Nitrogen-containing bisphosphonates (N-BPs), such as alendronate, are the most widely prescribed medications for diseases involving bone, with nearly 200 million prescriptions written annually. Recently, widespread use of N-BPs has been challenged due to the risk of rare but traumatic side effects such as atypical femoral fracture (AFF) and osteonecrosis of the jaw (ONJ). N-BPs bind to and inhibit farnesyl diphosphate synthase, resulting in defects in protein prenylation. Yet, it remains poorly understood what other cellular factors might allow N-BPs to exert their pharmacological effects. Here, we performed genome-wide studies in cells and patients to identify the poorly characterized gene, ATRAID. Loss of ATRAID function results in selective resistance to N-BP-mediated loss of cell viability and the prevention of alendronate-mediated inhibition of prenylation. ATRAID is required for alendronate inhibition of osteoclast function, and ATRAID-deficient mice have impaired therapeutic responses to alendronate in both postmenopausal and senile (old age) osteoporosis models. Last, we performed exome sequencing on patients taking N-BPs that suffered ONJ or an AFF. ATRAID is one of three genes that contain rare nonsynonymous coding variants in patients with ONJ or an AFF that is also differentially expressed in poor outcome groups of patients treated with N-BPs. We functionally validated this patient variation in ATRAID as conferring cellular hypersensitivity to N-BPs. Our work adds key insight into the mechanistic action of N-BPs and the processes that might underlie differential responsiveness to N-BPs in people.

INTRODUCTION
Nitrogen-containing bisphosphonates (N-BPs) are the standard treatment for osteoporosis and several other bone diseases (1, 2). Certain N-BPs (pamidronate and zoledronate) are also routinely prescribed to prevent skeletal complications in patients with multiple myeloma and with bone metastases from other malignancies, including breast and prostate cancer (3). However, because N-BPs cause rare yet serious side effects, such as atypical femoral fractures (AFFs) and osteonecrosis of the jaw (ONJ), many patients avoid taking them (1, 4–6), causing the number of prescriptions to plummet over 50% in the last decade (6, 7). A plan for addressing this crisis, developed by American Society for Bone and Mineral Research leadership, calls for action at the federal level. The ASBMR ADVANCE (Advancing Viable Options for Nondrug Alternatives to bisphosphonates) initiative was launched to both prevent and treat AFF and ONJ (6, 7). This research initiative has provided the much-needed data and understanding to make major medical advances and to improve patient care and quality of life (6, 7).

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for better pharmacogenomics to identify genetic factors that may underlie response to this class of drugs (6).

A goal of personalized medicine is to identify biomarkers that underlie drug responsiveness. For N-BPs, it can be said that there are limited personalization options owing to the limited number of genes implicated in the pharmacologic effects of N-BPs. Exposure of cells to N-BPs leads to inhibition of farnesyl diphosphate synthase (FDPS also known as FPPS) resulting in reduction in protein prenylation (8). On the basis of this observation, it is widely believed that N-BPs act therapeutically by impairing protein prenylation, ultimately leading to deficits in numerous cellular processes including differentiation, recruitment, and adhesion of osteoclasts (the major bone resorptive cell type) to bone and/or osteoclast cell death (9–11).

Recently, we performed CRISPRi-based, genome-wide screening and identified a poorly characterized gene, SLC37A3, that provides molecular details for how N-BPs reach their target, FDPS (12). As part of that work, we determined that SLC37A3 requires another poorly characterized protein, ATRAID, for its expression (12). Here, we independently identified ATRAID using a different genome-wide, mutagenesis strategy. We generated ATRAID-deficient mice and determined that it is required for the regulation of N-BPs on bone. We also performed exome sequencing in patients taking N-BPs and identified and functionally validated rare coding variants in ATRAID in patients that suffered side effects, namely, atypical femoral fractures and osteonecrosis of the jaw.

RESULTS
ATRAID is required for molecular responses to N-BPs
To provide insight into the mechanism(s) of N-BPs action, we performed a genetic screen to identify human genes required for the antiproliferative effects of N-BPs (Fig. 1A). We used a largely haploid human cell line of myeloid origin (KBM7) to generate a library of retroviral gene trap mutants (13) and then selected for clones that are resistant to cytotoxic concentrations of alendronate. The advantages of this cell line for genetic screening include the following: (i) Each gene is present as a single copy, enabling gene inactivation (except those genes on chromosome 8); (ii) KBM7 cells are human and of the hematopoietic lineage, increasing the likelihood that any genes we identify could be relevant to the natural context for N-BPs, the bones of human patients (14); and (iii) it is a different cell line and a different mutagenic approach than used previously with CRISPRi in K562 cells (12), which allows us to independently assess those results. Using this haploid approach, we identified ATRAID also known as APR-3/C2orf28 (15), as the gene most significantly enriched for insertions in alendronate-resistant cells compared to untreated cells [false discovery rate (FDR) corrected $P = 7.02 \times 10 ^{-6}$] (Fig. 1B; fig. S1, A and B; data file S1). Providing confidence in our screen, we also identified SLC37A3, as well as SNTG1, PLCLI1, and EPHB1, which have been previously connected to N-BP action on bone cells and/or human bone diseases (data file S1) (16–18).

ATRAID was named because it is a gene whose mRNA expression is strongly induced by the ligand all-trans retinoic acid (ATRA) (15). ATRAID is expressed in chondrocytes and contains a signal peptide, Toll-like receptor leucine rich repeat, epidermal growth factor-like domain, and a transmembrane domain (Fig. 1C and fig. SIA) (19, 20). The alendronate resistance phenotype of ATRAID-deficient cells [ATRAID_GT1 (gene-trap1) and ATRAID_GT2 (gene-trap2)] was reversed by the reintroduction of wild-type ATRAID splice variant 2 (v2) or splice variant 3 (v3) complementary DNA (cDNA), which differ in their N termini (Fig. 1D and fig. S1, A and B). To better understand the degree of alendronate resistance in ATRAID-deficient cells, we varied both cell number and drug concentration in a viability assay. ATRAID-deficient cells were resistant to alendronate over two to three orders of magnitude of drug concentration or cell number (fig. S1C). The growth of untreated wild-type and ATRAID-deficient cells did not differ (fig. S1D). Overexpression of full-length ATRAID (v2) sensitized cells to alendronate (fig. S1E). Last, ATRAID membrane targeting is required for the antiproliferative effects of alendronate, as ATRAID-deficient cells complemented with full-length ATRAID (v2) were sensitive to the cytotoxic effects of alendronate, whereas those expressing the membrane truncated form remained resistant (fig. S1F). Together, these data establish ATRAID as a genetic factor required for the growth inhibitory effects of alendronate.

N-BPs are part of a larger class of compounds known as bisphosphonates (BPs) that contain two phosphate moieties, each joined to a carbon atom by a carbon-phosphorus bond (Fig. 1E) (21). To determine whether the effects of ATRAID deficiency on alendronate resistance were specific to N-BPs, we tested the effect of several nitrogen-containing and non–N-BPs on wild-type and mutant ATRAID cells. ATRAID-deficient cells were resistant to the N-BPs, alendronate and zoledronate, but were as sensitive to the non–N-BPs, etidronate, and tiludronate, as control cells (Fig. 1F).

To determine whether ATRAID is required for the reduction in protein prenylation observed upon N-BP treatment, we monitored the prenylation of several proteins, including the heat shock protein Dnaj (Hsp40) homolog HDJ-2, and the Ras family protein Rap1a (22). Alendronate strongly inhibited prenylation of HDJ-2 and Rap1a in wild-type cells in a dose-dependent manner and had much less of an effect on prenylation of these proteins in ATRAID-deficient cells (Fig. 1G). Furthermore, the inhibitory effect of alendronate on prenylation was rescued when ATRAID cDNA variants (v2 or v3) were introduced (fig. S1G). We observed inhibition of prenylation resistance at N-BP doses where we did not see PARP-1 cleavage in ATRAID-deficient cells, suggesting that ATRAID can mediate the effect on prenylation independent of apoptosis (fig. S1G). Thus, these findings suggest that ATRAID functions as a positive regulator upstream of FDPS.

ATRAID is required for organismal responses to N-BPs
To determine whether ATRAID modulates organismal responses to N-BPs, we inactivated Atraid globally in mice (23). We confirmed deletion of Atraid exons 3 to 5 and determined that Atraid homozygous deleted AtraidKO mice [labeled knockout (KO), −/−] are viable, but their body weight is mildly reduced compared with litter-matched derived, wild-type animals (KO, +/−) are a viable, but their body weight is mildly reduced compared with litter-matched wild-type mice (fig. S2, A to C). We confirmed that tail fibroblasts derived from AtraidKO animals are resistant to the cytotoxic effects of alendronate (fig. S2, D and E). Before studying the effects of the N-BPs in the context of Atraid loss, we first characterized the basal role of Atraid in bones. We determined that Atraid mRNA expression was undetectable in the bones of AtraidKO animals and that AtraidKO mice had slightly smaller bones compared with litter-matched derived, wild-type control mice (fig. S2, F and G). To examine the effects of Atraid on bone structure, we performed microcomputed tomography (μCT) analysis (24). Atraid deficiency did not decrease either trabecular or cortical structural parameters (fig. S2, H and I, and data file S2). We measured bone strength using three-point bending tests (25). Some measures, such
**Fig. 1.** ATRAID is required for molecular responses to nitrogen-containing bisphosphonates (N-BPs). (A) Schematic of haploid mutagenesis screening pipeline. Sequencing-based identification of gene-trap insertion sites in alendronate-resistant human haploid KBM7 cells. Genomic DNA for sequencing was obtained from mutagenized KBM7 cells grown for 4 weeks after treatment with alendronate (165 μM). (B) Sequencing-based identification of gene-trap insertion sites in alendronate-resistant cells. N, number of unique insertions within the stated gene locus. False discovery rate (FDR)–corrected P values for ATRAID = 7.02 × 10−45, PLCL1 = 1.02 × 10−45, EPHB1 = 2.05 × 10−04, SNTG1 = 1.84 × 10−03. P values represent enrichment in alendronate-treated versus vehicle treated cells. (C) Schematic representation of structural features of human ATRAID protein and its mouse and frog orthologs. (D) Cell viability in wild-type control and ATRAID-deficient cells exogenously expressing or not expressing ATRAID cDNA. Cells were treated with alendronate (60 μM) and analyzed for cell viability. Cell viability was determined by measuring cellular ATP and is expressed as a ratio of that compared with untreated cells. Error bars indicate the SD for n = 4 (biological replicates), N.S., not significant; *P < 0.05, Student’s t test. v2, variant 2 (NM_080592.3); v3, variant 3 (NM_001170795.1) of the ATRAID gene, respectively. (E) Chemical structures for nitrogen-containing bisphosphonates (N-BPs) or non-N-BPs. (F) KBM7 cell viability in ATRAID-deficient (ATRAID_GT1 and ATRAID_GT2) and control (wild-type) KBM7 cells upon treatment with N-BPs or non-N-BPs. All cells were treated with the indicated concentration of the indicated N-BP (alendronate, zoledronate), BP (etidronate, tiludronate) for 72 hours. Cell viability was determined by measuring cellular ATP and is expressed as a ratio of that compared with untreated cells. All measurements were performed in quadruplicate (biological replicates). *P < 0.05, Student’s t test. (G) Immunoblots of cell lysates from ATRAID-deficient and ATRAID v3–reconstituted HEK-293T cells treated with the indicated dose of alendronate for 24 hours. Equal amounts of protein were loaded in each lane. This experiment was repeated three times (biological replicates) and was consistent all three times. Asterisk (*) indicates nonspecific band. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
as stiffness (Newtons/millimeter, N/mm) and postyield displacement (a measure of bone fragility, in millimeters, mm), were decreased by Atraid deficiency, whereas others, such as yield load (the point where bone bending goes from elastic to plastic, in Newtons, N), were not significantly altered (fig. S2, J to L, and data file S2; P > 0.05, Student’s t test).

Osteoclasts release degradation products from C-terminal telopeptides of type I collagen (CTX-I) from bone into blood (26), and CTX-I in serum was not significantly different in wild-type mice compared with AtraidKO mice (fig. S2M and data file S2; P > 0.05, Student’s t test). Histomorphometric measures of osteoclast function including osteoclast surface per bone surface (Oc.S/BS) (27), as judged by tartrate-resistant acid phosphatase (TRAP) staining (28), were also not statistically different (fig. S2N and data file S3). Consistent with a basal defect in osteoblast function (29), AtraidKO mice have reduced serum circulating Gla-Osteocalcin [the activated form of osteocalcin, incorporated in bone matrix (30)] and modestly reduced bone formation rate (BFR) as measured by double-labeling (fig. S2, O and P, and data file S2) (27).

To test the effect of alendronate in a model that mimics menopausal bone loss, the most common indication for the N-BPs, we performed ovariectomies (OVX) on adult female mice (Fig. 2A) (31). When ovaries are removed from females, the changes in estrogen cause a reduction in bone density triggered by disruption of the balance of osteoblast and osteoclast functions. This loss of bone density can be alleviated by treatment with N-BPs (32). The magnitude of trabecular bone loss in WT and AtraidKO sham mice 4 weeks after OVX is exemplified in the μCT three-dimensional reconstruction of the femoral proximal reconstruction (Fig. 2B). Consistent with alendronate preventing bone loss (32), femoral cortical and trabecular structural parameters, including cortical thickness and area, bone volume/trabecular volume (%), and trabecular thickness, were increased by alendronate treatment of WT OVX mice (Fig. 2, C to F, and data file S2; see WT OVX +/- alendronate). In contrast, alendronate had blunted effects in AtraidKO OVX mice (Fig. 2, C to F, and data file S2; see ATRAIDKO OVX +/- alendronate).

The same patterns of alendronate resistance in AtraidKO mice were observed for bone strength (Fig. 2, G and H). That is, alendronate increased bone strength as judged by stiffness and yield load in wild-type ovariectomized mice, but its effects were blunted in AtraidKO matched cohorts (Fig. 2, G and H, and data file S2). Together, these results suggest that Atraid is required for the beneficial effects of N-BPs in ovariectomized female mice.

To test an additional osteoporosis model, we examined senile (old age) osteoporosis using 18-month-old male WT and Atraid-deficient mice (33). After treating these mice weekly with alendronate or saline for 2 months, we found similar results to those in our OVX study. That is, measures of bone density were increased by alendronate but less so in the Atraid-deficient mice (fig. S2, Q and R, and data file S2). This further suggests that Atraid is key for responses to N-BPs in vivo.

**ATRAID is required cell autonomously for N-BP inhibition of osteoclast function**

Because N-BPs potentially affect osteoclasts and osteoblasts, we investigated whether Atraid deficiency would regulate the effects of alendronate in each cell type in our postmenopausal (OVX) and old-age (senile) osteoporosis models. Regarding osteoclasts, in wild-type mice, both serum and bone histological markers of osteoclast function, CTX-I, and osteoclast surface per bone surface (Oc.S/BS) and osteoclast number per bone surface (N.Oc/BS), respectively, were impaired by alendronate treatment (Fig. 3, A and B; fig. S3, A and B; data file S3) in both osteoporosis models. In contrast, in AtraidKO mice, alendronate was less effective on osteoclasts in both osteoporosis models (Fig. 3, A and B; fig. S3, A and B; data file S3). That osteoclast number was reduced by N-BPs in wild-type mice is consistent with our cell viability measurements in non-osteoclasts and with previous literature (32).

To provide insight into the effects of N-BPs on osteoblasts in our osteoporosis models, we measured BFR and mineral apposition rate (MAR) (27). Unlike BFR in which the rate is normalized by the amount of labeled bone surface, MAR is the rate of bone formation irrespective of how much of the bone is active (27). Alendronate did not affect trabecular MAR or BFR in either wild-type or AtraidKO mice in either osteoporosis model (fig. S3, C and D, and data file S3).

To determine whether Atraid is required in a cell autonomous manner for the N-BP–dependent effects on osteoclasts, we isolated macrophage colony-stimulating factor (M-CSF)–expanded bone marrow macrophages (BMMs) from both WT and AtraidKO mice and differentiated these cells into osteoclasts following a standard protocol (34). AtraidKO BMMs differentiated into osteoclasts as well as wild-type cells did irrespective of treatment with alendronate, yet BMM-derived AtraidKO osteoclasts were resistant to alendronate-induced apoptosis (Fig. 3, C and D). This suggests that Atraid is required cell autonomously in osteoclasts for the effects of N-BPs on cell number.

As an independent confirmation of our primary cell experiments, we generated AtraidKO RAW 264.7 cells and differentiated them to osteoclasts (fig. S3E). RAW 264.7 cells are a robust, well-characterized murine macrophage cell line that can be differentiated to osteoclast-like cells using RANKL (35). We treated both RAW 264.7 cells and the RAW 264.7 cells differentiated into osteoclasts with alendronate and found that Atraid deficiency, as expected, conferred resistance to doses that induced apoptosis in wild-type cells (Fig. 3E). Alendronate did not affect known markers of osteoclast differentiation in wild-type cells (Fig. 3C). Therefore, to pursue the mechanism of N-BPs on osteoclast function, we focused on prenylation. In alendronate-treated RAW 264.7 cells and osteoclasts differentiated from RAW 264.7 cells, we found that AtraidKO cells were resistant to alendronate-induced inhibition of prenylation (Fig. 3F).

We assessed whether wild-type osteoclasts might sensitize Atraid-deficient osteoclasts to N-BPs. We cultured primary wild-type osteoclasts with either WT or Atraid-deficient primary osteoclasts and treated these cocultures with alendronate or vehicle. As in the case of WT cells grown independently (Fig. 3, D to F), WT osteoclasts were more inhibited by alendronate than Atraid-deficient osteoclasts despite the presence of WT osteoclasts in both cases (Fig. 3, G and H). In total, these findings support that Atraid is required for the cell-autonomous effects of N-BPs on osteoclasts.

**Genetic factors involved in responses to N-BPs in patients**

We sought an unbiased approach to determine what genes might be relevant in patients treated with N-BPs. We performed whole-exome sequencing (WES) on two sets of patients taking N-BPs who experienced side effects: patients with osteoporosis who experienced AFFs (n = 27 patients), as well as patients with multiple myeloma or breast cancer patients who experienced ONJ (n = 8 patients) and 11 control patients taking N-BPs that did not experience AFF or ONJ (Fig. 4A and data file S4 for patient information). We also analyzed two
We then compared the patient data to three cell-based, genome-initiation of zoledronate treatment (Fig. 4A and data file S4) (patient died less than 1000 days versus greater than 2500 days after bone marrow disseminated tumor cells (DTCs), which reoccurred or the ONJ when taking N-BPs (36) and patients with breast cancer with N-BPs: patients with multiple myeloma who did or did not suffer published gene expression datasets involving patients who had taken N-BPs: patients with multiple myeloma who did or did not suffer ONJ when taking N-BPs (36) and patients with breast cancer with bone marrow disseminated tumor cells (DTCs), which reoccurred or the patient died less than 1000 days versus greater than 2500 days after initiation of zoledronate treatment (Fig. 4A and data file S4) (37). We then compared the patient data to three cell-based, genome-wide CRISPRi and CRISPRa screens we previously performed: alendronate and zoledronate CRISPRi and alendronate CRISPRa (data file S4) (12, 38). To identify genes involved in N-BP response across experimental paradigms, we generated a Venn diagram to visualize the overlap of "hits." In comparing the WES hits—genes that had the same rare coding variants (minor allele frequency < 0.05) in both patients with AFF and ONJ but not in controls—with our hits from our alendronate and zoledronate CRISPRi/a screens, we identified 64 genes in common including ATRAID, FDPS, and SLC37A3 (Fig. 4A). When comparing the WES hits with the gene expression hits, we identified 49 genes, whereas the CRISPRi/a and gene expression...
Fig. 3. Atraid is required cell autonomously for N-BP inhibition of osteoclast function. (A) CTX-I, a serum marker of osteoclast activity, was measured in WT and AtraidKO ovariectomized mice with or without alendronate treatment by ELISA. Each circle represents an individual animal. Circles offset to the right represent unique animals with similar values to those of another animal (offset for visual clarity). N = 8 to 13 mice per group. *P < 0.05, Student’s t test. (B) Osteoclast histomorphometric responses in WT and AtraidKO ovariectomized mice with or without alendronate treatment. Osteoclast surface to bone surface ratio (Oc.S/BS) was determined by tartrate-resistant acid phosphatase (TRAP)–assay reactivity. Each circle represents an individual animal. Circles offset to the right represent unique animals with similar values to those of another animal (offset for visual clarity). N = 5 to 7 mice per group. *P < 0.05, Student’s t test, and red lines indicate mean. (C) Quantitative polymerase chain reaction (PCR) to examine mRNA expression of markers of osteoclast differentiation, Ctsk, Tnfrsf11a (RANK), Acp5 (TRAP), in wild-type (WT) and AtraidKO M-CSF–expanded bone marrow macrophages (BMMs) differentiated with RANKL to osteoclasts. Expression is normalized to wild-type, undifferentiated BMM cells, using Actb and Rplp0 as control genes. Error bars represent the SD of technical triplicate reactions. (D) Percent of Annexin-V–positive cells after a 48-hour alendronate treatment of WT and AtraidKO BMMs differentiated into osteoclasts. Annexin V staining was assessed using flow cytometry. Each circle represents osteoclasts derived from an individual animal (split for treatment with 0, 10, and 30 µM alendronate). Red lines indicate mean. *P < 0.05, Student’s t test. (E) Percent of Annexin-V–positive cells after a 48-hour alendronate treatment (0, 30, and 80 µM) in wild-type and AtraidKO differentiated RAW 264.7 osteoclasts. Annexin V staining was assessed using flow cytometry. Error bars represent the SD of n = 3 experiments (biological replicates), *P < 0.05, Student’s t test. (F) Immunoblots of cell lysates of RAW wild-type (WT) and AtraidKO (KO) cells, and RAW 264.7–derived osteoclasts treated with alendronate for 48 hours. (Top) Immunoblot specific to the unprenylated version of Rap1a. (Bottom) Gapdh, serving as a loading control. Alendronate concentrations were 0, 20, and 80 µM. (G) Representative image of a six-well dish coculture of equal numbers of mouse primary osteoblasts and osteoclasts of the indicated genotypes with or without the indicated doses of alendronate for 4 days. The experiment was performed three independent times with a similar result. Red staining reflects TRAP-assay reactivity. (H) Image analysis of the samples in (G). Error bars represent the SD of n = 3 independent images (technical replicates). *P < 0.01, Student’s t test.
STUDIES HAD 76 GENES IN COMMON (FIG. 4A). THREE GENES, ATRAID, ATR, AND ZBTB4, HAD STATISTICALLY SIGNIFICANT HITS (FDR CORRECTED P < 0.05) IN A BP CELL-BASED CRISPRi/a SCREENING, DIFFERENTIALLY EXPRESSED IN BOTH GENE EXPRESSION DATASETS (ONJ AND DTCs), AND HAVE RARE MULTIPLE NONSYNONYMOUS CODING VARIANTS IN AFF AND ONJ CASES BUT NOT CONTROLS.

USING A SIMPLIFIED BINARY TEST WAS USED TO CALCULATE THE SIGNIFICANCE OF EACH VARIANT. *P < 0.05, STUDENT'S T TEST. "X"-ENRICHED REFERS TO THE FOLD-ENRICHMENT OF THE ALLELE COMPARED WITH A POPULATION WITH A SIMILAR GENETIC BACKGROUND AS THE CASES. FOR EXAMPLE, FOR ATRAID, THE D5G VARIANT IS PRESENT IN 2 OF 27 PATIENTS WITH AFF. ALTHOUGH THIS ALLELE WAS NOT DETECTED IN THE 11 CONTROL SAMPLES, IT IS PRESENT IN A POPULATION OF EUROPEAN AMERICANS (EA) AND ASIAN AMERICANS (AA) THAT IS REPRESENTATIVE OF THE STUDY POPULATION AT A PREVALENCE OF 0.0131. THEREFORE, THE D5G ALLELE IS (2/27)/0.0131 = 1.5. EXON SEQUENCE DATA FOR ATRAID VS. WT, P VAL < 0.05 FOR THE INDICATED CELL LINES, STUDENT'S T TEST. "X"-ENRICHED REFERS TO THE FOLD-ENRICHMENT OF THE ALLELE COMPARED WITH A POPULATION WITH A SIMILAR GENETIC BACKGROUND AS THE CASES. FOR EXAMPLE, FOR ATRAID, THE D5G VARIANT IS PRESENT IN 2 OF 27 PATIENTS WITH AFF. ALTHOUGH THIS ALLELE WAS NOT DETECTED IN THE 11 CONTROL SAMPLES, IT IS PRESENT IN A POPULATION OF EUROPEAN AMERICANS (EA) AND ASIAN AMERICANS (AA) THAT IS REPRESENTATIVE OF THE STUDY POPULATION AT A PREVALENCE OF 0.0131. THEREFORE, THE D5G ALLELE IS (2/27)/0.0131 = 1.5.

WE Sought TO DETERMINE THE FUNCTIONAL RELEVANCE OF DECREASED ATRAID mRNA EXPRESSION AND THE ATRAID D5G/G32R VARIANT, BOTH OF WHICH ARE ASSOCIATED WITH BAD OUTCOMES OF N-BP TREATMENT (FIG. 4A AND B). TO TEST THE FORMER, WE EXPRESSED ATRAID mRNA AT SUB-ENDOGENOUS QUANTITIES IN ATRAID-DEFICIENT CELLS SUCH THAT THE EXPRESSION WAS SIMILAR TO THE REDUCED EXPRESSION SEEN IN PATIENTS THAT EXPERIENCED ONJ OR DTCs (~50% COMPARED TO WILD-TYPE CONTROLS) (FIG. 4C). WE REFER TO THESE ATRAID PARTIALLY RESTORED CELLS AS "ATRAID LOW EXP." ATRAID-DEFICIENT CELLS CONFERRED RESISTANCE TO...
alendronate as expected, whereas ATRAiD low expr. cells were hyper-sensitized to alendronate (Fig. 4D). Similarly, the ATRAiD DS5/G32R variant, which we identified in both the patients with AFF or ONJ, conferred hypersensitivity to alendronate compared with wild-type ATRAiD (Fig. 4, E and F). Together, this suggests that bad patient outcomes might reflect cellular hyperresponse to N-BPs. In total, these findings support the importance of ATRAiD in N-BP responsiveness in humans.

**DISCUSSION**

This work focused on the physiological impact of ATRAiD as a positive regulator genetically upstream of FDPS. Here, we use prenylation as an output of FDPS function. Recently, we linked FDPS to DNA synthesis and damage (39). This was intriguing in light of earlier studies in the context of ONJ where N-BPs regulated p63—a well-known mediator of DNA damage (40)—in a mevalonate pathway-dependent manner (41). Considering that each of the three top genes from the patient analysis, ATRAiD, ATR, and ZBTB4, are involved in p53 responses—a better known p63-related mediator of DNA damage (42–45)—it will be interesting to determine whether these genes mediate their effects on p53/p63 signaling via FDPS.

The molecular effects of N-BPs on FDPS require the transporter SLC37A3 (12). The SLC37A family member (46) SLC37A2 is mutated in dogs and gives rise to a bone overgrowth phenotype that resembles the human disease Caffey syndrome (47, 48). This phenotype is particularly interesting because it suggests that natural ligands or drugs that inhibit the SLC37A family might phenocopy N-BP treatment in increasing bone density.

ATRAiD binds NELL-1, a secreted protein that promotes bone mineralization in mice and potentiates osteoblast differentiation in an ATRAiD-dependent manner (29, 49). It is also notable that NELL-1 is in preclinical testing for the treatment of osteoporosis (50). In future studies, it will be interesting to determine whether NELL-1 affects the responses to N-BPs we observe upon manipulating ATRAiD. NELL-1 has a related family member, NELL-2. This family member has been the subject of high profile studies in the field of axon guidance (51). It is unknown whether ATRAiD signals to NELL-2 and if so what role it may play in the brain.

There are several limitations of our study. Because we used a global KO strategy with Atraid, we cannot definitively conclude that it is required in vivo in osteoclasts—the target cell type for the N-BPs. We identified ATRAiD in screening in leukemia cells not in osteo-
clasts. Therefore, it is possible that a screen in a cell type more rele-
vant to the N-BPs would yield additional genes important to bone. There are relatively modest numbers of DNA samples in existence from patients with ONJ or AFF. More samples need to be collected and analyzed to further test our findings, as identifying those patients who might experience these consequences when taking N-BPs is of paramount importance.

**MATERIALS AND METHODS**

**Study design**

The objective of this study was to identify and subsequently charac-
terize factors involved in the on- and off-target effects of the osteo-
porosis drugs, N-BPs. To address this, we performed a genome-wide haploid cell screen and identified the gene ATRAiD. To assess the cellular role of ATRAiD in the response to N-BPs, we treated a va-
riety of cell lines including human 293T and KBM7 cells, murine macro-
phage RAW 264.7 cells differentiated into osteoclasts, and primary cells derived from mice, with the N-BPs (including alendronate and zoledronate) or other drugs, and assessed cell viability/growth/fitness by measuring cellular ATP, as well as protein prenylation by immunoblot. These analyses established that ATRAiD is re-
quired for the cellular responses to N-BPs. We investigated the
in vivo role of ATRAiD by using two mouse models of osteoporosis
[ovariectomy (OVX) on 3.5-month-old female mice as a model of postmenopausal osteoporosis and 18-month-old male mice as a model for senile osteoporosis] treated with alendronate. The effects on wild-type and Atraid KO mice were assessed by profiling bone structure using µCT (OVX; senile, n = 6 to 11; n = 5 to 8), strength using a three-point bending assay (OVX, n = 6 to 11), histo-
morphometry using TRAP staining and double-labeling (OVX; senile, n = 5 to 7; n = 4 to 7), and serum bone proteins using enzyme-linked immunosorbent assay (ELISA) (OVX, n = 8 to 13). These analyses established that ATRAiD is required for the organismal responses to N-BPs. To translate our findings to humans, we integrated human cell line- and patient-based unbiased genome-scale molecular data
where N-BPs were used, including data from patients that experienced DTCs, an AFF, or ONJ while being treated with N-BPs. These analyses established that ATRAiD is potentially important for N-BP responses in humans. For our animal studies, mice were randomized to treat-
ment groups, and subsequent analyses were blinded to the extent possible. All experiments involving mice were performed with pro-
tocols approved by the Harvard and Washington University Animal Studies Committees. The details of study design, sample sizes, experimental replicates, and statistics are provided in the corresponding figures, figure legends, data files, and Material and Methods.

**Statistical analysis**

Unless otherwise specified, group means were compared by one-tailed Student’s t test for unpaired samples. Data on repeated mea-
ures were analyzed by analysis of variance (ANOVA), followed by a post hoc multiple Holm-Sidak method t test. All data are expressed as the mean ± SD with numbers of samples indicated in figure legends. P values are indicated in each figure legend, and values less than 0.05 were considered significant (alpha) with ≥80% power, unless indicated otherwise. We estimated the cohort sizes we would need for this study based on our prior study that involved a similar exper-
imental paradigm in using N-BPs and the OVX osteoporosis model in BL/6 mice (32). All code used to generate statistics and correlations for this project can be found at https://github.com/tim-peterson/ ATRAiD (DOI: 10.5281/zenodo.3739576).

**SUPPLEMENTARY MATERIALS**

stm.sciencemag.org/cgi/content/full/12/544/eaav9166/DC1

Materials and Methods

Fig. S1. ATRAiD is required for the cellular responses to N-BPs.

Fig. S2. Generation and skeletal characterization of Atraid KO mice.

Fig. S3. Atraid is required cell autonomously for the effects of N-BP on osteoclasts in two models of osteoporosis.

Data file S1. Results of haploid genomic screen for genes required for the response to alendronate.

Data file S2. Statistics for Atraid KO mice basal characterization and statistics for bone structure and strength of ovariectomized wild-type and Atraid animals treated with alendronate.

Data file S3. Statistics for bone histomorphometry and serum bone proteins in ovariectomized and senile wild-type and Atraid animals treated with alendronate.

Data file S4. Gene expression, sequencing, and cell growth phenotype data for ONJ, DTCs, AFF, and CRISPRi/a studies.
Evidence for altered osteogenesis in germ cells.
performed the statistical analysis for the CRISPRi screens. S.M., J.C.B., M.M., M.H., S.D., V.N.B., R.C., M.I.G., C.M.M., W.M.R., C.A.G., and K.D. performed the exome sequencing on the patients with AFF. G.H. and C.C.G. performed statistical analysis for the exome sequencing of the patients with AFF. D.A.H., C.L.C., K.M.S., N.R., T.B.D., and T.R.P. performed the exome sequencing on the patients with ONJ. G.H. performed the statistical analysis for the exome sequencing of the patients with ONJ. M.V., K.B., J.E.C., T.R.B., D.M.S., and J.L. performed and/or provided assistance with the cell-based genomic screening. T.R.P., J.P., and L.E.S. wrote the paper. Competing interests: ATRAID, SNTG1, EPHB1, and PLCL1, the genes identified here, are part of a Whitehead-Harvard patent on which T.R.P., T.R.B., and D.M.S. are inventors (US8748097B1). No authors received consulting fees related to this work. Data and materials availability: All data associated with this study are present in the paper, the Supplementary Materials, or will be available at NCBI BioProject ID: PRJNA624650. Shared reagents are subject to a materials transfer agreement.

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**ATRAID** regulates the action of nitrogen-containing bisphosphonates on bone


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**Screening for side effect susceptibility**

Osteoporosis is typically treated with nitrogen-containing bisphosphonates (N-BPs), which inhibit bone resorption; however, N-BPs can cause serious side effects including osteonecrosis of the jaw and fracture. To understand susceptibility to these side effects, Surface et al. investigated the role of the gene **ATRAID** in mediating responses to N-BPs. *Atraid*-deficient mice had weaker bones and did not respond to N-BP treatment in models of osteoporosis, and patients with coding variants in **ATRAID** taking N-BPs presented with atypical fractures and osteonecrosis of the jaw. The authors determined that **ATRAID** is necessary for inhibition of osteoclast function by N-BPs. Results suggest that it may be prudent to screen patients for variants in **ATRAID** to avoid N-BP side effects.