomes and are required for releasing N-BP molecules that have trafficked to lysosomes through fluid-phase endocytosis into the cytosol. Our results elucidate the route by which N-BPs are delivered to their molecular target, addressing a key aspect of the mechanism of action of N-BPs that may have significant clinical relevance.

Introduction: N-BPs are the most commonly prescribed drugs used to treat osteoporosis (Drake et al., 2008). They have two negatively charged phosphonate groups that bind to hydroxyapatite crystals with high affinity and enable efficient accumulation of N-BPs on the bone surface (Drake et al., 2008). Osteoclasts, the major cell type responsible for bone resorption, release N-BPs from the bone matrix by dissolving bone mineral and then take up N-BPs through fluid-phase endocytosis (Drake et al., 2008; Thompson et al., 2006). N-BPs subsequently inhibit farnesyl diphosphate synthase (FDPS) in the mevalonate pathway and reduce protein prenylation, an essential post-translational lipid modification required for the function of

numerous proteins such as Ras, Rab and Rho, thereby inducing apoptosis in osteoclasts and diminishing their bone-resorption activities (Drake et al., 2008; Dunford et al., 2001; Fisher et al., 2000; Hughes et al., 1995; Kavanagh et al., 2006; Luckman et al., 1998a; Luckman et al., 1998b; van Beek et al., 1999). However, it is not known how highly charged N-BPs exit the endocytic pathway to target FDPS, which is localized to the cytosol and peroxisomes (Martin et al., 2007). It has been proposed that the acidification of endocytic compartments might neutralize the negative charges on the phosphonate groups and allow N-BPs to diffuse across the vesicle membrane (Thompson et al., 2006), but this model does not address the issue that the amine groups in N-BPs become positively charged in acidic environments. An alternative model is that a transporter exists that facilitates the exit of N-BPs from endocytic vesicles.

Results and Discussion: To gain further insight into the mechanism of action of N-BPs, including the mechanism by which N-BPs are delivered to their molecular target, we implemented an unbiased genome-wide screening approach based on CRISPR-mediated interference (CRISPRi) (Figure 1A) (Gilbert et al., 2014). We transduced a genome-scale CRISPRi single-guide RNA (sgRNA) library into K562 human myeloid leukemia cells that stably express a dCas9-KRAB fusion protein, which functions as an sgRNA-guided transcription inhibitor. The cells were split into a population treated with alendronate (ALN), a representative N-BP, and an untreated control population. Through deep sequencing, we quantified the enrichment/depletion of each sgRNA in the treated population compared to the control population (Figure 1B, Figure 1 - figure supplement 1A), and designated the target genes of those sgRNAs enriched in the

treated population as resistance hits and those depleted as sensitizing hits (Figure 1B, Figure 1 - figure supplement 1B and Supplementary Data Table 1). Consistent with the current model for the action of N-BPs, enzymes, co-factors, and regulators of the mevalonate pathway are enriched in top hits (Figure 1C-D and Figure 1 - figure supplement 1C-D). Particularly, in accordance with the model that N-BPs induce cell death through the reduction in protein prenylation, inhibition of *FDPS* and *GGPPS1*, the enzymes that produce the substrates required for protein prenylation, strongly sensitized cells to ALN (Figure 1C-D).

Amongst the resistance hits not known to be involved in the mevalonate pathway, the gene that conferred the strongest resistance was *SLC37A3* (Figure 1C), which is predicted to encode a membrane protein with 12 transmembrane segments (Chou et al., 2013). *SLC37A3* also appeared as a top resistance hit in a second CRISPRi screen using zoledronate, another representative N-BP, as the selection agent (Figure 1 - figure supplement 1E and Supplementary Data Table 2), further supporting its role in the mechanism of action of N-BPs. Little is known about the function of *SLC37A3*, but it is predicted based on sequence homology to be a sugar-phosphate antiporter (Chou et al., 2013). Intriguingly, a recent human protein interactome study reported an interaction between *SLC37A3* and *ATRAID* (Huttlin et al., 2017), a type I transmembrane protein that was identified as an N-BP target in a previous work (L. E. Surface et al.) and our zoledronate CRISPRi screen (Figure 1 - figure supplement 1E), suggesting *SLC37A3* and *ATRAID* might be functionally related.

To investigate the roles of *SLC37A3* and *ATRAID* in the action of N-BPs, we generated *SLC37A3*-knockout (*SLC37A3*^{KO}) and *ATRAID*-knockout (*ATRAID*^{KO}) cells in

K562 cells, human embryo kidney (HEK) 293T cells and murine macrophage-like RAW 264.7 cells (Figure 2 - figure supplement 1A-E) (L. E. Surface et al.; Ran et al., 2013), with K562 and HEK 293T cells serving as human cell models that represent distinct lineages, and RAW 264.7 macrophages as a mouse cell model that can be differentiated into mature osteoclasts (Collin-Osdoby and Osdoby, 2012). Consistent with the CRISPRi screen and our previous results, knockout of *SLC37A3* and *ATRAID* in all cell types conferred resistance to ALN (Figure 2A-C) (L. E. Surface et al.). Similarly, mature osteoclasts differentiated from knockout RAW cells are more resistant to ALN compared to those differentiated from wild-type cells (Figure 2D and Figure 2 - figure supplement 2A) (L. E. Surface et al.). We also measured the reduction in protein prenylation as a readout of N-BP toxicity and verified that ALN treatment had significantly less of an effect on protein prenylation in *SLC37A3*^{KO} and *ATRAID*^{KO} cells compared to wild-type cells (Figure 2E). Complementation with epitope-tagged *SLC37A3* or either isoform of *ATRAID* (a short isoform, UniProt Q6UW56-1, and a long isoform, UniProt Q6UW56-3) in *SLC37A3*^{KO} or *ATRAID*^{KO} HEK 293T cells, respectively, restored sensitivity to ALN (Figure 2 - figure supplement 2B-D), confirming that the resistance to ALN observed in the knockout cells is indeed caused by the lack of *SLC37A3* or *ATRAID* expression, and that the epitope-tagged versions of the two proteins are functional.

The knockout cells are also resistant to N-BPs other than ALN (Figure 2 - figure supplement 2E) but not to non-nitrogen-containing bisphosphonates (non-N-BPs), which do not target FDPS (Figure 2 - figure supplement 2F) (Drake et al., 2008). Interestingly, knockout of *SLC37A3* and *ATRAID* did not protect cells from lovastatin

(LOV), a statin drug that also targets the mevalonate pathway (Figure 2 - figure supplement 2G-I) (Tiwari and Khokhar, 2014). The distinctive responses of *SLC37A3*^{KO} and *ATRAID*^{KO} cells to N-BPs, non-N-BPs, and LOV indicate that the roles of *SLC37A3* and *ATRAID* in the mechanism of action of N-BPs are not related to the mevalonate pathway but are instead specific to N-BPs. As knockout of *SLC37A3* and *ATRAID* in different cell types conferred similar responses, we focused on HEK 293T cells for further studies as they host various tools for molecular biology.

To probe the epistatic relationship between the two genes, we generated double knockout (*ATRAID*^{KO}; *SLC37A3*^{KO}, abbreviated as KO²) cells (Figure 2 - figure supplement 1F). We observed that *SLC37A3*^{KO} cells are more resistant to ALN compared to *ATRAID*^{KO} cells, and the knockout of *ATRAID* in addition to *SLC37A3* did not further protect cells from the drug (Figure 2F and Figure 2 - figure supplement 2J), indicating that *ATRAID* and *SLC37A3* are indeed functionally related and that *SLC37A3* is epistatic to *ATRAID*.

Next, we investigated the mechanism underlying the observed functional relationship between *ATRAID* and *SLC37A3*. As protein localization can often provide clues to protein function and functionally linked proteins frequently share subcellular distribution patterns, we expressed functional, epitope-tagged *SLC37A3* and *ATRAID* from near-endogenous levels of transcripts (Figure 2 - figure supplement 2B-C and Figure 3 - figure supplement 1A) and characterized their localization with immunofluorescence (IF). We observed that *SLC37A3* co-localizes with LAMP2, a lysosomal marker, but not with Na⁺/K⁺-ATPase or EEA1, which mark the plasma membrane and early endosomes, respectively (Figure 3A, B and Figure 3 - figure

supplement 1C). Both isoforms of ATRAID also predominantly localize to lysosomes but not to the plasma membrane or early endosomes (Figure 3C-D and Figure 3 - figure supplement 1D, F-H). When we co-expressed functionally-tagged SLC37A3 and ATRAID (Figure 3 - figure supplement 1A-B), we observed that both isoforms of ATRAID predominantly co-localize with SLC37A3 (Figure 3E and Figure 3 - figure supplement 1E). To investigate whether the observed co-localization between SLC37A3 and ATRAID represents a physical interaction, we performed reciprocal co-immunoprecipitation (co-IP) experiments in HEK 293T cells over-expressing the two proteins. We detected an interaction between SLC37A3 and ATRAID in both pull-down directions (Figure 3F), confirming that SLC37A3 and ATRAID physically interact, likely forming a lysosomal complex.

As it has been reported that the expression levels of certain solute carriers depend on the presence of accessory proteins (Makrides et al., 2014), we investigated the possibility that the functional relationship between ATRAID and SLC37A3 is due to the impaired expression of SLC37A3 in the absence of ATRAID. Indeed, when we expressed SLC37A3 in the KO² cells, we observed a substantial decrease in the protein level of SLC37A3 compared to that when expressed in the *SLC37A3*^{KO} background (Figure 3G and Figure 3 - figure supplement 2A), even though the mRNA levels of *SLC37A3* in both backgrounds are similar (Figure 3 - figure supplement 1A). The protein level of SLC37A3 was restored by complementation with either isoform of ATRAID (Figure 3G). In reciprocity, we also observed reduced protein levels of both isoforms of ATRAID in the KO² background that cannot be explained by changes in transcript levels (Figure 3H, Figure 3 - figure supplement 2B-C and Figure 3 - figure

supplement 1A). As the translation efficiency of *SLC37A3* transcripts is not significantly altered in the absence of *ATRAID* (Figure 3 - figure supplement 2D-E), the decreased *SLC37A3* protein level in the KO² background is likely caused by shortened protein half-life, suggesting that *ATRAID* and *SLC37A3* are mutually dependent for their stability. Intriguingly, the deletion of *ATRAID* also altered the glycosylation pattern of *SLC37A3*. In the absence of *ATRAID*, the mature, glycosylated population of *SLC37A3* (around 50kD) became undetectable, whereas a population of un-glycosylated *SLC37A3* (around 40kD) emerged (Figure 3G and Figure 3 - figure supplement 2F). Moreover, in cells overexpressing *SLC37A3*, although a significant proportion of *SLC37A3* remained un-glycosylated, only the glycosylated population of *SLC37A3* interacted with *ATRAID* (Figure 3 - figure supplement 2G), suggesting that the interaction with *ATRAID* is crucial for the expression of correctly modified *SLC37A3*.

Finally, we explored the mechanism by which the knockout of *SLC37A3* and *ATRAID* conferred resistance to N-BPs. Given the predicted function of *SLC37A3* as a transporter, we hypothesized that *ATRAID* and *SLC37A3* transport N-BP molecules across the lipid bilayer to inhibit FDPS. This hypothesis is consistent with our finding that the role of *SLC37A3* and *ATRAID* in the mechanism of action of N-BPs is specific to the chemical properties of N-BPs. To test this hypothesis, we incubated wild-type, *ATRAID*^{KO} and *SLC37A3*^{KO} cells with radioactive ALN (³H-ALN) and measured total intracellular radioactivity. However, we observed no significant difference in accumulation of radioactivity between the knockouts and wild-type cells (Figure 4A). As a previous study showed that N-BP molecules traffic to acidified endocytic compartments through fluid-phase endocytosis (Thompson et al., 2006), we further

hypothesized that N-BP molecules traffic to lysosomes and that SLC37A3 and ATRAID, together as a lysosomal complex, might function to release N-BP molecules from the lumen of lysosomes into the cytosol. This model predicts that the total amount of intracellular ^3H -ALN will remain the same in wild-type, *SLC37A3*^{KO} and *ATRAID*^{KO} cells, but ^3H -ALN will not be able to exit lysosomes in knockout cells. To detect this potential shift in the subcellular distribution of ^3H -ALN in knockout cells, we used digitonin to selectively permeabilize the plasma membrane of ^3H -ALN treated cells and generated a cytosolic fraction and a membranous fraction, which contained intact membrane-bound organelles (Figure 4 - figure supplement 1A) (Liu and Fagotto, 2011). We observed that the distribution of radioactive signal changed from being primarily in the cytosolic fraction in wild-type cells to being predominantly in membranous fractions in *SLC37A3*^{KO} and *ATRAID*^{KO} cells (Figure 4B), suggesting that ^3H -ALN cannot be released from membrane-bound organelles in knockout cells. As our model specifically predicts that ^3H -ALN will be trapped in lysosomes in the absence of SLC37A3 or ATRAID, we affinity-purified lysosomes from ^3H -ALN treated cells (Figure 4 - figure supplement 1B-D) (Abu-Remaileh et al., 2017; Wyant et al., 2017) to assess the lysosomal accumulation of ^3H -ALN. As our model predicted, we observed a significant enrichment of ^3H -ALN in lysosomes purified from knockout cells compared to those from wild-type cells (Figure 4C). Additionally, consistent with the observation that ATRAID is required for the stable expression of SLC37A3, *ATRAID*^{KO} cells phenocopied *SLC37A3*^{KO} cells in these uptake assays. Taken together, our results suggest that N-BPs traffic to lysosomes after internalization through endocytosis, and SLC37A3 and

ATRAID form a lysosomal transporter complex that releases N-BP molecules from the lumen of lysosomes into the cytosol.

In summary, this study elucidates the route by which N-BPs enter the cytosol and inhibit their molecular target. As a recent study has proposed that patients who harbor a genetic variant of *GGPS1* might be more prone to the side-effects of N-BP treatment (Roca-Ayats et al., 2017), it is possible that patients with variants of *SLC37A3* or *ATRAID*, which are genes crucial for the action of N-BPs, might also exhibit non-canonical responsiveness to the drugs. Therefore, our results may bear significant relevance to the clinical applications of N-BPs.

Materials and Methods

Materials. Reagents were obtained from the following sources: antibodies against LAMP2 (mouse, sc-18822), Ran BP3 (mouse, sc-373678) and Rap 1A (goat, sc-1482) were from Santa Cruz Biotechnology; antibodies against GAPDH (rabbit, 2118), V5-tag (rabbit, 13202) and EEA1 (rabbit, 2411) were from Cell Signaling Technology; antibodies against Na⁺/K⁺-ATPase (mouse, ab7671) and Lamin B1 (rabbit, ab16048) were from Abcam; antibodies against V5-tag (mouse, R960-25) and HDJ-2 (mouse, MS-225-P0) were from ThermoFisher Scientific; antibodies against Caveolin-1 (rabbit, C3237) and HA-tag (rat, 11867423001) were from Sigma-Aldrich; antibody against EEA1 (mouse, 610456) was from BD Biosciences; antibody against LMAP1 (mouse, H4A3) was from DSYB (Developmental Studies Hybridoma Bank); antibody against Ubiquitin (mouse, 05-944) was from Millipore-Sigma; alendronate (A4978), lovastatin (PHR1285), zoledronate (SML0223), ibandronate (I5784), etidronate (P5248), tiludronate (T4580), chloroquine (C6628), puromycin (P8833), polybrene (H9268), anti-

242 HA (A2095) and anti-V5 (A7345) agarose affinity matrix, poly-L-lysine solution (P4707),
 243 fibronectin solution (F0895), Triton X-100 (T8787), saponin (47036), Bovine Serum
 244 Albumin (BSA, A9647) and cOmplete protease inhibitor cocktail (11836170001) were
 245 from Sigma-Aldrich; HRP-conjugated anti-rat secondary antibody (31470), Alexa 488
 246 and Alexa 647-conjugated secondary antibodies (A21208, A32728, A11034 and
 247 A32733), 0.1 µm TetraSpeck™ microspheres (T7279), SlowFade™ Diamond mounting
 248 medium (S36968), Halt™ protease-phosphatase inhibitor cocktail (78443), BCA protein
 249 assay kit (23225), SuperSignal™ west femto substrate (34095), TOPO TA cloning kit
 250 (450030), TRIzol™ and TRIzol™ LS reagents (15596018 and 10296028),
 251 SuperScript™ IV (18090050), RNase-free Turbo DNase (AM2238), SUPERase-In™
 252 RNase Inhibitor (AM2694), SYBR Green qPCR master mix (A25742), Hygromycin B
 253 (10687010), DMEM (11965118), RPMI (11875093), Fetal Bovine Serum (FBS,
 254 16000044) and Lipofectamine 3000 reagent (L3000008) were from ThermoFisher
 255 Scientific; HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (1706515
 256 and 1706516) were from Bio-Rad; PNGase F (P0704) and Endo H_f (P0703) were from
 257 New England Biolabs (NEB); cell line Nucleofector® kit V was from Lonza (VACA-
 258 1003); CellTiter-Glo® kit (G7572) was from Promega; mouse RANK ligand (RANKL,
 259 462-TEC-010) was from R&D systems; IMDM (30-2005) was from ATCC (American
 260 Type Culture Collection); digitonin (300410) was from Millipore-Sigma; QuickExtract™
 261 DNA extraction solution (QE0905T) was from Epicentre; tritium-labeled alendronate
 262 (MT-1727) was from Moravek; pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng
 263 Zhang (Addgene plasmid # 48138); AAVS1-Puro-PGK1-3xFLAG-TwinStrep was a gift
 264 from Yannick Doyon (Addgene plasmid # 68375); pLenti PGK Hygro DEST (w530-1)

was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 19066); psPAX2 and pMD2.G were gifts from Didier Trono (Addgene plasmid # 12260 and # 12259).

Cell lines and tissue culture. K562 human myeloid leukemia cells, Human Embryo Kidney (HEK) 293T cells and RAW 264.7 murine macrophage-like cells were obtained from ATCC and tested for mycoplasma contamination. K562 cells were cultured in RPMI supplemented with 25 mM HEPES, 2mM L-glutamine, 2 g/L NaHCO₃, 10% FBS and Penicillin/Streptomycin; HEK 293T cells and RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and Penicillin/Streptomycin. All cultures were maintained at 37°C and 5% CO₂.

Unbiased whole-genome CRISPRi screen. K562 cell line generation, genome-scale library design and cloning, virus production, and bioinformatic analysis were conducted as previously described (Gilbert et al., 2014; Jost et al., 2017). In summary, K562 cells stably expressing dCas9-KRAB were transduced in duplicate with the v1 CRISPRi sgRNA library to achieve ~30% infection to ensure no more than one viral integration event per cell. Two days after transduction, cells were selected with 0.75 µg/mL of Puromycin for 2 days and then kept with fresh Puromycin-free medium for 2 days for recovery. At this point (t_0), 250 million cells (ensuring a minimum of 1000× library coverage) were harvested from each replicate and the remaining cells in each replicate were split into two populations for untreated growth and alendronate-treated growth. For alendronate treatment, cells were cultured in medium containing 250 µM alendronate for 24 hours, spun down to remove the drug and re-suspended in fresh medium. Cells were cultured for another 13 days to allow the untreated population to double 7 more times than the alendronate-treated population. 250 million cells were then harvest from each

group (two replicates for each condition, 4 groups in total). Cells were maintained at a density of 500,000 to 1,000,000 cells/mL in 2-liter cultures to ensure a library coverage of at least 1000 cells per sgRNA during the entire screening period. Genomic DNA was collected from all harvested samples and the genomic regions containing the inserted sgRNAs were amplified for 20 cycles by PCR and sequenced at 800x coverage on Illumina HiSeq-2500 using custom primers as previously described (Kampmann et al., 2013).

For data analysis, sequencing reads were aligned to the v1 CRISPRi library sequences, counted, and quantified using the Python-based ScreenProcessing pipeline (Horlbeck et al., 2016). Sensitivity phenotypes (ρ) were calculated by computing the log2 difference in enrichment of each sgRNA between the treated and untreated samples, subtracting the equivalent median value for all non-targeting sgRNAs, and dividing by the number of population doubling differences between the treated and untreated populations (Gilbert et al., 2014; Jost et al., 2017). Similarly, untreated growth phenotypes (γ) were calculated from the untreated endpoint samples and t_0 samples, dividing by the total number of doublings of the untreated population. Phenotypes from sgRNAs targeting the same gene were collapsed into a single sensitivity phenotype for each gene using the average of the top three scoring sgRNAs (by absolute value) and assigned a P -value using the Mann-Whitney test of all sgRNAs targeting the same gene compared to the non-targeting controls (Supplementary Data Table 1). For genes with multiple independent transcription start sites (TSSs) targeted by the sgRNA libraries, phenotypes and P -values were calculated independently for each TSS and then collapsed to a single score by selecting the TSS with the lowest Mann-Whitney P -value.

Replicate-averaged sensitivity phenotype and *P*-value for each gene were obtained by performing the above computations on the average of sgRNA phenotype values calculated from both replicates and used for illustration. The CRISPRi screen was performed only once.

Generation of knockout cell lines in K562, HEK 293T and RAW cells. Genome editing experiments were designed based on an established protocol (Ran et al., 2013). For the human *ATRAID* locus, one sgRNA targeting exon 3 and another targeting exon 5 were used to act simultaneously and remove part of exon 3, the entire exon 4 and part of exon 5. For the human *SLC37A3* locus, one sgRNA targeting intron 5 and another targeting intron 6 were used to act simultaneously and remove the entire exon 6, which contains 146bp of CDS. For murine *SLC37A3* locus, two sgRNAs targeting exon 2 were designed to cause microdeletions and frameshifts in the CDS. sgRNAs were cloned into PX458 for co-expression with Cas9.

sgRNA_human_ <i>ATRAID</i> _exon3_1:	GCCTGATGAAAGTTTGGACC
sgRNA_human_ <i>ATRAID</i> _exon3_2:	CCCTGGTCCAACTTTCATC
sgRNA_human_ <i>ATRAID</i> _exon5:	GTCCTGGAGGAATTAATGCC
sgRNA_human_ <i>SLC37A3</i> _intron5_1:	GTGTGAGTGTATCCTTCACG
sgRNA_human_ <i>SLC37A3</i> _intron5_2:	GCCAGTGCCTGTAAGTCACG
sgRNA_human_ <i>SLC37A3</i> _intron6:	GTAGCAAGTCAGAGTTGTTCA
sgRNA_mouse_ <i>SLC37A3</i> _exon1_1:	TCTCTGCAAAAATCGTGGCC
sgRNA_mouse_ <i>SLC37A3</i> _exon1_2:	TGTTCTGCTCACGTTCTTC

For K562 and HEK 293T cells, on day one, 500,000 cells were seeded onto a 6 cm dish. 24 hours later, cells were transfected with 1.25 µg of PX458 construct for each

sgRNA (total of 2.5 µg DNA) using the Lipofectamine 3000 reagent according to manufacturer instructions. Medium was replenished on the following day. 48 hours after transfection, cells were trypsinized, filtered through a 50 µm strainer into ice-cold FACS buffer (PBS containing 1% FBS) and sorted with a flow cytometer for GFP-positive cells. Single GFP-positive cells were seeded into the wells of a 96-well plate containing 150 µL of DMEM in each well. 12 to 14 days later, each surviving clone was split into two wells, with one well saved for expansion and the other for genotyping. For genotyping, genomic DNA was extracted from each confluent clone with QuickExtract™ solution and used to perform genomic PCR using a pair of primers flanking the target region. Successful deletion events were identified by a significant decrease in the size of PCR products. Clones with deletions on all alleles of the target gene were further expanded and stored. Genomic PCR products from clones with homozygous deletions were inserted into TOPO-TA cloning vectors and sequenced to identify clones that have frameshifts on all alleles of the target gene. *ATRAID*^{KO}; *SLC37A3*^{KO} cells are generated by knocking out *ATRAID* in *SLC37A3*^{KO} cells.

human_*ATRAID*_KO_verification_forward: CTGAAAAGGGGGTTGTGTAGTCAA
human_*ATRAID*_KO_verification_reverse: GGGTTATAGCCCCAGAACTCTGAA
human_*SLC37A3*_KO_verification_forward: GTTGGAGGGCTGATAGCTTAATG
human_*SLC37A3*_KO_verification_reverse: AAAAATTGAGACCTCCTGCCTTG

For RAW 264.7 cells, 2 million cells were electroporated with 2 µg of PX458 harboring the sgRNAs of interest (1 µg per sgRNA construct) in 100 µL of Nucleofector® solution V on a Nucleofector® device (Lonza) using program D-023. Cells were allowed to recover for two days before they were single-cell sorted into a 96-

well plate. Clonal expansion and genotyping were then performed as described above for HEK 293T cells.

Mouse_*SLC37A3*_KO_verification_forward: CCCACAGGCAGAAGACAAGA

Mouse_*SLC37A3*_KO_verification_reverse: TGTAAGTCAGTCACTGGGAGGA

Differentiation of RAW macrophages into osteoclasts. Differentiation of RAW cells to osteoclasts was achieved following an established protocol (Collin-Osdoby et al., 2012). Briefly, RAW 264.7 cells were seeded into a 24-well plate and treated with 35 ng/mL RANKL for 6 days to reach a large, multi-nucleated morphology that is characteristic of osteoclasts. For experiments with alendronate, the drug was added at the indicated concentrations 48 hours prior to harvesting. RAW cell differentiation was repeated independently for three times.

Cell viability assays. On day one, cells were seeded at 8000 cells per well for K562 and HEK 293T cells or 4000 cells per well for RAW 264.7 cells in a 96-well plate and treated with a series of doses of the desired drug. Three wells were prepared for each combination of cell line and drug concentration. Forty-eight hours later, the total cellular ATP level in each well was measured using the CellTiter-Glo® luminescent assay following manufacturer instructions. Relative ATP levels were then plotted as percentages of ATP levels in the untreated samples and interpreted as a proxy for cell viability under drug treatments. Each viability curve was repeated independently for at least two times.

Generation of HEK 293T cells stably expression epitope tagged ATRAID and *SLC37A3*. V5-tagged ATRAID and HA-tagged *SLC37A3* were constructed by appending codon-optimized sequences of V5 (sequence: GGA AAG CCC ATA CCG

AAT CCT CTC CTT GGG TTG GAT AGC ACT) and HA tags (sequence: TAC CCC TAT GAT GTT CCT GAT TAC GCG) to the C-termini of *ATRAID* (both the short isoform, 229 a.a., and the long isoform, 284 a.a.) and *SLC37A3* CDSs, respectively. A GGGGSGGGGS flexible linker (sequence: GGT GGA GGG GGA AGT GGC GGA GGA GGT TCA) was added between each CDS and its epitope tag.

To generate HEK 293T cells that stably express sATRAID-V5, IATRAID-V5 or SLC37A3-HA at sub-endogenous levels, CDSs were cloned into pPGK-AAVS-Puro (derived from Addgene # 68375) and inserted into human *AAVS1* locus via CRISPR-Cas9 mediated homologous recombination. For genome integration, a 40% confluent dish of *ATRAID*^{KO}, *SLC37A3*^{KO} or KO² HEK 293T cells was transfected with 1.5 µg of pPGK-AAVS-Puro carrying the desired CDS and 1 µg of PX458 expressing sgRNA_*AAVS1* (see below) using Lipofectamine 3000 reagent. Culture medium was replenished on the next day. 48 hours after transfection, cells were re-plated in a 10 cm dish in DMEM containing 2 µg/mL puromycin to select for successfully edited cells. Puromycin selection was continued for a week, with medium replenished every other day. After selection, expression levels of epitope-tagged proteins were analyzed by immunoblotting and RT-qPCR as described below.

sgRNA_*AAVS1*: GGGGCCACTAGGGACAGGAT

To generate HKE 293T cells that co-express ATRAID-V5 and SLC37A3-HA at near-endogenous levels, the CDS of sATRAID-V5 or IATRAID-V5 was cloned into w530-1 and transduced into KO² + SLC37A3-HA cells. To generate the lentiviruses, on day one 150,000 HEK 293T cells were seeded into a well of a 6-well plate. 24 hours later, cells were transfected with 2 µg w530-1 construct, 0.8 µg psPAX2 and 0.4 µg pMD2.G using

Lipofectamine 3000 reagent. On the next day culture medium was replenished. 48 hours after transfection, supernatant from the culture was collected and filtered through a 0.45 μ m filter. 150 μ L of the viral medium was added to a 40% confluent well of KO² + SLC37A3-HA cells in a 6-well plate in the presence of 8 μ g/mL Polybrene to a total volume of 2 mL. 48 hours later cells were re-plated into a 6 cm dish in DMEM containing 200 μ g/mL hygromycin. Selection was continued for one week, with medium replenished every other day. After selection, expression levels of epitope-tagged proteins were analyzed by immunoblotting and RT-qPCR as described below.

Immunoblot assays. Cells were washed once with PBS and lysed on ice by scraping into ice-cold RIPA buffer supplemented with Halt™ protease-phosphatase inhibitor. The lysate was then cleared by centrifuging at 20,000 $\times$ g, 4°C for 15 min. Protein concentration in the lysate was determined with BCA protein assays. Loading samples were prepared by mixing lysates with SDS loading buffer and incubating at 37°C for 15 minutes. (Higher denaturing temperatures may cause SLC37A3 to aggregate and prevent it from entering the gel.) SDS-PAGE electrophoresis and protein transfer onto nitrocellulose membranes were performed according to standard protocols. Membranes were blocked in TBST containing 2.5% BSA and 2.5% skim milk for 1 hour and incubated with primary antibodies overnight in TBST containing 5% BSA. (Primary antibody concentrations: α -HA, 100 ng/mL; α -GAPDH and α -HDJ-2, 250 ng/mL; α -V5 (mouse), α -LAMP1, and α -Lamin B1, 500 ng/mL; α -Rap 1A, α -Ran BP3, α -EEA1 (rabbit) and α -Caveolin-1, 1 μ g/mL.) Membranes were then washed 3 $\times$ 5 min in TBST and incubated with either HRP-conjugated or fluorophore-conjugated secondary antibodies (1:2000 dilution for all secondary antibodies) in TBST containing 5% skim

426 milk for 1 hour. Membranes were then washed again for 3×5 min in TBST and
427 visualized with either SuperSignal™ substrate or a Typhoon scanner. Each blot was
428 repeated independently for two times.

429 **RT-qPCR.** RNA was extracted from near-confluent 3 cm dishes using TRIzol reagent
430 following manufacturer instructions. Purified RNA was reverse transcribed using
431 SuperScript™ IV and oligo d(T)₂₀ following manufacturer instructions. qPCR reactions
432 were performed with SYBR Green qPCR master mix and primers listed below using a
433 CFX96 or CFX384 Real-Time PCR machine (Bio-Rad). Ct values were calculated for
434 each transcript using triplicate measurements, and relative mRNA levels were
435 determined for each gene using *TBP* (TATA binding protein) and *RPLP1* (60S acidic
436 ribosomal protein P1) as loading references for human transcripts and *Actb* (β actin)
437 and *Rplp0* (60S acidic ribosomal protein P0) as loading references for mouse
438 transcripts. Each qPCR measurement was repeated independently for two times.

439	human_ <i>TBP</i> _forward:	ATAAGAGAGCCACGAACCACG
440	human_ <i>TBP</i> _reverse:	TGCCAGTCTGGACTGTTCTTC
441	human_ <i>RPLP1</i> _forward:	AGCCTCATCTGCAATGTAGGG
442	human_ <i>RPLP1</i> _reverse:	TCAGACTCCTCGGATTCTTCTTT
443	human_ <i>ATRAID</i> _forward:	CAGAAGGGCACCATCTTGGG
444	human_ <i>ATRAID</i> _reverse:	ACCTTTGAGGGGGTTTGCTT
445	human_ <i>SLC37A3</i> _forward:	GCTGCCTGTGGATTGTGAAC
446	human_ <i>SLC37A3</i> _reverse:	AAAATGTTGCCCACCGAAGC
447	mouse_ <i>Actb</i> _forward:	TGTCGAGTCGCGTCCA
448	mouse_ <i>Actb</i> _reverse:	ATGCCGGAGCCGTTGTC

449 mouse_*Rplp0*_forward: TGCTCGACATCACAGAGCAG
 450 mouse_*Rplp0*_reverse: ACGCGCTTGTACCCATTGAT
 451 mouse_*Ctsk*_forward: CCTTCCAATACGTGCAGCAG
 452 mouse_*Ctsk*_reverse: CATTTAGCTGCCTTTGCCGT
 453 mouse_*Rank*_forward: GCAGCTCAACAAGGATACGG
 454 mouse_*Rank*_reverse: TAGCTTTCCAAGGAGGGTGC
 455 mouse_*Trap*_forward: AAGAGATCGCCAGAACCGTG
 456 mouse_*Trap*_reverse: CGTCCTCAAAGGTCTCCTGG

457 **Immunofluorescence assays.** On day one, coverslips are placed into wells of 6-well
 458 plates and coated for 1 hour at 37°C with 0.01% Poly-L-lysine solution supplemented
 459 with 10 µg/mL fibronectin. Coating solution was aspirated and 120,000 HEK 293T cells
 460 were plated into each well. Twenty-four hours later, the coverslips were rinsed two times
 461 with PBS⁺⁺ (PBS containing calcium and magnesium) and fixed in 4% formaldehyde in
 462 PBS⁺⁺ for 15 min at room temperature. The coverslips were rinsed three times with
 463 PBS⁺⁺ and cells were permeabilized/blocked with 0.1% Saponin and 2% BSA in PBS⁺⁺
 464 for 30 min. After rinsing briefly with PBS⁺⁺, the coverslips were transferred to a humidity
 465 chamber and incubated overnight at 4°C in PBS⁺⁺ containing 2% BSA and desired
 466 primary antibodies. (Antibody concentrations: α-HA and α-EEA1 (mouse), 500 ng/mL; α-
 467 LAMP2, 1 µg/mL; α-V5 (rabbit), 2 µg/mL; α-Na⁺/K⁺-ATPase, 5 µg/mL.) On the next day,
 468 the coverslips were washed 3x5 min in PBS⁺⁺ and incubated for 1 hour with Alexa-488
 469 and Alexa-647-conjugated secondary antibodies diluted in PBS⁺⁺ containing 2% BSA.
 470 The coverslips were again washed 4x5 min in PBS⁺⁺ and mounted onto slides in
 471 SlowFade™ Diamond anti-fade mountant supplemented with DAPI, and the edges of

the coverslips were sealed with nail polish. (We do not recommend curing mountants such as ProLong™ Gold, as they distort/flatten the samples. We also do not recommend Vectashield if Alexa 647 is chosen as a fluorophore.)

Images were acquired on a Zeiss AxiObserver Z.1 microscope equipped with a Zeiss α Plan-APO 100 $\times$ (NA1.46) oil-immersion objective, a 561 nm Hamamatsu Gemini 2C beam splitter, dual Hamamatsu Image-EM 1K EM-CCD cameras and CoolLED P4000 light sources. The ZEN blue software package (Zeiss) was used to control the hardware and image acquisition conditions. An LED-DA/FI/TR/Cy5-A quadruple band-pass filter set was installed in the microscope turret and used for all channels. Excitation wavelength (EW), excitation filters (ExF) and emission filters (EmF) used for each channel are: DAPI channel, EW 385 nm, ExF 387/11 nm, EmF 525/45 nm; Alexa 488 channel, EW 470 nm, ExF 473/10 nm, EmF 525/45 nm; Alexa 647 channel, EW 635 nm, ExF 635/6 nm, EmF 682/40 nm. Images were sampled above the Nyquist limit with a voxel size of 81.25 nm $\times$ 81.25 nm $\times$ 220 nm. Each IF sample was independently prepared and imaged for two times.

With each batch of samples, one slide of 0.1 μ m TetraSpeck™ microspheres immersed in SlowFade™ Diamond was also prepared and imaged with the same settings. The point-spread function (PSF) of each channel was distilled from the microsphere images and used to deconvolute sample images using Huygens Professional deconvolution software (Scientific Volume Imaging). Chromatic aberration (shift, rotation and scaling) were estimated by correlating different channels of the microsphere images. The estimated aberration parameters were then used to align different channels of deconvoluted images using Huygens Profession software. Only images that have been

deconvoluted and aligned were used for analysis. Images displayed in figures are representative single Z-slices.

Reciprocal co-immunoprecipitation. 10 cm dishes of near-confluent KO² HEK 293T cells over-expressing the proteins indicated in Figure 3f were lysed by scraping on ice into 1 mL of ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl pH 8.0, 150 mM NaCl and 2 mM EDTA in ddH₂O) supplemented with cOmplete protease inhibitors. The lysates were cleared as described above. 10 µL of each cleared lysate was saved for input analysis. The rest of the lysates were transferred to Eppendorf tubes containing 20 µL (settled volume) of anti-HA or anti-V5 agarose beads that had been blocked overnight in lysis buffer containing 2% BSA. Lysates were incubated with the beads for 90 min at 4°C, washed three times in low-salt wash buffer (0.1% Triton X-100, 10 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM EDTA in ddH₂O) supplemented with protease inhibitors and three times in high-salt wash buffer (0.1% Triton X-100, 10 mM Tris-HCl pH 7.5, 300 mM NaCl and 1 mM EDTA in ddH₂O) supplemented with protease inhibitors, and eluted in 100 µL elution buffer (1% Triton X-100, 10 mM Tris-HCl pH 7.5 and 150 mM NaCl) containing 2 mg/mL HA or V5 peptide. Inputs and eluates were then analyzed by immunoblotting as described. The co-IP experiment was repeated independently for two times.

Analysis of protein glycosylation. Protein lysates and IP eluates were prepared as described above. Once obtained, samples (lysates containing 20-40 µg of total protein or IP eluates that correspond to 60 µg of total protein input) were either left untreated, or treated with Peptide-N-Glycosidase F (PNGase F) or Endo-glycosidase H (Endo H) following manufacturer instructions (NEB) using non-denaturing conditions (without

adding SDS or boiling for denaturation). The reactions were incubated at 37°C overnight and subsequently analyzed by SDS-PAGE electrophoresis and immunoblotting.

Inferring translation efficiency with polysome profiling. For each cell line of interest, one near-confluent 15 cm dish of cells was lysed on ice by scraping into 300 μ L of ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl pH 7.0, 20 mM Tris-HCl pH 8.0, 15 mM $MgCl_2$, 150 mM NaCl, 5 mM $CaCl_2$, 25 U/mL RNase-free Turbo DNase, 500 U/mL SUPERase[•]In[™] RNase Inhibitor and 0.1 mg/mL cycloheximide in RNase-free water) supplemented with protease inhibitors. Lysates were homogenized by passing through a 22-gauge needle for 10 times and then cleared as described above. 10% - 50% Sucrose gradients were composed by mixing 6 mL of 10% Sucrose solution (20 mM Tris-HCl pH 7.0, 20 mM Tris-HCl pH 8.0, 15 mM $MgCl_2$, 150 mM NaCl, 0.1 mg/mL cycloheximide and 10% (w/v) Sucrose in RNase-free water) with 6 mL of 50% Sucrose solution (20 mM Tris-HCl pH 7.0, 20 mM Tris-HCl pH 8.0, 15 mM $MgCl_2$, 150 mM NaCl, 0.1 mg/mL cycloheximide and 50% (w/v) Sucrose in RNase-free water) on a Gradient Master device (BioComp). 450 μ L of each cleared lysate was loaded onto a 10% - 50% Sucrose gradient and centrifuged at 35,000 rpm, 4°C for 2.5 hours using a SW 41 Ti rotor (Beckman). At the same time 50 μ L of each cleared lysate was used to extract total RNA. The density-separated RNA samples were loaded onto a Gradient Master device, collected from the top (low density) to the bottom (high density) and analyzed by measuring A_{254} . Fractionation of samples was carried out manually by visually determining the boundaries between desired fractions during sample analysis. RNA was then extracted from each fraction using TRIzol[™] LS reagent following manufacturer instructions. Levels of *SLC37A3* transcripts in collected fractions were then measured

by RT-qPCR as described above and the distribution of *SLC37A3* transcript in the differentially translated fractions was plotted and interpreted as a proxy for translation efficiency. The polysome profiling experiment was repeated independently for two times.

Radioactive uptake assays. For whole-cell uptake assays, on day one 1.5 million WT, *ATRAID*^{KO} or *SLC37A3*^{KO} HEK 293T cells were seeded into each poly-L-lysine coated 6 cm dish in DMEM containing 1 μ Ci/mL ³H-alendronate. 24 hours later, cells were washed 3 times with PBS⁺⁺, each time thoroughly removing the PBS. Cells were then trypsinized, pelleted and re-suspended in PBS. Cell density in each sample was measured on a Multisizer 3 Coulter Counter (Beckman Coulter). Total intracellular radioactivity in 2 million cells from each sample was measured by scintillation counting on a LS6500 Liquid Scintillation Counter (Beckman Coulter). Three plates were used as triplicates for each cell line.

Fractionation-based uptake assays were designed based on an established protocol (Liu et al., 2011). On day one 6 million WT, *ATRAID*^{KO} or *SLC37A3*^{KO} HEK 293T cells were seeded into each Poly-L-lysine coated 10 cm dish in DMEM containing 1 μ Ci/mL ³H-alendronate. 24 hours later, dishes were transferred to a cold room and washed three times with 5 mL of PBS⁺⁺, each time thoroughly removing the PBS. 3 mL of permeabilization buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 2 mM MgCl₂, 2 mM DTT and 42 μ g/mL digitonin in ddH₂O) supplemented with protease inhibitors was then added to each dish, and the dishes were incubated on an orbital shaker (100 rpm) at 4°C for 10 min. 2.5 mL supernatant was collected from each dish as the cytosolic fraction, the rest of the supernatant was thoroughly aspirated. The

permeabilized cells were then washed again in 5 mL of PBS⁺⁺ and lysed in 500 µL of RIPA buffer supplemented with protease inhibitors. The RIPA lysates were cleared as described above and collected as membranous fractions. Radioactivity in cytosolic and membranous fractions was measured by scintillation counting and normalized to protein concentrations measured by BCA protein assays. Two plates were used as duplicates for each cell line.

Lysosome purification-based uptake assays were adapted from established protocols (Abu-Remaileh et al., 2017; Wyant et al., 2017). Three 15 cm dishes of ~ 35 million WT, *ATRAID*^{KO} or *SLC37A3*^{KO} HEK 293T cells expressing Tmem192-3xHA were used for each experiment. Cells were incubated in RPMI containing 1 µCi/mL ³H-alendronate and incubated for 3 hours, quickly rinsed twice with PBS, scraped in 1 mL of KPBS (136 mM KCl, 10 mM KH₂PO₄, pH 7.25 was adjusted with KOH), pelleted by centrifuging at 1000×g, 4°C for 2 min and re-suspended in 950 µL KPBS. The cell suspension was then homogenized with 20 strokes of a 2 mL dounce homogenizer and centrifuged at 1000×g, 4°C for 2 min. The supernatant was then incubated with 150 µL of KPBS-prewashed anti-HA magnetic beads for 3 min. The beads were then gently washed three times with KPBS and then re-suspended in 50 µL ice-chilled extraction buffer (80% methanol and 20% ddH₂O) and incubated for 5 min on ice. The lysosome extract was then centrifuged at 1000×g, 4°C for 2 min. Radioactivity in the supernatant was then measured by scintillation counting.

All uptake assays were repeated independently for two times.

Statistical tests. *P*-values assigned to individual genes in the CRISPRi screen were calculated using non-parametric Mann-Whitney *U* test (11, 23). *P*-values in Figure 4b

were calculated using unpaired two-way ANOVA test. *P*-values in Figure 4c were calculated using two-tailed unpaired t-test assuming equal variance. Degrees of freedom, *F* values and *t* values are reported in figure legends.

Data availability. The data that support the findings in this study are available within the paper and its supplementary information files.

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Competing interests: The authors declare no competing financial interests.

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670

Figure 1 An unbiased CRISPRi screen identifies genetic targets of alendronate. (A)

Schematic illustrating the workflow of the genome-wide CRISPRi screen. The IC₅₀ of alendronate in K562 cells is 250 μ M. **(B)** Volcano plot showing, for each gene, a p score that averages the normalized fold enrichment (in the treated population compared to the untreated control) of the gene's three most effective sgRNAs, and a Mann-Whitney P -value for fold enrichment (Gilbert et al., 2014). The dashed lines represent thresholds used to identify significant hits. Positive p scores correspond to resistance hits and negative scores to sensitizing hits. **(C)** Gene names and annotated functions of the top 7 resistance and sensitizing hits. Genes are sorted by the absolute values of their p scores

in descending order. **SLC37A3** is marked in bold. **(D)** Diagram of the mevalonate pathway, with genes in the pathway that were identified as significant hits marked with their p scores. Resistance hits are color-coded in red and sensitizing hits in blue.

Figure 1 – figure supplement 1 Additional analysis of the whole-genome CRISPRi screen.

(A) Evaluation of the reproducibility of the CRISPRi screen. The enrichment score (ρ) of each sgRNA was calculated separately from two biological replicates of the CRISPRi screen and compared in a scatter plot. Data points corresponding to negative control sgRNAs are colored in gray. (B) Quantile-quantile plot comparing the distribution of observed average sgRNA enrichment scores (ρ scores) of each gene in the genome with a Gaussian distribution that has the same mean and standard deviation. The dashed gray line represents the predicted location of data points if the distribution of ρ scores is indeed Gaussian. The large deviations from the gray line observed at the two ends of the distribution indicate that the silencing of those genes has stronger effects than expected by pure Gaussian noise and is therefore likely to be biologically meaningful. The dotted lines are arbitrary thresholds set to select resistance hits (red dotted line) and sensitizing hits (blue dotted line) that deviate significantly from Gaussian predictions. 398 resistance hits and 28 sensitizing hits passed the thresholds. (C-D) Gene Ontology (GO) pathway enrichment analysis of the top 100 resistance hits (C) and the top 30 sensitizing hits (D) identified in the CRISPRi screen. Only the most specific subclasses that are statistically significant are shown. Both fold enrichment of pathway genes and P -values of fold enrichment are displayed. Fold enrichment values were clipped at 100 fold. P -values were corrected for multiple testing using Bonferroni correction. Note that genes involved in the mevalonate pathway, which includes IPP biosynthesis, geranyl phosphate synthesis and farnesyl phosphate synthesis, are significantly enriched in top hits from the screen. IPP: isopentenyl pyrophosphate. (E) Volcano plot showing the results from a second CRISPRi screen using zoledronate, another representative N-BP, as the selection agent. Plot layout is the same as in Figure 1B. *SLC37A3* and *ATRAID* are highlighted in red and cyan, respectively. Significant hits were defined as genes that had a fold enrichment with an absolute value larger than 0.1, and a P -value smaller than 0.05.

Figure 2 *SLC37A3* and *ATRAID* are functionally related genes required for the mechanism of action of N-BPs. (A-D) Dose response curves of wild-type, *ATRAID*^{KO} and *SLC37A3*^{KO} K562 cells (A) and HEK 293T cells (B), and wild-type and *SLC37A3*^{KO} RAW cells (both undifferentiated macrophages, C, and differentiated osteoclasts, D) to alendronate. Cells were treated with a series of doses of alendronate (x-axis) for 48 hours. Relative cell viability was determined by measuring post-treatment total cellular ATP levels and normalizing to those in untreated cells (y-axis). Data depict mean with s.d. for biological triplicate measurements. (E) Immunoblots measuring alendronate-induced reduction in protein prenylation in wild-type and knockout K562 and HEK 293T cells. Cells were treated with indicated doses of alendronate for 24 hours before analysis by immunoblotting. (F) Immunoblots comparing alendronate-induced reduction in protein prenylation in single and double-knockout HEK 293T cells. Experimental procedure is as in C. Note that higher alendronate doses were used in F compared to E to induce detectable levels of unprenylated proteins. ALN: alendronate.

Figure 2 – figure supplement 1 Genotypes of knockout cells used in this study. (A-F) Sequences of the *ATRAID* (A, C and F) or *SLC37A3* locus (B, D and E) in *ATRAID*^{KO} (A) and *SLC37A3*^{KO} (B) K562 cells, *ATRAID*^{KO} (C), *SLC37A3*^{KO} (D) and *KO*² (F) HEK 293T cells, and *SLC37A3*^{KO} (E) RAW 264.7 cells, showing that truncations in *ATRAID* and *SLC37A3* coding sequences (CDS) have caused frame shifts in the knockout cell lines. The *SLC37A3* locus in *KO*² HEK 293T cells is identical to that in *SLC37A3*^{KO} HEK 293T cells. Introns and exons are not drawn to scale. Note that more than 2 alleles are present for the *SLC37A3* locus in HEK 293T cells as these cells are hypo-triploidic. *KO*²: *ATRAID*^{KO}; *SLC37A3*^{KO}.

Figure 2 – figure supplement 2 Additional evidence that validates *SLC37A3* and *ATRAID* as functionally related genes required for the mechanism of action of N-BPs. (A) Expression of osteoclast markers (*Ctsk*, *Rank* and *Trap*) in undifferentiated RAW macrophages (Mφ) and

differentiated RAW osteoclasts (OC), demonstrating successful differentiation of RAW cells. **(B-C)** Dose responses to alendronate in *SLC37A3*^{KO} or *ATRAID*^{KO} HEK 293T cells complemented with epitope tagged *SLC37A3* or *ATRAID*, respectively, compared with those in wild-type, *ATRAID*^{KO} and *SLC37A3*^{KO} cells. **(D)** Immunoblot comparing the short isoform of *ATRAID* (s*ATRAID*) with the long isoform of *ATRAID* (l*ATRAID*), showing that the long isoform of *ATRAID* is expressed as the same protein as the short isoform of *ATRAID*. Note that unequal amount of protein was loaded in each lane to obtain even exposure. The amount of protein loaded from left to right was: 100 µg, 40 µg, 10 µg and 4 µg, respectively. **(E-F)** Dose response curves of wild-type, *ATRAID*^{KO} and *SLC37A3*^{KO} HEK 293T cells to nitrogen-containing-bisphosphonates **(E)** and non-nitrogen-containing-bisphosphonates **(F)**. **(G-H)** Dose response curves of wild-type, *ATRAID*^{KO} and *SLC37A3*^{KO} K562 cells **(G)** and HEK 293T cells **(H)** to lovastatin. **(I)** Immunoblots measuring lovastatin-induced reduction in protein prenylation in wild-type, *ATRAID*^{KO} and *SLC37A3*^{KO} K562 cells and HEK 293T cells. **(J)** Dose response to alendronate in KO² HEK 293T cells, compared with those in wild-type, *ATRAID*^{KO} and *SLC37A3*^{KO} cells. For **B-C**, **E-H** and **J**, data depict mean with s.d. for biological triplicate measurements. LOV: lovastatin. KO²: *ATRAID*^{KO}; *SLC37A3*^{KO}. PNGase F: peptide: N-glycosidase F, an enzyme that removes asparagine (N)-linked sugar modifications on glycoproteins.

Figure 3 SLC37A3 and ATRAID form a lysosomal complex and are inter-dependent for their stable expression. (A-D) Localization of HA-tagged SLC37A3 (SLC37A3-HA) (A-B) and V5-tagged short isoform of ATRAID (sATRAID-V5) (C-D) shown with markers for lysosomes (LAMP2, A and C) and the plasma membrane (Na⁺/K⁺-ATPase, B and D). (E) Co-localization of SLC37A3-HA and sATRAID-V5. Nuclei were stained with DAPI in blue. Scale bars represent 10 μ m. Each image displayed is the representative example chosen from at least five similar images. (F) Reciprocal co-IP of SLC37A3-HA and sATRAID-V5 in KO² HEK 293T cells stably overexpressing both proteins. In each negative control cell line, one of the two tagged proteins was replaced with GFP tagged with the same epitope. (G) Immunoblots measuring SLC37A3-HA protein levels in various cells, showing that deletion of *ATRAID* significantly reduces the protein level of SLC37A3-HA. The un-glycosylated population of SLC37A3 that appears in the absence

of ATRAID is marked with an asterisk. (H) Immunoblots measuring ATRAID-V5 protein levels in various cells, demonstrating that deletion of *SLC37A3* significantly reduces the protein level of ATRAID-V5. IP, immunoprecipitation. IB, immunoblot. KO²: *ATRAID*^{KO}; *SLC37A3*^{KO}.

Figure 3 – figure supplement 1 Additional evidence supporting that SLC37A3 and ATRAID form a lysosomal complex. (A) mRNA levels of *ATRAID* and *SLC37A3* in various HEK 293T cells used in this study. Data depict mean and s.d. for technical triplicate measurements. (B) Dose responses to alendronate in *KO*² HEK 293T cells complemented with either only *SLC37A3*-HA or both *ATRAID*-V5 and *SLC37A3*-HA, compared with those in wild-type and *KO*² HEK 293T cells. Data depict mean and s.d. for biological triplicate measurements. (C-H) Localization of *SLC37A3*-HA (C), *sATRAID*-V5 (D) or *IATRAID*-V5 (F-H) shown with LAMP2 (F), EEA1 (C, D and H) and Na⁺/K⁺-ATPase (G), and co-localization of *SLC37A3*-HA and *IATRAID*-V5 (E). Scale bars represent 10 μm. Each image displayed is the representative example chosen from at least five similar images. Note that in E, there exists a subpopulation of *IATRAID*-V5 that localizes to the plasma membrane but not with *SLC37A3*-HA. Such distribution is likely a result of a higher-than-endogenous expression level of *IATRAID*-V5 in *KO*² + *SLC37A3*-HA + *IATRAID*-V5 HEK 293T cells (A), as such distribution is not observed in *ATRAID*^{KO} + *IATRAID*-V5 HEK 293T cells (G), which express *IATRAID*-V5 at a lower-than-endogenous level (A). *KO*²: *ATRAID*^{KO}; *SLC37A3*^{KO}. *sATRAID*: short isoform of *ATRAID*. *IATRAID*: long isoform of *ATRAID*.

Figure 3 – figure supplement 2 Additional evidence supporting that SLC37A3 and ATRAID depend on each other for stable expression. (A-C) Analysis of IF images comparing the expression levels of SLC37A3-HA (A) or both isoforms of ATRAID-V5 (B and C) in their respective single knockout backgrounds and the *KO*² background. The two images in each sub-figure were acquired with the same setting and adjusted to the same contrast. In each image a background

area (turquoise or blue, inside nuclei, where no stain should be present) and a signal area (orange or red) were selected and the distribution of pixel values within each area was plotted in a histogram. The outlines in the histogram are color-coded to match the borders of selected areas. **(D-E)** Polysome profiling experiment assessing the translation efficiency of *SLC37A3* transcripts in *SLC37A3*^{KO} and KO² backgrounds. Lysates from indicated cell lines were analyzed on a gradient station and fractionated into five fractions: untranslated transcripts (UT), small and large ribosome subunits (SL), lowly translated transcripts (LT), medially translated transcripts (MT) and highly translated transcripts (HT). The level of *SLC37A3* transcripts relative to the level of *TBP* (TATA-binding protein) transcripts in each fraction was measured and plotted. The SL fraction was excluded from the analysis. A total RNA fraction was included as a reference. No overall shift was observed in the distribution of *SLC37A3* transcripts in the KO² background compared to that in the *SLC37A3*^{KO} background, suggesting that the translation efficiency of *SLC37A3* is not affected by the absence of *ATRAID*. **(F)** Immunoblot comparing the glycosylation patterns of *SLC37A3* in *SLC37A3*^{KO} and KO² backgrounds. Lysates from *SLC37A3*^{KO} + *SLC37A3*-HA (lane 1-3) and KO² + *SLC37A3*-HA (lane 4-6) HEK 293T cells were left untreated (lane 1 and 4), treated with PNGase-F (lane 2 and 5), or with Endo H (lane 3 and 6). The band corresponding to an unglycosylated population of *SLC37A3* that is present in the absence of *ATRAID* but not in the presence of *ATRAID* is marked with an asterisk. **(G)** Immunoblot comparing the glycosylation patterns of total cellular *SLC37A3* and the sub-population of *SLC37A3* that interacts with *ATRAID*. In KO² HEK 293T cells over-expressing *SLC37A3*-HA and s*ATRAID*-V5, proteins that interact with s*ATRAID*-V5 were purified with immuno-precipitation against V5 epitope and compared with total proteins in the lysate. The pre-IP total lysate (lane 1-2) and anti-V5 IP eluate (lane 3-4) were either left untreated (lane 1 and 3) or treated with PNGase F (lane 2 and 4) and analyzed by blotting against *SLC37A3*-HA. IF: immunofluorescence. IB: immunoblot. KO²: *ATRAID*^{KO}; *SLC37A3*^{KO}. PNGase F: peptide: N-glycosidase F, an enzyme that removes all asparagine (N)-linked sugar modifications from glycoproteins. Endo H: endoglycosidase H, an enzyme that only removes high mannose sugar moieties on ER glycoproteins that have not been processed by the Golgi apparatus.

Figure 4 SLC37A3 and ATRAID transport alendronate from the lumen of lysosomes into the cytosol. (A) Radioactive uptake assay measuring total intracellular radioactivity in indicated HEK 293T cells treated with ^3H -alendronate. Data depict mean and s.d. for biological triplicate measurements. (B) Radioactive uptake assay measuring levels of radioactivity in subcellular fractions in indicated HEK 293T cells treated with ^3H -alendronate. Data depict mean with s.d. for biological duplicate measurements. Significance was determined using unpaired two-way ANOVA test. Effect of genotype: $F(2,6) = 74.93$, $P < 0.0001$; effect of subcellular location: $F(1,6) = 864.9$, $P < 0.0001$; effect of interaction between genotype and subcellular location: $F(2,6) = 312.4$, $P < 0.0001$. (C) Radioactive uptake assay measuring levels of radioactivity in lysosomes purified from indicated HEK 293T cells treated with ^3H -alendronate. Data depict mean and s.d. for biological triplicate measurements. Significance was determined using two-tailed unpaired t-test with equal s.d. Comparison between wild-type and ATRAID^{KO} cells: $df = 4$, $t = 36.24$, $P < 0.0001$. Comparison between wild-type and SLC37A3^{KO} cells: $df = 4$, $t = 17.96$, $P < 0.0001$. HEK 293T cells were treated with $1 \mu\text{Ci/mL}$ ^3H -alendronate for 24 hours in A-B and 3 hours in C. ****: $P < 0.0001$.

Figure 4 – figure supplement 1 Quality controls for the radioactive uptake assays. (A) Immunoblot against markers for cytosol (GAPDH), plasma membrane (Caveolin1), early endosome (EEA1), lysosome (LAMP1), nucleosol (Ran-BP3), and nucleoskeleton (LaminB1) in cytosolic fractions and membranous fractions in wild-type, *ATRAID*^{KO} and *SLC37A3*^{KO} HEK 293T cells, demonstrating successful subcellular fractionation in the fractionation-based radioactive uptake assay. Cyt: cytosolic fraction. Mem: membranous fraction. Tot: total cell lysate. **(B-D)** Localization of HA tagged TMEM192 (TMEM192-HA), a lysosomal protein we expressed in wild-type **(B)**, *ATRAID*^{KO} **(C)** and *SLC37A3*^{KO} **(D)** HEK 293T cells and used as a handle to immunoprecipitate lysosomes, shown with a lysosomal marker, LAMP2, demonstrating correct localization of TMEM192-HA to lysosomes and, consequently, successful purification of lysosomes in the lysosome-purification-based uptake assay. Scale bars represent 10 μ m. Each image displayed is the representative example chosen from at least five similar images.